1977 Vinyl Chloride

REVIEW ARTICLES

VINYL CHLORIDE—PART 1: METABOLISM

The period of phrenetic activity—or at least sustained debate—on the status of vinyl chloride (VC) seems to have passed. Since technological feasibility eventually formed the basis of the first generation of VC legislation, the authorities are now biding their time, pending the next generation of biological data, which will allow an assessment of the hazards associated with the currently permitted levels. We last reviewed VC early in 1976 (BIBRA Bull. 1976, *15*, 6 & 72). In the intervening period the biochemists and microbiologists have demonstrated particular interest in this monomer.

Dose-related metabolism

A preliminary study by Dow Chemical USA on the fate of unlabelled VC in the rat indicated that its metabolism was dependent on the level of exposure (Hefner *et al.* Ann. N.Y. Acad. Sci. 1975, *246*, 135). In the range 50-105 ppm the half-life was 86 min, whereas in the range 220-1167 ppm the half-life was 261 min. Following up this work, the same group investigated the metabolism of ^{14}C-labelled VC in the rat (Watanabe *et al.* Toxic. appl. Pharmac. 1976, *37*, 49). Rats exposed to 10 ppm VC for 6 hr eliminated 68% of the absorbed radioactivity in their urine and 2% (as VC) in the expired air over 72 hr; 6-hr exposures to 1000 ppm resulted in 56% of the dose appearing in the urine with 12% in the expired air as VC. The pulmonary excretion of the VC followed first-order kinetics with similar half-lives at both treatment levels: 20·4 min at 10 ppm and 22·4 min at 1000 ppm. Elimination of ^{14}C in the urine was more complex, being described by a two-exponential equation; but the rates of the initial phase were comparable; at 10 ppm the half-life was 4·6 hr while at 1000 ppm it was 4·1 hr. After 72 hr, 14-15% of the administered radioactivity of both the high and low doses was recovered from the carcases, indicating, when correction is made for the amount of VC metabolized, that there is increased storage of radioactivity at higher doses.

The metabolism of VC administered by other routes appears also to be dose-dependent. Indeed an even greater disparity between the metabolism of high and low doses seems to follow oral administration. In another study by Watanabe *et al.* (*ibid* 1976, *36*, 339) from 59 to 68% of an oral dose of 0·05 or 1 mg VC/kg was converted to non-volatile urinary metabolites, with 9-13% appearing as expired CO_2 and only 1-2% being excreted by the lungs as unchanged VC. By contrast, 11% of the highest dose of 100 mg/kg was excreted in the urine, and 67% was excreted as VC (and 3% as CO_2) in the expired air. The pulmonary excretion at 100 mg/kg was biphasic, with half-lives of 14·4 and 40·8 min, whereas at 0·05 and 1 mg/kg it was monophasic with a half-life of about 55 min.

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Similar findings were reported by the ICI workers, Green & Hathway (Chemico-Biol. Interactions 1975, *11*, 545), low intragastric doses of VC being eliminated principally in the urine whilst higher doses were mainly excreted unchanged via the lungs. About three-quarters of an intubated dose of 250 μg [^{14}C]VC/kg was converted to non-volatile urinary metabolites and 12-15% was excreted in the expired air as CO_2 together with a few per cent as unchanged monomer. By contrast, at a dose of 450 mg/kg pulmonary excretion predominated, with 90% of the radioactivity being associated with unchanged VC and less than 1% with CO_2. The pulmonary elimination of monomer, shown to be proportional to the log of reciprocal dose, was completed within 5 hr, whereas both the excretion of CO_2 via the lungs and the elimination of non-volatile metabolites in the urine continued over 72 hr. Similar excretion kinetics were observed in rats that had been pretreated for 60 days with unlabelled VC at doses up to 300 mg/kg/day (Green & Hathway, *loc.cit.*).

Withey (J. Toxicol. envir. Hlth 1976, *1*, 381) was unconvinced that intubation was an acceptable method of administering VC in a metabolic investigation, for he observed wide inter-animal variability in his studies in which rats with a cannulated jugular vein were intubated with aqueous and oil solutions of VC. The uptake of VC was rapid, peak concentrations being achieved within 10 min, with blood levels varying from 6 to over 40 μg VC/ml.

The metabolism of VC given by iv and ip injection was also studied at ICI (Green & Hathway, *loc.cit.*). Almost all of a small (250 μg/kg) iv dose was found in the expired air as VC. About 43% of the same dose given by ip injection was excreted in the expired air as VC, another 43% being converted to urinary metabolites and 11% to CO_2. An ip dose of 450 mg VC/kg was almost totally excreted (96%) as VC in the expired air, with only 2·6% being metabolized to non-volatile species excreted in the urine.

