SPECIFIC COVALENT BINDING AND TOXICITY OF ALIPHATIC HALOGENATED XENOBIOTICS

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1. INTRODUCTION

In the past four decades there has been a tremendous increase in the production and use of a series of halogenated short chain hydrocarbons. They are used in vast amounts as industrial solvents for degreasing, dry-cleaning, food processing and paint removing purposes. In addition, some of them are industrially important plastic monomers; others are lead scavengers (in petrol) or are used on a large scale basis in agriculture for a variety of purposes. Most of these compounds are released in increasing quantities into the environment. Residues of technically important substances have been detected in drinking water (detailed information on production, use and occurrence is included in references /1, 2, 3/).

The chemical and physical properties of halogenated xenobiotics are well documented. As can be seen from Fig. 1, most of the halogenated hydrocarbons in question are C_1 or C_2 compounds.

In general, their chemical reactivity is relatively low, but (unlike many halogenated aromatic hydrocarbon pesticides) many of these compounds (Fig. 1) are readily metabolized. Some technically important products have long been known as hepatotoxic agents. However, it was not until the discovery of the carcinogenicity of vinyl chloride that research on the mode of action of these compounds was greatly stimulated.

According to the present data, halogenated aliphatic hydrocarbons exert a variety of toxic effects which have been attributed to the formation of reactive metabolic intermediates. Such metabolites are capable of alkylating nucleophilic sites of different cellular constituents, resulting in "covalent binding", i.e., in chemical modification of targets, the biochemical function of which may be essential for the cell. Causal relationship between covalent binding of reactive halogenated xenobiotic metabolites to cellular proteins and/or lipids and tissue necrosis has been suggested /4,5/. Initiation of peroxydative lipid membrane breakdown by halogenated reactive radical intermediates /6/ may be responsible for parenchymal damage observed. Mutagenicity of halogenated aliphatic xenobiotics has been thoroughly investigated in different "short term assays" /7,8,9/.

With regard to DNA-alkylation, the believed primary event in the process of chemical carcinogenesis, covalent binding of halogenated hydrocarbon metabolites to DNA has been examined *in vitro* and *in vivo* /10,11/. Depletion of cellular glutathione has also been investigated.

Fig. 1. Structural formulas of the halogenated short chain hydrocarbons reviewed.

Very recently, an increased acetone exhalation by rats exposed to various halogenated alkanes and alkenes has been related to a covalent binding of reactive metabolic intermediates of these compounds to cellular CoASH /11,12/.

In addition, considerations connecting structures and metabolic reactivities of the chlorinated ethylenes have been published /13,14/. For most of the substances of concern, results of long-term animal carcinogenicity tests are now available /1,2,3/. The oncogenic effects of different halogenated ethylenes have been quantitated on the basis of histochemical examination of preneoplastic nucleoside-5′-triphosphatase (ATPase) deficient foci in rats exposed to these chemicals from the time of birth on /14/. Furthermore, the pharmacokinetics of these compounds have been investigated /15/. This now extends the data base for a toxicological evaluation of haloalkanes and haloalkenes.

2. HALOALKANES

In contrast to the haloalkenes C_1 and C_2 haloalkanes differ widely from each other in terms of mechanisms of reactive metabolite formation, and the chemical structure of the reactive metabolites. Thus, these compounds, from a mechanistic point of view, cannot be regarded as a uniform toxicological entity.

2.1. Methylene chloride (Dichloromethane)

The non-flammability of methylene chloride which is widely used in paint removers and as a solvent in food processing, makes this compound an attractive substitute for a replacement of other, more hazardous, solvents /16/. Neither nephrotoxicity nor hepatotoxicity could be observed after acute i.p. dosage of methylene chloride to mice /17,18/, and in a one year inhalation study (3500 ppm) with rats only minimal hepatocellular alterations were reported /19/. Hepatotoxic effects were observed in mice only with ultimately lethal doses /20/ and after continuous inhalation of 5000 ppm.

A carcinogenicity study in male mice by i.p. injection /21/ revealed no significant difference in the results from treated and control animals. No malignant tumour increase was found in a two year inhalation study in male or female hamsters, nor in female rats, at exposure concentrations of 500, 1,500 and 3,500 ppm (6 hours per day, 5 days a week). However, an apparent exposure related association between an increased incidence of malignant salivary gland tumours in male rats and pro-

longed exposure to 3,500 ppm methylene chloride was reported. This observation was attributed to a viral infection of the salivary glands, which affected all rats at the age of about 2-3 months /19/ and was not interpreted as of toxicological significance in the light of the extensive data on toxicity of methylene chloride. With respect to the importance of this compound, several further studies are in progress /22/. In contrast to earlier observations which showed a low teratogenic potential (see /23/) methylene chloride was reported to be neither embryotoxic nor teratogenic in mice and rats /24,25/.

Since the findings that exposure to methylene chloride resulted in elevated carboxyhemoglobin levels /26,27/ which were dependent on the dose level administered /28/ the metabolism of this compound has been extensively investigated *in vitro* and *in vivo* /29-34/. Various lines of evidence show that the greatest portion of absorbed methylene chloride is metabolically eliminated in rats and mice via dose dependent (saturable) pathways /30,31/.

Most probably, methylene chloride is mainly metabolized by a cytochrome P-450 mediated oxidative reaction (see Fig. 2) to a hydroxydichloromethane intermediate. HCl elimination of the latter leads to formyl chloride, which decomposes, yielding the end product carbon monoxide.

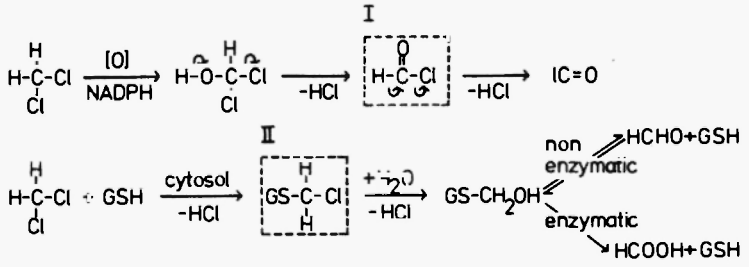

Fig. 2: Metabolism of methylene chloride according to Anders *et al.* /32/ (proposed reactive metabolites framed).

As a second metabolic pathway glutathione-transferase-mediated nucleophilic attack of glutathione is suggested, leading to a chloromethylglutathione intermediate (Fig. 2). Rapid hydrolysis of this chloromethylglutathione leads to S-hydroxymethyl-glutathione which, *via* enzymic

reduction and hydrolysis, yields formic acid and glutathione. By the non-enzymic hydrolysis of S-hydroxymethyl-glutathione, glutathione and formaldehyde are generated, the latter of which is readily metabolized to formate and ultimately to carbon dioxide /34/.

Covalent binding of methylene chloride metabolites to microsomal proteins and lipids has been demonstrated *in vitro* /32/ and radioactivity associated to serine residues of (liver) proteins has been reported after administration of ^{14}C-methylene chloride /34, 4/. However, the overwhelming portion of this macromolecular radioactivity observed after administration of the compound *in vivo*, is derived from utilization of radioactive one-carbon fragments by the intermediary metabolic pathways /34/.

Formyl chloride (I) and S-chloromethyl-glutathione(II), a reactive α-halomethyl-thioether, are thought to be responsible for the "true" covalent protein binding observed.

The formation of two reactive intermediates in the metabolism of methylene chloride is also supported by mutagenicity of methylene chloride in bacterial test systems /31, 36/. In addition, increased acetone exhalation, eventually an indicator for reactive metabolite formation, was found during exposure of rats to methylene chloride /12/. However, oral administration of 1,000 mg/kg to rats had no visible effect on hepatic glutathione levels /37/.

Although reactive intermediates are formed in the course of methylene chloride metabolism, that covalently bind to microsomal proteins and lipids *in vitro* and *in vivo*, there is yet no evidence for alkylation of DNA. The toxicity of methylene chloride relies on the fact that about 50% of the compound is converted in the organism to carbon monoxide /29/. Current occupational and environmental standards for methylene chloride in different countries are based upon this metabolic feature.

2.2 Chloroform

Prior to the mid-1940s, chloroform had been widely in use as an anaesthetic agent and, until recently, it was present in some pharmaceutical preparations. Today, the overwhelming amount of chloroform produced is used as an intermediate in the synthesis of polytetrafluorethylene and, to a lesser extent, as an industrial solvent. The toxicological literature on chloroform is extensive and specific reviews have been published /38, 3/. The acute toxicity of chloroform is species-, strain- and sex-dependent. Acute oral doses of chloroform produce fatty infil-

tration and necrosis of both liver and kidney in rats /39/ and male mice /40/; female mice are reported to develop hepatic but no renal damage.

Chloroform was identified as a possible carcinogen in mice more than 30 years ago by Eschenbrenner and Miller /41/. This was confirmed by subsequent investigations, also in other strains and species. Hepatocellular carcinoma in male and female mice /4, 38, 41/ and kidney tumours in rats were found /4/ at high dose levels which also produced /41/ necrotic changes of the target organ.

Chloroform is metabolically transformed to CO_2 by laboratory animals and humans. Regardless of strain, mice showed the highest conversion rates (about 80% of an orally administered dose) followed by rats which exhaled 60% of the dose administered as CO_2. For monkeys, a conversion rate of about 18% was determined whilst 79% appeared as unchanged chloroform in the exhaled air. By human volunteers about 50% of a chloroform dose was exhaled as CO_2 /4, 38/. Various lines of evidence suggest /42/ that chloroform is transformed by monooxygenase(s), *via* trichloromethanol to phosgene (see Fig. 3). Phosgene as a reactive electrophilic intermediate may then react with water to yield CO_2, with intracellular glutathione or with cellular macromolecules.

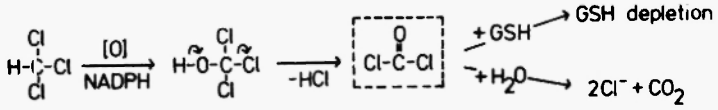

Fig. 3: Metabolism of chloroform according to Pohl *et al.* /42/ (proposed reactive metabolite framed).

Covalent binding of reactive metabolites of chloroform to tissue molecules is thought to be responsible for the necrotic changes evoked by this chemical. After administration of ^{14}C-chloroform to mice, extensive covalent binding to proteins of liver and kidneys occurred, in parallel with the extent of necrosis in these organs /5/. In rat liver, chloroform metabolites bound covalently to protein, especially at SH-groups of methionine and, to a lesser extent, to phospholipids /4, 10/. Covalent binding of ^{14}C-chloroform to microsomal proteins and lipids has also been demonstrated *in vitro* with microsomal preparations from rat, mouse, rabbit and human liver /10/, thus confirming the investiga-

tions *in vivo*. Furthermore, metabolic activation of chloroform in isolated hepatocytes caused protein alkylation, glutathione depletion and a loss in the hepatocytes' ability to replenish GSH from amino acid precursors /43/; in these experiments, glutathione depletion was followed by lipid peroxydation. However, the potency of chloroform to induce lipid peroxydation *in vivo*, as indicated by determinations of pentane production in the rat, was only about 1/10 that of carbon tetrachloride /44/.

Although irreversible binding of reactive chloroform metabolites to proteins clearly occurs, so far all investigations reported /4, 10, 45, 46/ have failed to detect any significant binding to liver RNA or DNA from rats and mice after (oral) administration of ^{14}C-chloroform. [Extremely low RNA binding was observed when RNA was incubated with liver microsomes and labelled chloroform /7/.]

In accordance with these results, chloroform is not mutagenic in *S. typhimurium*, with or without metabolic activation /7/, and it was thought that the reactive intermediate generated in the biological system may not reach the target (the DNA of the test bacteria), due to a very short half-life. Such a view is eventually supported by metabolism of chloroform to genetically active compounds in *Saccharomyces cerevisae*, an indicator organism containing a cytochrome P-450 dependent monooxygenase system /9/.

With regard to the carcinogenic properties of chloroform, there is evidence that chronic tissue injury always precedes tumour development /45/ and that tumours do not develop after doses of chloroform that are not sufficient to cause tissue necrosis. The virtual absence of DNA-binding (in rodents) suggests an epigenetic mechanism of tumorigenicity by chloroform /45/.

2.3. Carbon tetrachloride

Carbon tetrachloride, which is used in the industrial production of fluorocarbons and as an industrial solvent, is the most hepatotoxic chlorinated methane /47, 20/. However, its nephrotoxic potency is much less than that of chloroform /48, 49/.

As early as one hour after oral administration of a sufficiently high carbon tetrachloride dose, biochemical alterations indicative of hepatotoxicity are visible /4/. Such changes include early centrilobular suppression of glucose-6-phosphatase, lipid peroxydation, triglyceride accumulation, increase of serum enzyme activities due to membrane dam-

age, fatty degeneration and centrilobular necrosis /4, 10, 50/. In humans, inhalation of high, but unknown levels of carbon tetrachloride has predominantly led to kidney injury, whilst oral intoxications have resulted mainly in hepatic necrosis /51/.

Long-term administration of high levels of carbon tetrachloride to mice, rats and hamsters result in formation of hepatocellular carcinomas /3, 52, 53/. Two early studies in mice have demonstrated the impact of dose and dose intervals /52, 53/, and a correlation was found between the degree of necrosis and the incidence of hepatomas observed /53/.

Most probably, carbon tetrachloride is metabolized by cytochrome P-450 enzymes of the liver; an initial reductive dehalogenation step leads to a trichloromethyl radical (see Fig. 4) /54, 55/. This may be oxygenated by the microsomal mixed-function oxidase system to yield a trichloromethanol intermediate and, after HCl-elimination, phosgene /55/. Hydrolytic dechlorination of phosgene would then result in carbon dioxide formation, a major end product in carbon tetrachloride metabolism, *in vivo* /56, 57/ and *in vitro* /57, 58/.

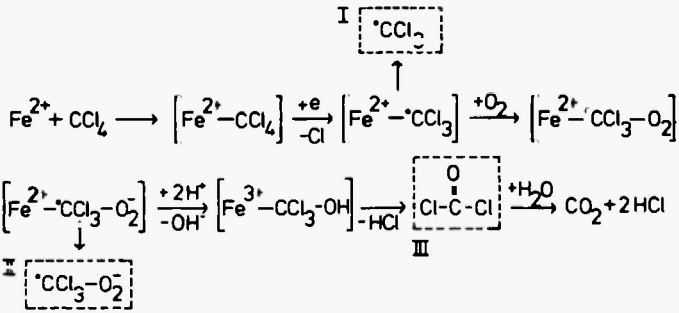

Fig. 4: Metabolism of carbon tetrachloride according to Sah *et al.* /55/ (proposed reactive metabolites framed).

Trichloromethyl radicals, released during carbon tetrachloride metabolism, may either abstract a hydrogen atom from its environment to yield chloroform, combine to hexachloroethane, or bind covalently to proteins and lipids /10, 55/. Covalent binding of carbon tetrachloride in rat liver *in vivo* results in about the same extent of protein alkylation,

as chloroform. However, lipid alkylation by carbon tetrachloride is about three times that of chloroform; and covalent binding of CCl_4 is probably unspecific towards various lipid components /4/.

It is well known that the abstraction of a hydrogen atom from unsaturated lipids, e.g. by carbon tetrachloride derived radicals, initiates lipid peroxydation, and both the $CCl_3^{\cdot}$-(I) and the CCl_3-$O_2^{\cdot}$- radical (II) have been suggested as being responsible for the lipoperoxydative membrane damage observed /50, 55/.

However, it is still not clear whether the resulting cell necrosis is due to covalent binding of the radical(s) to microsomal proteins and lipids, or to radical induced peroxydation of membrane-bound lipids. As a second reactive metabolite of CCl_4, phosgene (III) /55/ may be involved in covalent binding to proteins and lipids.

Investigations on nucleic acid alkylation by reactive carbon tetrachloride metabolites revealed inconsistent and contradictory results. No covalent binding of radioactivity to RNA was reported after incubation of ^{14}C-carbon tetrachloride with RNA and rat liver microsomes /10/. When incubated with liver microsomes from mice pretreated with methylcholanthrene, ^{14}C-carbon tetrachloride was covalently bound to various polynucleotides and DNA. After chromatographic separation of hydrolysates of this DNA, two distinct peaks of radioactivity, not coinciding with the elution of the natural bases, were described /59/.

Administration of ^{14}C-carbon tetrachloride to rats did not result in significant amounts of covalent binding to nucleic acids of liver /4/. In contrast, low amounts of carbon tetrachloride derived radioactivity could be detected in liver DNA of mice, after pretreatment of the animals with methylcholanthrene /59/ and after administration of a toxic dose of non-radioactive carbon tetrachloride, together with the labelled compound /60/. However, in both sets of experiments, no detectable radioactivity was eluted on chromatographic separation of the hydrolyzed DNA. Also, covalent binding to cytoplasmic RNA was not detected in these experiments.

This led the authors to speculate that reactive carbon tetrachloride metabolites, covalently binding to DNA, may probably be generated by cytochrome P-450 enzymes of the nuclear envelope /60/.

On the basis of these investigations *in vivo* it is difficult to decide whether carbon tetrachloride radioactivity is in fact bound to the nucleic acid, or whether binding occurred to minute co-isolated protein impurities. In future experiments, proof of a definitely characterized alkylation product should be attempted.

Carbon tetrachloride was non-mutagenic in *S. typhimurium* and *E. coli*, both in the presence and absence of a microsomal activation system /3, 7/. As already suggested for chloroform, carbon tetrachloride metabolites, generated outside of the test organisms, may also be too short-lived to reach the genetic material of the indicator micro-organism.

The mechanism of tumour formation by carbon tetrachloride shows similarities to that described for chloroform. Like chloroform, carbon tetrachloride produces tumours only in animals treated with doses that are high enough to evoke hepatocellular necrosis.

In the light of these results, it seems most likely that carbon tetrachloride, like chloroform, acts as a tumorigen mainly *via* an epigenetic mechanism, which is triggered by chronic hepatic injury.

2.4 1,2-Dichloroethane, 1,2-Dibromoethane

Because of its use as an intermediate in the production of vinyl chloride, 1,2-dichloroethane is among the "top 50 chemical products" (ranked according to the U.S. production volume /61/). 1,2-dibromoethane is much less used. Both haloethanes are applied as lead scavengers in petrol and as grain fumigants, and widespread human exposure has been suggested.

A large amount of work has been done on mutagenicity, carcinogenicity and metabolism of these two haloethylenes. Recently, a detailed review on genetoxic effects of 1,2-dichloroethane and 1,2-dibromoethane /62/, and a report on 1,2-dichloroethane in book form have been published /63/. A brief synopsis of major toxicological manifestations and metabolism of the two compounds, with special reference to covalent binding, is given below.

The chemicals show acute hepatotoxic effects in inhalation experiments with rats (order : 1,2-dibromoethane > 1,2-dichloroethane), especially in fasted animals /64/. Chronic exposure to 1,2-dichloroethane caused liver and kidney necrosis. Anti-fertility effects of 1,2-dibromoethane in various species have been reported /62/. Both chemicals have been found carcinogenic in laboratory animals on various routes of administration. 1,2-dichloroethane was not as tumorigenic as 1,2-dibromoethane. 1,2-dibromoethane (but not 1,2-dichloroethane) produced tumours at the application sites: oral administration resulted in squamous cell carcinomas of the stomach, skin application in skin carcinomas. By contrast, skin application of 1,2-dichloroethane produced lung tumours in mice. Hemangiosarcomas in male and mammary adeno-

carcinomas in female rats were also reported. Male mice showed some increase in hepatocellular tumours, and both sexes developed lung tumours. From the only inhalation study with mice and rats no carcinogenic effect of 1,2-dichloroethane could be deduced.

Most probably the two 1,2-dihaloethanes are metabolized *via* two major metabolic pathways (see Fig. 5). One of these involves conjugation with glutathione by cytosolic glutathione transferases giving rise to a "half-sulphur-mustard" (Ia) which may rearrange to an episulfonium ion (Ib). Further attack of glutathione on the episulfonium ion of S-(2-haloethyl) glutathione would lead to S,S'-ethylene-bis-glutathione or ethylene, whereas S-(2-hydroxyethyl) glutathione could be formed by hydrolysis of the episulfonium ion. A second suggested pathway also leads to mercapturic acids: it involves microsomal oxydation at one of the carbon atoms, to form a highly unstable *gem*-chlorohydrin which would spontaneously dehydrohalogenate to the 2-halo-acetyldehyde (II). Both the haloacetaldehyde and the episulfonium ion are electro-

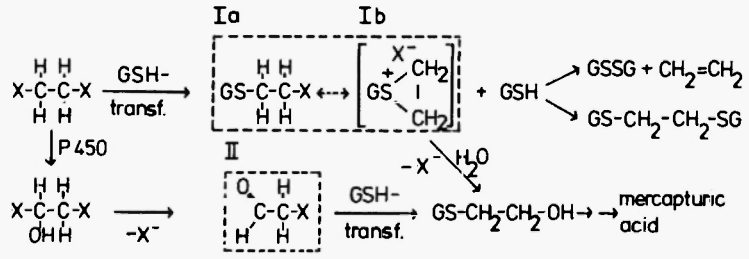

Fig. 5: Major metabolic pathways of 1,2-dichloroethane (X=Cl) and 1,2-dibromoethane (X=Br) according to Rannug *et al.* /62/ (proposed reactive metabolites framed).

philic reactive metabolites, probably responsible for covalent binding to proteins and nucleic acids and for the observed mutagenicity. In general, lower concentrations of 1,2-dibromoethane, compared with 1,2-dichloroethane are needed for achieving hepatotoxic, mutagenic or carcinogenic effects; this may be due to the higher reactivity of the bromine- compared with chlorine- substituted compound. The direct mutagenic effect of both haloethanes on *S. typhimurium*, in the absence of an exogenous metabolizing system, has been attributed to their activa-

tion to the S-haloethyl conjugates, because these bacteria exhibit glutathione-S-transferase activity /62/. However, for 1,2-dibromoethane direct chemical alkylation of p-nitro-benzylpyridine and considerable binding to microsomal protein by a non-enzymic reaction has been reported. So, in addition to its action *via* reactive metabolic intermediates, a direct reaction of 1,2-dibromoethane as such cannot be excluded.

Studies to determine whether covalent binding of the two haloethylenes is dependent on microsomal or cytosolic activation have revealed contradictory results.

In the presence of microsomal preparations from mouse, stomach or liver, radioactivity of ^{14}C-1,2-dichloro-, or ^{14}C-1,2-dibromoethane was covalently bound to microsomal proteins and added DNA; binding was not significant with denatured microsomes /65/.

Furthermore, it was demonstrated that binding of 1,2-dichloroethane to lung microsomal proteins was significantly higher when lung microsomes from B6C3F1 mice, a species susceptible to dichloroethane-induced pulmonary tumorigenesis, were used, as opposed to Osborne-Mendel rats which are resistant to lung tumorigenesis by dichloroethane /66/. Lung cytosol did not catalyze dichloroethane binding, and the addition of glutathione inhibited irreversible binding of dibromo- and dichloroethane in microsomal systems of mouse liver /66/. This would suggest that microsomal activation plays a prominent role in covalent binding of the two haloethanes, probably *via* the P-450 mediated metabolic pathway (see Fig. 5). However, from a second study *in vitro* on covalent binding of dichloroethane it was concluded that in the presence of glutathione most of the DNA-binding results *via* cytosolic glutathione-transferase /67/.

After administration of ^{14}C-dibromoethane to rats, significant amounts of radioactivity became covalently bound to protein RNA and DNA of all major tissues, with largest amounts of bound radioactivity in the liver and kidneys /68/. So far, no specific reaction product of haloethane metabolites with DNA or protein has been characterized. However, investigations on the specific type of lesion, after treatment with dibromo- or dichloroethane *in vivo* would be valuable in elucidating the relative importance of the two major metabolic pathways in haloethane mediated toxicity and carcinogencity.

2.5. *1,1,1-Trichloroethane ("methyl chloroform"), 1,1,2-trichloroethane*

1,1,1-Trichloroethane and 1,1,2-trichloroethane are chemical intermediates in the synthesis of vinylidene chloride; 1,1,1-trichloroethane is also extensively used as an industrial cleaning solvent /3/.

In contrast to its isomer, 1,1,1-trichloroethane ("methyl chloroform") exhibits a very low toxic potential /3/, liver damage occurring only after exposure of animals to nearly lethal levels. The oral LD_{50} of 1,1,1-trichloroethane in rats and mice is about 11 g/kg; and death occurs by central nervous depression (narcotic effect). Long-term carcinogenicity assays with 1,1,1-trichloroethane have revealed no increased incidence of tumours in rats and mice, and also no embryotoxicity and teratogenicity was observed in inhalation experiments with that species. More than 98% of 1,1,1-trichloroethane administered is exhaled unchanged /69/ and the absence of specific organ toxicity may be viewed along with the low metabolic rate of the compound. There are also no reactive metabolites in the metabolic pathway yet proposed for 1,1,1-trichloroethane.

Most probably, 1,1,1-trichloroethane is hydroxylated by cytochrome P-450 enzymes to trichloroethanol, followed by subsequent conjugation with glucuronic acid to yield trichloroethanol glucuronide, or by oxidation to trichloroacetate, both being major urinary metabolites of 1,1,1-trichloroethane /70/.

A slight mutagenic potential, described for 1,1,1-trichloroethane in *S. typhimurium* with and without metabolic activation, has been attributed to contamination with other potentially carcinogenic compounds /71/. An elevated acetone exhalation during exposure of rats to 1,1,1-trichloroethane, as probable proof of formation of reactive metabolites has not been observed /12/.

At present there is no evidence for mutagenic or carcinogenic effects of pure 1,1,1-trichloroethane /71/. This is in agreement with the mentioned insignificant metabolic rate and an absence of reactive metabolites and may recommend this compound as a substitute for other (more toxic) halogenated hydrocarbons.

Compared to 1,1,1-trichloroethane, the oral LD_{50} of 1,1,2-trichloroethane in rats and mice is about 10 times lower /49/.

The hepatotoxic potency of 1,1,2-trichloroethane is considerably less than that of carbon tetrachloride and chloroform; however, like chloroform, 1,1,2-trichloroethane exerts marked nephrotoxic effects /48, 49/.

In long-term carcinogenicity tests administration of (technical grade) 1,1,2-trichloroethane has increased the incidence of hepatocellular tumours and adrenal phaeochromocytomas in mice. In rats, no significant increase in tumour incidence was observed /3/. No data are available on teratogenicity or embryotoxicity of the compound. 1,1,2-Trichloroethane is readily metabolized so that small amounts only are eliminated by exhalation of the unchanged compound /72/.

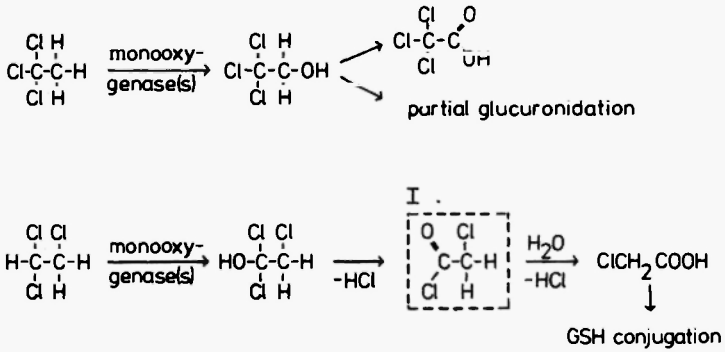

Fig. 6: Metabolism of 1,1,1-trichloroethane (top) and 1,1,2-trichloroethane (bottom) according to Ivanetich *et al.* /70/ (proposed reactive metabolite framed).

Several lines of evidence suggest that 1,1,2-trichloroethane is metabolized by cytochrome-P-450 enzymes (see Fig. 6, bottom) *via* a highly unstable chlorohydrin, which after spontaneous dehydrohalogenation, yields the reactive chloroacetylchloride (I). Hydrolysis of chloroacetylchloride then leads to chloroacetate, which is eliminated in urine or is conjugated with glutathione, leading to urinary elimination of thiodiglycolic acid /70/.

The formation of a reactive acylating metabolite (I) is consistent with the observed hepatotoxic and nephrotoxic properties of 1,1,2-trichloroethane, but so far no data are available on covalent macromolecular binding of this compound. 1,1,2-Trichloroethane was not mutagenic in *S. typhimurium* with and without metabolic activation /73/. Hepatic tumour formation occurred experimentally with doses that also led to damage of the liver parenchyma. There is some similarity in the toxi-

cological patterns of both 1,1,2-trichloroethane and chloroform; also, the suggested reactive metabolic intermediates both have acylating properties.

2.6. 1,2-Dibromo-3-chloropropane

1,2-Dibromo-3-chloropropane had gained widespread acceptance as a nematocide and was used as a soil fumigant. It was also present as an impurity in tris(2,3-dibromopropyl)phosphate that was used as a flame retardant additive in synthetic textiles /3/.

In 1977, employees at a chemical plant in California, who had manufactured 1,2-dibromo-3-choropropane were found azoospermic or oligospermic. After subsequent surveys the U.S. Environmental Protection Agency in 1979 banned this compound for agricultural use in the USA /74/.

Acute and subchronic inhalation studies with 1,2-dibromo-3-chloropropane in different species revealed toxic lesions in lung, liver, kidney and testes /76/. Severe atrophy and degeneration of the testes were observed in rats, guinea pigs and rabbits /75/. Following repeated treatment with 1,2-dibromo-3-chloropropane, number and viability of spermatozoa were decreased in male rats and oestrus was inhibited in females /3/.

1,2-Dibromo-3-chloropropane has carcinogenic properties in rats and mice with mainly a local action. It produces squamous cell carcinomas of the forestomach in both species and adenocarcinomas of the mammary gland in female rats /3/. Chronic inhalation studies with 1,2-dibromo-3-chloropropane resulted in carcinomas of the nasal cavity in rats and mice /76/. In a two-stage carcinogenesis assay 1,2-dibromo-3-chloropropane was an initiator of skin tumour when phorbol myristate acetate was applied as a promoter /77/. However, repeated skin application to mice resulted also in lung and stomach tumours, indicating that 1,2-dibromo-3-chloropropane acts not only at the site of application /77/.

When rats were orally treated with 20 mg/kg of ^{14}C-1,2-dibromo-3-chloropropane, 99% of the dose was metabolized and the metabolites eliminated in urine, bile and expired air. Radioactivity expired consisted mainly of $^{14}CO_2$ /78/. As urinary metabolites the mercapturic acids of S-(2,3-dihydroxypropyl)cystine and 1,3-(bis-cysteinyl)propan-2-ol, β-bromolactate and β-chlorolactate, have been identified /79/. Because notably epichlorohydrin (I) and epibromohydrin (I) produce the same

urinary metabolites, the epihalohydrins were suggested as metabolic intermediates of dibromo-chloropropane /79/.

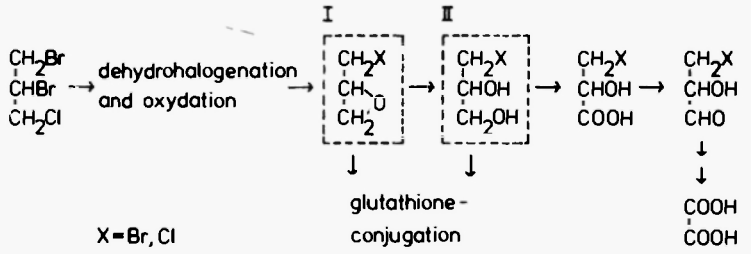

Fig. 7: Metabolism of 1,2-dibromo-3-chloropropane according to Jones *et al.* /79/ (proposed reactive metabolites framed).

It has been speculated (see Fig. 7) that dehydrohalogenation of dibromo-chloropropane, followed by oxidation *via* monooxygenases, may result in reactive aliphatic epihalohydrins (I). Opening of the epoxide ring by hydrolysis would produce α-halohydrins (II) which are known to undergo oxidation to β-haloacetate and, *via* the respective aldehydes, to oxalate.

This means that four reactive metabolites, epichlorohydrin (I), epibromohydrin (I), α-chlorohydrin (II) and α-bromohydrin (II) have been suggested /79/.

1,2-Dibromo-3-chloropropane showed little direct mutagenicity in the *Salmonella* test system. However, when activated to an ultimate mutagen with S-9 preparation (pure) dibromo-chloropropane was clearly mutagenic. The microsomal S-9 fraction alone was responsible for metabolic activation of the compound, and glutathione inactivated rather than activated dibromo-chloropropane /80/, much in contrast to the findings with 1,2-dihaloethanes (see above).

In addition, covalent binding of 1,2-dibromo-3-chloropropane to microsomal protein *in vitro* is dependent on enzymatically active microsomes /81/.

3. HALOALKENES

The data available on metabolism of haloethenes /9, 13/ indicate that compounds of this chemical class are uniformly biotransformed to

the corresponding epoxides (oxiranes) by microsomal monooxygenases, located at endoplasmic membranes. The oxiranes undergo rearrangement to halogenated aldehydes or acyl halides, which can be further converted to halogenated acetic acids. At different metabolic levels conjugation with glutathione may take place, resulting in final excretion of sulphur-containing metabolites (see Fig. 8) /11, 118/.

According to the present data, reactive metabolic intermediates of haloethenes are represented by the epoxides, and probably also by rearrangement products thereof /9, 13/. It is well established that individual members of the haloethene class differ widely in terms of both reactivities of their metabolites and toxic actions /9, 11, 13/. As toxic and carcinogenic effects of individual haloethenes have been attributed to formation of reactive metabolic intermediates, differences in toxicities may partly be due to different metabolic rates of the compounds.
pounds.

Extensive studies on pharmacokinetics of the haloethenes in question have now revealed that the metabolic elimination of these compounds is a saturable, dose-dependent process /15, 85/. When rats are exposed to an atmospheric concentration of a halogenated ethene which exceeds "a point of saturation", elimination is determined by a zero-order law, i.e., its rate is independent of the tissue concentration of the compound. Below saturation, metabolic elimination is described by normal first order kinetics /15, 85/. In Wistar rats, under conditions of saturation, the metabolic rates of the different haloethenes (V_{max}, see Fig. 8), and hence the rates of formation of reactive metabolites, range over two orders of magnitude. This clearly demonstrates that for quantitative toxicological considerations the rates of metabolic transformation of these compounds must be taken into account /14, 15, 84/.

Preliminary considerations connecting the structure of chlorinated ethenes and the activities of the initial epoxide intermediates have been published by Henschler and coworkers. These authors have suggested that reactivities and hence toxicities of the individual chloro-oxirane intermediates depend on the type of chlorine substitution, in that symmetric substitution renders the epoxides relatively stable and not mutagenic, whilst asymmetric substitutions cause unstable and therefore mutagenic epoxides /13, 83/. Trichloroethylene was exempted from this general rule, because its epoxide, although asymmetrically substituted and reactive, is immediately further transformed (at the cytochrome P-450 site) to trichloroacetaldehyde. From this point of view, halogenated ethenes may be divided into two classes, the symmetrical ones

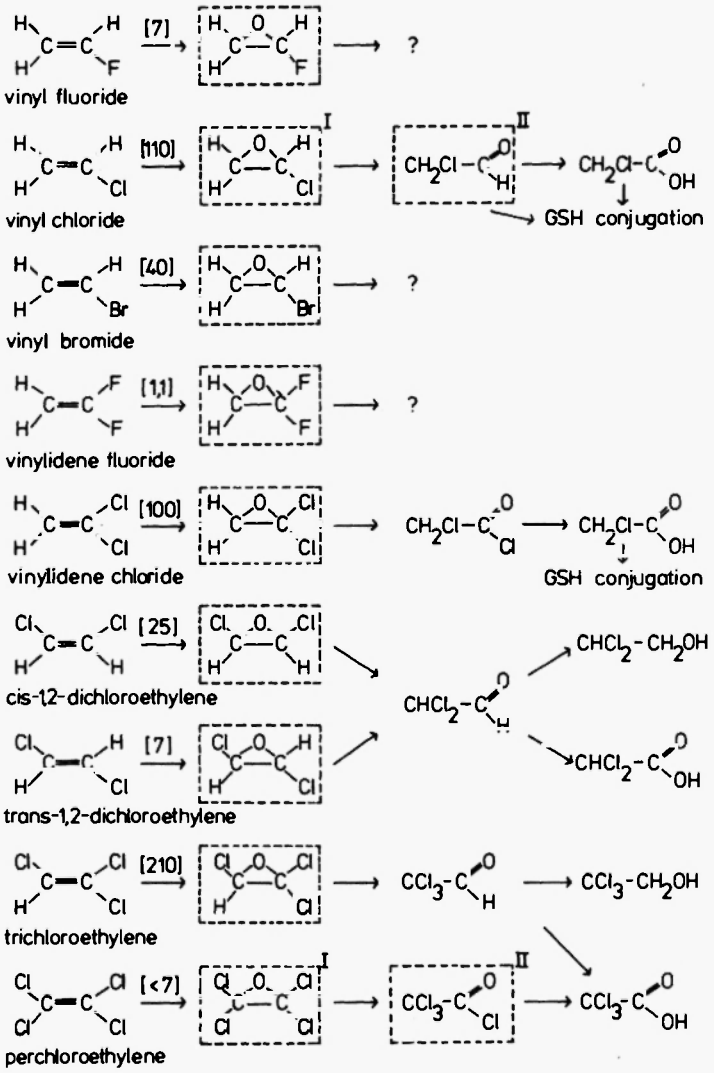

Fig. 8: Metabolic scheme for different haloethenes according to Bonse and Henschler /13/ and Bartsch *et al.* /8/. Metabolic rates v_{max} [$\frac{\mu \cdot Mol}{h \cdot kg}$] according to Filser and Bolt /15/.

(*cis*- and *trans*-1,2-dichloroethylene; perchloroethylene) which form stable epoxides and are not mutagenic and the unsymmetrical compounds (vinyl chloride, vinyl fluoride, vinyl bromide, vinylidene chloride and vinylidene fluoride), with trichloroethylene as an exception (see Fig. 8).

3.1. Vinyl fluoride, vinyl chloride, vinyl bromide

Vinyl fluoride, vinyl chloride and vinyl bromide are used in the manufacture of synthetic polymers in the plastics industry. By contrast to the vinyl chloride, only small amounts of vinyl bromide are used as a co-monomer in plastic fibres. Vinyl fluoride is used in the industrial synthesis of polymers with improved chemical and physical stabilities.

These three halogenated ethenes do not show acute hepato- or nephrotoxicity in laboratory animals (except in one study with mice) /2, 86/; pretreatment with selected inducers of the hepatic monooxygenase system being necessary for injury to become manifest /86/. After pretreatment with polychlorinated biphenyls the relative hepatotoxic potencies of these compounds have been found similar; also, the spectrum of morphologic changes was the same /86/.

The detection of carcinogenic properties of vinyl chloride in laboratory animals /87/ and in man /88/ has prompted many publications dealing with various toxicological and biochemical aspects. Literature on toxicity, mutagenicity, metabolism and carcinogenicity of vinyl chloride in animals and humans has been thoroughly reviewed, and vinyl chloride is now considered a human and animal carcinogen, with liver, brain, lung and haemo-lymphopoietic system being target organs. Furthermore, it has been suggested that vinyl chloride is mutagenic to humans /2/. Preliminary results of an inhalation study with vinyl bromide in rats reported on increased incidences of liver angiosarcomas and zymbals gland carcinomas /8/. No data are available on long-term carcinogenicity studies with vinyl fluoride. However, after exposure of newborn rats to either vinyl fluoride, vinyl chloride or vinyl bromide, the potency of vinyl fluoride to induce hepatocellular preneoplastic "ATP-ase" deficient foci has been demonstrated /14/.

Metabolic pathways for vinyl chloride in man /89/ and in the rat /2/ have been investigated by several research groups. All the available evidence shows /90/ that vinyl chloride is epoxidized by monooxygenase(s) (see Fig. 8) to chloroethylene oxide which then rearranges to chloroacetaldehyde. Chloroacetaldehyde is oxidized to chloroacetic acid.

Conjugation of these primary intermediates with glutathione is followed by modification of the peptide moiety of the glutathione conjugates, leading to sulfur containing urinary excretion products.

Chloroethylene oxide (I) and chloroacetaldehyde (II), both being reactive electrophilic intermediates in vinyl chloride metabolism, may be responsible for the toxicity and carcinogenicity observed /11/. No details are available on the metabolic pathway of vinyl bromide subsequent to epoxidation, although the implication of bromoethylene oxide in the metabolism of this compound has been suggested /8/.

Several lines of evidence suggest that covalent binding of the reactive mono-halooxiranes and haloacetaldehydes might be involved in tissue damage observed. After exposure of rats to ^{14}C-vinyl chloride, covalent binding of this ^{14}C radioactivity to proteins of various tissues, with preferential binding in the liver, has been demonstrated /91, 92, 93/. Further studies on protein alkylation by reactive vinyl chloride metabolites revealed a preferential alkylation of SH-groups of cysteine *in vitro* /94/ and the attachment of a 2-oxoethyl group to the 1-N and 3-N positions of histidine in experiments in mice *in vivo* /95/.

After incubation of ^{14}C-vinyl bromide in a rat liver microsomal system, reactive metabolites of vinyl bromide also became covalently bound to microsomal protein /96/.

Depletion of hepatocellular glutathione, most probably due to covalent interaction of the latter with reactive metabolites of vinyl chloride /97/ and vinyl bromide /98/ has been reported, and considerations of the mechanistic background of interaction of both compounds with glutathione have been published /99/.

Exposure of rats to vinyl chloride resulted in deactivation of certain cytochrome P-450 species /100, 101/.

Very recently the possibility of covalent binding of reactive vinyl chloride metabolites to coenzyme A has been proven /11/ and evidence was obtained for specific binding by a thioether linkage. This, and electrochemical data on coenzyme A reactivity compared to that of glutathione, lends support to the suggestion that coenzyme A might be an even better target for reactive haloethene metabolites than glutathione /11/.

Both reactive vinyl chloride metabolites (chloroethylene oxide and chloroacetaldehyde) react with nucleic acid moieties, leading to defined alkylation products /11/ (see Fig. 9). Targets for alkylation in nucleic acids are adenine, cytosine and guanine residues of DNA and/or RNA.

In RNA, both *in vitro* and *in vivo* the principal alkylation products

Fig. 9: Modified bases derived from covalent binding of reactive vinyl chloride metabolites to DNA and/or RNA.

are 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine /102, 103/. Furthermore, small amounts of 7-oxoethylguanine have been demonstrated in rat liver RNA after exposure of the animals to ^{14}C-vinyl chloride /104/.

After chemical reaction of chloroacetaldehyde with DNA, N^2,3-ethenoguanine was formed as the only guanine alkylation product within the nucleic acid /105/, whereas chemical reaction of chloroethylene oxide with deoxyguanosine led to 7-(2-oxoethyl)-deoxyguanosine (I) by introduction of a 2-oxoethyl group at the 7-N position of the molecule /106/. This compound may normally be present as O^6-7-(1'-hydroxyethano)-deoxyguanosine (II) /106/, thus representing a cyclic hemiacetal form, which affects the 7-N as well as the O^6-position, an important aspect in view of the often dissimilar biological roles of O^6- and 7-N alkylation of guanine.

7-(2-Oxoethyl)guanine, the major alkylation product, 1,N^6-etheno-deoxyadenosine and 3,N^4-etheno-deoxycytidine are readily formed *in vitro* on incubation of DNA with labelled vinyl chloride and rat liver microsomes /104/. However, the nature of the vinyl chloride-DNA adducts formed *in vivo* are still a matter of controversy. Evidence for the occurrence of 1,N^6-etheno-deoxyadenosine and, 3,N^4-etheno-deoxycytidine in the liver DNA of rats, after receiving vinyl chloride for two years in their drinking water, has been presented /107/. Othher research groups have detected only 7-(2-oxoethyl)guanine in liver DNA of rats /104/ and mice /95/ after acute exposures of the animals to ^{14}C-vinyl chloride. N^2,3-Etheno-guanine has not yet been identified as a product *in vivo*.

The similarity of vinyl bromide with vinyl chloride is evident as both form the same alkylation products with RNA /108/, *in vitro* after microsomal activation, and *in vivo* and with DNA *in vitro* /109/.

Experiments in various systems have been carried out /110-113/ to determine whether these adducts have miscoding properties or would alter the processes of transcription and translation. Translation of poly A containing m-RNA in wheat germ cell free systems, resulted in a different pattern of the synthesized proteins when the m-RNA was isolated from livers of rats, 8 hours after exposure of the animals to vinyl chloride or vinyl bromide /110/. Transcription of different synthetic templates which contained 1,N^6-ethenoadenine and/or 3,N^4-ethenocytosine by DNA- or DNA-dependent RNA polymerase revealed evidence for miscoding properties of these alkylation products, and it was suggested that the "promutagenic lesions" evoked by 1,N^6-ethenoade-

nine and 3,N^4-ethenocytosine residues may represent the initial steps in vinyl chloride induced carcinogenesis /111/.

The available data on covalent binding to proteins and nucleic acids are consistent with the formation of haloethene oxide and haloacetaldehyde as reactive metabolites of vinyl chloride and vinyl bromide. Significant direct bacterial mutagenicity was observed with chloroethylene oxide and chloroacetaldehyde, but the former was approximately 20 times more effective than the aldehyde, on an equimolar basis /114/.

Subcutaneous injections of chloroethylene oxide to mice revealed local sarcomas /115/. Chloroethylene oxide induced skin tumours in an initiation-promotion experiment whereas chloroacetaldehyde did not /115, 77/. Furthermore, chloroacetaldehyde-diacetal has been reported to be non-carcinogenic /116/ and bis-chloroethylether, a substance partly metabolized to chloroacetaldehyde in the organism, showed no carcinogenicity in rats after oral administration /117/.

Although chloroacetaldehyde has not yet been studied in long-term carcinogenicity tests, the above arguments support the more or less exclusive role of chloroethylene oxide as ultimate carcinogen in vinyl chloride induced carcinogenicity.

3.2. Vinylidene fluoride, vinylidene chloride

Vinylidene chloride is widely used for copolymerisation with other monomers. Vinylidene fluoride has been found suitable for industrial synthesis of polymers of improved stability /119/; however, sufficient toxicological data on this monomer are lacking. Acute inhalation tests in rats /120, 121/ are inconsistent, but suggest a much lower degree of acute toxicity of this compound compared to vinylidene chloride /2/. This may be partly due to the slow metabolic rate of vinylidene fluoride which is about 1/100 that of vinylidene chloride in the rat /122/.

Vinylidene chloride exerted a marked toxic effect in a series of species which was dependent on dietary parameters (fed or fasted animals) and the hepatic glutathione content /2, 123-125/. Liver and kidney injury has been reported in animals exposed to 50 ppm vinylidene chloride (8h/day, 5days/week) for several months /123/. A continuous 90-day inhalation exposure to 48 ppm produced death in monkeys and guinea pigs, occurrence of morphological changes in livers from monkeys, dogs and rats, and kidney changes in rats /124/. In fasted rats, liver parenchymal cell injury was observed after a 4-hour exposure to 200 ppm vinylidene chloride /126/. Acute inhalation exposure to vinylidene

chloride resulted in a marked decrease in liver glutathione concentrations /127/. No teratogenic effect of vinylidene chloride was seen in either rats or rabbits, and some evidence of embryotoxicity and fetotoxicity was associated with maternal toxic levels of exposure /2, 128/. In a long-term carcinogenicity study in rats, after oral administration of vinylidene fluoride, liposarcomas were reported /129/, the biological significance of which is not clear. Carcinogenicity of vinylidene chloride has not been observed in several long-term studies with rats /130/; but another study reported about hemangiosarcomas in two rats, probably related to this compound /131/. Vinylidene chloride induced kidney tumours have been reported in mice, with a higher sensitivity of males /3, 11/. However, vinylidene chloride inhalation induced also bronchioalveolar adenomas and angiosarcomas of the liver in this species /131/. Its potency to induce preneoplastic hepatocellular ATPase deficient foci in the rat was about one order of magnitude higher than that of vinylidene fluoride, but ranged about three orders of magnitude below that of vinyl chloride /132/.

Both asymmetrically substituted 1,1-dihaloethenes are bioactivated .o mutagenic metabolites, but to much different extents.

In experiments with vinylidene fluoride, a borderline liver-microsome-dependent mutagenic effect was observed, whereas vinylidene chloride showed a more pronounced mutagenic response in *S. typhi. murium*, especially when activated with liver and kidney fractions from mice /8, 83/.

Most probably, vinylidene chloride is metabolically activated *via* its epoxide (2,2-dichlorooxirane) which rearranges to chloroacetylchloride (see Fig. 8) /133, 13/. The latter compound is hydrolyzed to chloroacetic acid, which after combination with glutathione and further degradation of the glutathione residue leads to thiodiacetic acid as a major metabolic end product /134/. No data are available on vinylidene fluoride metabolism. 2,2-Dichlorooxirane and chloroacetylchloride, re active intermediates in vinylidene chloride metabolism, have been pro posed as responsible for acute and chronic toxicity of the compound observed in rodents. Furthermore, chloroacetic acid was proposed to lead to cellular damage by a biochemical mechanism of lethal synthesis (see 4.2) /127/.

After inhalation exposure of rats to ^{14}C-vinylidene chloride, the radioactivity covalently bound to liver protein of the animals was greater in fasted than in fed rats, in parallel to the hepatotoxic effect, although fasted animals metabolized less vinylidene chloride than did fed

rats /135/. Disulfiram pretreatment, which reduced the acute lethal and hepatotoxic effect of inhaled vinylidene chloride, also reduced the levels of covalently bound radioactivity in the liver and kidney of mice after i.p. administration of ^{14}C-vinylidene chloride to the animals /136/. This led the authors to conclude that an increase in the covalent binding of reactive vinylidene chloride intermediates to target tissues may be associated with vinylidene chloride toxicity /135, 136/.

Covalent binding of the epoxide and/or its derivatives to ethanolamine moieties of membrane lipids has been suggested as being responsible for the parenchymal damaging effect of vinylidene chloride; and methyl-thioacetaminoethanol, a urinary metabolite of vinylidene chloride in the rat, was thought to be derived *via* this reaction /133/.

DNA alkylation in liver and kidneys of rats and mice after exposure of the animals to ^{14}C-vinylidene chloride has been reported, and a low level of DNA repair in the kidneys of mice exposed to vinylidene chloride could be measured /130/. Furthermore, these experiments revealed a 25-fold increase in DNA replication in the kidneys of mice, probably due to the tissue damage observed in the same organs /130/. This led the authors to the conclusion that vinylidene chloride appears to induce tumours primarily through epigenetic mechanisms. However, their results do not necessarily indicate that the tumours observed in mice exposed to vinylidence chloride, might arise through effects of the chemical on non-genetic components of the cell, but may suggest that tumorigenic doses of this compound could range in the same order of magnitude, where tissue damage is to be expected.

3.3. cis-*1,2-Dichloroethylene,* trans-*1,2-dichloroethylene*

trans- and *cis-* 1,2-Dichloroethylene are used as industrial solvents. In various countries the TLV/MAK value of both isomers has been set to 200 ppm, a concentration which is based on earlier publications on the narcotic properties of these compounds /137/. Animal experiments have shown that exposure of rats to 200 ppm *trans-* or *cis-* 1,2-dichloroethylene inhibits drug metabolism by the hepatic mixed function oxidase /138/. Furthermore, exposure of rats to 200 ppm *trans-*1,2-dichloroethylene for various time periods induced fatty degeneration of hepatocytes and of Kupffer cells /139/. Both haloethenes have been reported to be weakly nephrotoxic to the mouse, and *cis-*1,2-dichloroethylene seemed to be more toxic than the *trans* isomer /17/. This may

partly be due to the higher metabolic rate of *cis*-1,2-dichloroethylene /15/. (See Fig. 8).

Most probably, both isomers are metabolized *via* primary intermediate oxiranes which rearrange to dichloroacetaldehyde /13/. The latter may then be oxidized to dichloroacetic acid or reduced to dichloroethanol; both metabolites have been identified in perfusates of an isolated rat liver preparation /140/. Although a reactive metabolic intermediate (1,2-dichlorooxyrane) is suggested, no mutagenic activity of *cis*- or *trans*- 1,2-dichloroethylene could be detected in *E. coli* K12 with and without metabolic activation /83/. This is in accordance with the above-mentioned rule on structure activity relationships /13, 140/, which predicts for those chlorinated ethenes which are metabolized *via* symmetrically substituted epoxides no mutagenic or carcinogenic activity.

3.4. Trichloroethylene

Today, the overwhelming amount of trichloroethylene produced is used as a solvent, e.g. for degreasing of metals or in the dry cleaning industry.

Due to its wide applications, an extensive literature on the toxicity of this compound is available which has been reviewed by several authors (see /3/). Although acute and long-term exposure has disclosed some cellular damage in liver and kidney of experimental animals /3, 141/ no pronounced hepato- or nephrotoxicity could be attributed to this compound. However, pretreatment of the animals with agents that induced hepatic monooxygenases, resulted in acute hepatic injury after exposure to high trichloroethylene concentrations /142, 143/.

In a long-term carcinogenesis assay by gavage to rats and mice, trichloroethylene was reported to induce malignant liver tumours in mice but not in rats /144/. In this study a technical grade preparation of trichloroethylene was used, which contained substantial amounts of epichlorohydrin and 1,2-epoxybutane, two well-established mutagens and carcinogens /145/. A recent inhalatory carcinogenicity study in three animal species revealed no indication for carcinogenicity of pure trichloroethylene /146/. Furthermore, trichloroethylene did not induce preneoplastic hepatocellular foci after inhalation exposure to rats, from time of birth onwards /147/.

Studies in rats and mice revealed no embyrotoxic or teratogenic potential of the compound /3/.

All the available evidence shows that trichloroethylene is metabolized by mixed function oxydases to 2,2,3-trichloroethylene oxide /13, 148/, which is further converted to chloral /13, 149/. Chloral is in part reduced to trichloroethanol or oxidized to trichloroacetic acid, the major urinary metabolites of trichloroethylene in animals and in man /13, 3/ (see Fig. 9). Glutathione conjugates have not been identified as metabolites of trichloroethylene so far /150/, although an hepatic glutathione decrease has been reported in phenobarbitone pretreated animals after exposure to this compound /150/. For the apparently exclusive rearrangement of trichloroethylene epoxide to chloral *in vivo*, which is in contrast to the expectation from the thermal rearrangement behaviour, an explanation has been provided /149/. This suggests an immediate Lewis acid-type catalyzed rearrangement of the reactive epoxide to the non-reactive chloral within the hydrophobic environment of the monooxygenases, which would be consistent with the lacking carcinogenic potential of trichloroethylene. After microsomal activation, trichloroethylene is mutagenic in *E. coli, S. typhimurium* and several strains of *Saccharomyces cerevisiae* /3, 83/. In one study the pure compound showed no mutagenic activity, in presence or absence of a rat liver microsomal activation system /145/. On incubation of ^{14}C-trichloroethylene in liver microsomal systems from rats and mice, ^{14}C-radioactivity was covalently bound to liver endoplasmic protein /151, 152/ and the binding was related to the activity of hepatic monooxygenases in the different species /152, 153/. Binding was decreased by addition of inhibitors of the monooxygenase, and enhanced by inhibition of the microsomal epoxide hydrolase with trichloropropene oxide /152/. Microsomal trichloroethylene metabolites were bound not only to sulphydryl groups, but also to amine groups of proteins which were added to the incubation mixture /151/.

After exposure of rats to ^{14}C-trichloroethylene vapour, radioactivity was irreversibly attached to hepatic proteins /151/. An i.p. administration of the labelled compound to mice revealed a distinct binding pattern in the different cellular compartments, with highest values in microsomal and lowest in cytosolic proteins /153/, pointing also to P-450 centred rearrangement reaction.

Incubations of ^{14}C-trichloroethylene with salmon sperm DNA in the presence of microsomal preparations resulted in covalent binding of reactive metabolites to the DNA. The amount of covalently bound radioactivity was much higher in the presence of microsomal proteins of male than female mice, and could be enhanced by pretreatment of the

animals with phenobarbital *in vivo* or by addition of trichloropropene oxide to the incubate /152/. After microsomal incubation of the nucleic acids with ^{14}C-trichloroethylene, chromatographic separation of DNA or RNA hydrolysates revealed some radioactive peaks, but no major alkylation product could be detected in positions of the eluate which were characteristic for alkylation products of the chemically related vinyl chloride /147, 154/.

The possibility has been mentioned that the positive mutagenic results may have been partly due to impurities in the test samples /3/ and that small amounts of reactive impurities also in the commercial radioactive trichloroethylene preparations may have been the cause of "apparent" covalent binding /5/. However, purified ^{14}C-trichloroethylene also showed a considerable microsomal binding when incubated *in vitro* /153/. Possible structural differences of the metabolic activation system *in vitro*, compared with the *in vivo* situation, may have facilitated the escape of the epoxide as such from the hydrophobic rearrangement site, which may be partly responsible for the effects observed *in vitro*.

Very recently, small amounts of covalently bound radioactivity have been reported in rat and mice liver DNA after i.p. administration of ^{14}C-trichloroethylene to the animals /155/. In future experiments, proof of a definitely characterized alkylation product should be attempted.

3.5. Perchloroethylene

Due to its non-flammability and its excellent solvent properties, perchloroethylene is used as a dry-cleaning, fabric finishing and metal degreasing agent /3/.

Perchloroethylene is absorbed mainly through the lungs and is primarily eliminated unchanged in the expired air. With repeated exposures, it is stored particularly in fatty tissues, and is retained unchanged within the body for prolonged periods of time /156, 157/.

In laboratory animals and in man, the predominant effect of acute perchloroethylene exposure is depression of the central nervous system /158, 159/ probably due to the pre-narcotic and narcotic action of the unchanged compound /158/. Although perchloroethylene is generally regarded as being of low toxicity /159/, liver and kidney damage has been reported in laboratory animals after high acute dosage, /3, 159/ or after pretreatment with polychlorinated biphenyls /160/. Daily admin-

istration of high oral doses of perchloroethylene produced an increase of hepatocellular carcinoma in mice. In rats, in the same study /161/ perchloroethylene was not tumorogenic. In addition, perchloroethylene did not induce preneoplastic hepatocellular foci after exposure of newborn rats to the compound /163/.

Most probably, perchloroethylene is metabolized by microsomal monooxygenase(s) to tetrachlorooxyrane /13, 140, 157, 164/. This epoxide may rearrange to trichloroacetyl chloride, which is subsequently hydrolyzed to trichloroacetic acid /13, 164/. Another suggested pathway, involving nucleophilic attack by water, or enzymic reduction of the epoxide by epoxide hydratase, would lead to a tetrachlorinated diol intermediate. Spontaneous dehalogenation of the latter and hydrolysis of the resulting acyl chloride would yield oxalic acid /157/. Trichloroacetic acid and oxalic acid have both been reported as major urinary metabolites of perchloroethylene /157, 165/. No glutathione conjugates have been identified, and perchloroethylene did not show glutathione depletion, which has been attributed to the very low metabolic rate of the compound.

Tetrachlorooxirane (I) and/or trichloroacetylchloride (II), both reactive metabolic intermediates of perchloroethylene, have been suggested to acylate cellular constituents /140, 164/. On incubation of ^{14}C-perchloroethylene in a rat or mouse liver microsomal system, ^{14}C-radioactivity was covalently bound to microsomal protein of both species /163, 164/ and microsomal incubations of the non-labelled compound revealed significant amounts of the trichloroacyl moiety bound in ester or amide linkage to the microsomal protein /164/. Similar results have been obtained in the isolated perfused rat liver preparation, where 3-5% of the total uptake of perchloroethylene was covalently bound to rat liver tissue as acyl chloride. This type of covalent binding was suggested to undergo spontaneous or enzyme-catalyzed hydrolytic cleavage /140/. After exposure of rats and mice to ^{14}C-perchloroethylene, the greater extent of irreversibly bound ^{14}C-radioactivity in hepatic macomolecules of the mouse was attributed to the higher metabolic rate of the compound in this species /166/. Perchloroethylene was neither mutagenic when tested in *E. coli* K12 /83/ or *S. typhimurium* /8/ in the presence of liver microsomal fractions, nor active as skin tumour initiator in a two-stage carcinogenesis assay on mouse skin /77/. Investigations on an anticipated differential sensitivity of the mouse (B6C3F1) and the rat to perchloroethylene revealed no covalent binding of perchloroethylene metabolites to purified mouse liver DNA /166/. However, ap-

proximately a two-fold increase in DNA synthesis and histopathological changes were observed in the liver of mice but not in rats after repeated and oral administration of perchloroethylene, at dose levels which are tumorigenic to mice in lifetime studies. This led the authors to the conclusion that the spontaneous incidence of liver tumours (in this highly susceptible mouse strain) has been enhanced by recurrent cytotoxicity, and that levels of perchloroethylene which do not induce organ toxicity "are not likely to pose a carcinogenic risk to man".

4. CONCLUSIONS

A comparison of the haloalkanes and haloalkenes in question reveals that these compounds, although chemically closely related, differ widely in their toxicities which are preferentially directed towards liver and kidney as the main target organs.

4.1. Specific covalent binding and carcinogenicity

Factors that influence the oncogenic potential are primarily the extent of formation of reactive metabolic intermediates and the reactivity or stability of the reactive metabolite(s) within the physiological environment. For the halooxiranes, it has been reasonably suggested that successful DNA alkylation requires an optimum between stability (to reach the DNA target) and reactivity (to react with it); from that optimum a further decrease in stability may render the oxirane too short-lived to reach the DNA. Similar considerations may be relevant for reactive haloalkane metabolites which show a high (microsomal and cytoplasmic) protein binding even when DNA binding is negligible or absent. In general, the intracellular distance may become more important with increasing reactivity of the metabolic intermediate.

Long-term carcinogenicity assays in rats and mice revealed liver and kidney as principal target organs (except for compounds acting directly at the site of application; substances for which significant and specific covalent binding to liver and/or kidney DNA has been demonstrated have also been found carcinogenic in these species. To-date, chemically defined adducts of DNA alkylation for vinyl chloride (and vinyl bromide) only have been identified and related to the genotoxic and carcinogenic properties. No, or at least questionable, covalent binding to liver DNA could be attributed to some halocarbons which have been found to be hepatocarcinogenic.

This has raised the question as to whether an "epigenetic" mechanism of halocarbon-induced tumour formation, possibly triggered by cellular injury due to chronic administration of cytotoxic doses, does exist.

Recurrent cytotoxicity, leading to increased cell division during tissue regeneration, has been considered to be the responsible for a promotion of inherent or spontaneous mutations, thus enhancing the tumorigenic process.

An "epigenetic" mechanism of tumour formation, linked to enhanced cellular proliferation evoked by tissue injury would imply the necessity for differential risk assessment procedures for "epigenetically" acting halocarbons compared to those causing tumours by genetic damage. For the "epigenetic" mechanism threshold principles should apply, in that exposure to doses which fail to induce tissue injury should be unlikely to cause cancer. However, the presently available data do not yet provide a sufficiently sound basis for such a procedure of risk assessment, but ideas in this direction are being developed /171/.

4.2. Specific covalent binding and (acute) toxicity

In many publications on this subject toxicity of halocarbons has been attributed to the covalent binding of reactive metabolic intermediates to proteins. As quantitative measures the radioactivity covalently attached to total (tissue, cytoplasmic, microsomal) protein, and binding of reactive metabolites to free sulphydryl groups were most commonly used. After induction of halocarbon metabolism, the enhanced toxicity of the single compounds was paralleled by the increase in covalent binding to cellular protein constituents. However, when related to the dose metabolized, the amount of covalent protein binding did not coincide with the toxicity observed and often, halocarbons of low toxicity showed a protein binding comparable to that of the acute toxic ones. Thus, covalent protein binding seems to represent a measure of the amount of reactive metabolic intermediates generated, rather than of toxic potencies of the latter.

Chloroform, carbon tetrachloride, 1,1,2-trichloroethane and vinylidene chloride are regarded as highly toxic compounds evoking acute liver and kidney injuries in experimental animals. Lipid peroxidation is thought to be responsible for carbon tetrachloride-produced cellular damage. However, out of the substances evaluated here, only chloroform seems to induce, to a much lesser extent than carbon tetrachlo-

ride, a lipoperoxidative mechanism. Furthermore a general relationship between lipoperoxidation and hepatotoxicity is not established /167/.

To explain the vinylidene chloride induced cellular damage, Jaeger /127/ has suggested that chloroacetate (as a metabolite of this compound) could block the citric acid cycle in mitochondria by the mechanism of "lethal synthesis". This was hypothesized in analogy to the well-known biochemical effect of fluoroacetate, which involves an enzymic "activation" of the latter to fluoroacetyl-CoA before entering the citric acid cycle as fluorcitrate and blocking *cis*-aconitase (see Fig. 10, 1) /168/. However, the relative LD_{50} potency ratio of chloroacetate/fluoroacetate in rats is 21,6 /169/ which points to a much lower extent of enzymic activation or a dissimilar binding mechanism of chloroacetate (Fig. 10, 2a,b). Furthermore, vinylidene chloride toxicity in rats was reduced by simultaneous exposure to vinyl chloride, which is of low acute toxicity but generates chloroacetate as a metabolic intermediate /64/. This raises some doubt as to a major role of chloroacetate in the mechanism of acute toxicity.

A common feature in the metabolism of chloroform, carbon tetrachloride, 1,1,2-trichloroethane and vinylidene chloride, which they only share with perchloroethylene is the generation of halogenated acylating intermediates like phosgene and chloroacetylchloride.

With regard to the theory of Jaeger /127/, these haloacylating intermediates would be reactive enough to generate "activated" haloacyl-coenzyme A derivatives by direct chemical acylation (Fig. 10, 3a,b). The resulting haloacyl-coenzyme A derivatives, possessing a reactive chlorine function, would represent "active site-directed agents" and might be able to reach key positions in the extremely important metabolic functions of "activated" coenzyme A derivatives. There, specific covalent binding mediated by the reactive chlorine function may block metabolic reactions of vital importance of the cell.

Although forming an haloacylating metabolic intermediate, perchloroethylene may not share the acute toxic action of the other compounds due to its very low metabolic rate and/or the low alkylating potency of the trichloromethyl residue (Fig. 10, 3c).

Recently, increasing attention was drawn to a covalent binding of reactive metabolic intermediates to coenzyme A. A mechanistic treatise had arrived at the conclusion that the sulphydryl group of coenzyme A should be a preferred target for electrophilic reactive metabolites /170/, and the possibility of covalent binding of reactive vinyl chloride metabolites to coenzyme A (in a thioether linkage) has been proved /11/. In-

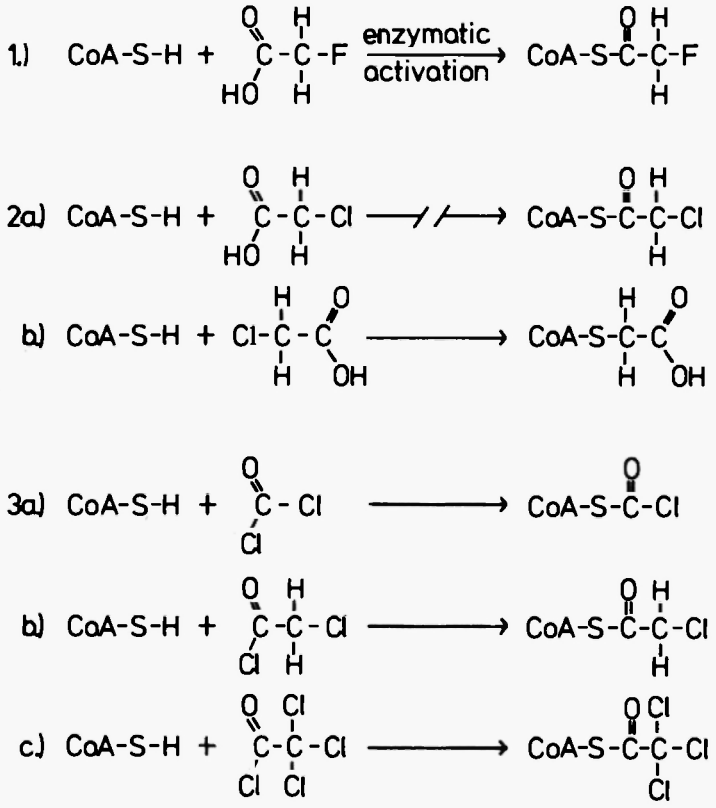

Fig. 10: Specific covalent binding of haloacyl-derivatives to coenzyme A-SH (see text).

creased acetone exhalation by rats exposed to various halogenated al kanes and alkenes has been related to a depletion of cellular coenzyme A by reactive metabolic intermediates of these compounds /12/.

In view of these data, the hypothesis on the involvement of a specific coenzyme-binding in acute toxicity of haloalkanes and haloalkenes may provide a basis for productive further investigations on toxicities evoked by halogenated aliphatic xenobiotics.

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NOTE ADDED IN PROOFS

During the time of editorial processing of the manuscript, two experimental studies on covalent binding of trichloroethylene to DNA have published or accepted for publication /172, 173/.

The authors' report an extremely high covalent binding of radioactivity to proteins of the liver nuclear pellet, after administration of ^{14}C-trichloroethylene to experimental animals /173/. The small amount of radioactivity associated with liver DNA of these animals /172, 173/ could be further diminished by subsequent procedures of DNA purification /173/. Analysis in different chromatographical separation systems of liver DNA hydrolysates revealed DNA associated radioactivity either as physiologically incorporated into the natural nucleosides (bases) or in the early fractions where protein contaminants of DNA were expected to elute /172, 173/. Furthermore, DNA fragmentation in the alkaline sucrose gradient, indicative of phosphotriester formation, could not be observed after administration of trichloroethylene to mice /173/. This further supports the view, that there is no indication of a genotoxic action of trichloroethylene.

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