Identity of metabolites

Identification of the urinary metabolites was attempted in both the Dow and ICI studies. In the oral and inhalation studies of Watanabe *et al.* (*loc.cit.*), most of the radioactivity excreted in the urine of rats was associated with three compounds, the proportions of which were unaffected by the dose or by the route by which the VC was administered. *N*-Acetyl-*S*-(2-hydroxyethyl)cysteine accounted for 29-40% and thiodiglycollic acid for 18-26% of the radioactivity detected in the urine, with the unidentified third compound being present at levels of from 30 to 39%. In the oral experiments, the three metabolites represented from 91 to 95% of the total radioactivity excreted in the urine, whilst in the inhalation experiments the corresponding figure was 96-97%.

The first report by Green & Hathway (*loc.cit.*) of their investigations of the metabolism of orally administered [^{14}C]VC (150 mg/kg) in the rat, noted that the three major urinary metabolites were thiodiglycollic acid (47% of the urinary radioactivity), *S*-(2-chloroethyl)cysteine (23%) and *N*-acetyl-*S*-(2-chloroethyl)cysteine (23%). The remaining radioactivity in the urine was associated with urea (6%), glutamic acid (0·5%), chloroacetic acid (0·5%), methionine (trace) and serine (trace). Watanabe *et al.* (Toxic. appl. Pharmac. 1976, *36*, 339) were critical of these results, suggesting that the chloroethyl compounds were artefacts formed

by the reaction of the hydroxy analogues with the hydrochloric acid gas used in the derivatization procedure. This criticism was accepted by Green & Hathway who reported in their most recent paper (Chemico-Biol. Interactions 1977, *17*, 137) that the major urinary metabolites of VC in the rat were *N*-acetyl-*S*-(2-hydroxyethyl)cysteine, the related *N*-acetyl-*S*-vinylcysteine and thiodiglycollic acid.

Further elucidation of VC's metabolic pathway(s) was attempted by Green & Hathway (1977, *loc.cit.*), who measured the yields of thiodiglycollic acid in the urine of rats given the various possible metabolites intragastrically at high doses. Thiodiglycollic acid in 0·5% theoretical yield was isolated in the urine of rats dosed with *S*-(2-hydroxyethyl) cysteine, a result said to be highly significant in view of the cysteine derivative's instability in even mild reaction conditions. Furthermore, as high yields of the acid were found in those animals dosed separately with chloroacetaldehyde, chloroacetic acid and *S*-(carboxymethyl)cysteine, it was suggested that all these compounds might lie on a common metabolic pathway linking VC with thiodiglycollic acid. Nevertheless, the fact that only small amounts of chloroacetic acid (<0·1%) were detected in the body fluids of animals treated with the high doses of VC, backed by evidence from the metabolism of chloroacetaldehyde (and vinylidene chloride), suggested that chloroacetic acid is not a major VC metabolite. Green & Hathway (1977, *loc.cit.*), believed that in rats, chloroethylene oxide was formed from VC and might then be transformed spontaneously into chloroacetaldehyde. The chloroacetaldehyde or chloroethylene oxide reacts principally with glutathione, catalysed by glutathione-*S*-epoxide transferase, to form *S*-(2-acetal)cysteine with subsequent formation of *S*-(2-hydroxyethyl)cysteine and its *N*-acetyl derivative, *S*-(carboxymethyl)-cysteine and thiodiglycollic acid.

It was the view of Hefner and his colleagues (*loc.cit.*) in 1975 that VC was metabolized at low exposures, below 100 ppm, to 2-chloroethanol, chloroacetaldehyde and monochloroacetic acid via the alcohol dehydrogenase pathway. Only small amounts of the chloroacetic acid were formed at these low doses, due to the rapid reaction of chloroacetaldehyde with the sulphydryl groups of glutathione and cysteine. It was thought that at higher exposures (200-1000 ppm) direct epoxidation by microsomal oxidases occurred, with rearrangement of the resulting chloroethylene oxide to chloroacetaldehyde. However, the more recent study of Bolt *et al.* (Arch. Tox. 1976, *35*, 153) showed that in rats exposed to the monomer at an initial atmospheric concentration of 50 ppm in a closed system, the uptake of VC was completely blocked initially by 3-bromophenyl-4(5)-imidazole or 6-nitro-1,2,3-benzothiadiazole, both inhibitors of cytochrome *P*-450-dependent metabolism and was increased by DDT pretreatment, suggesting that the microsomal oxidases may be operative even at low doses. Weaker inhibitors of this enzyme system, such as SKF-525A or 5,6-dimethyl-1,2,3-benzothiadiazole, produced a measurable but lower order of inhibition. In the original Dow work (Hefner *et al. loc.cit.*), SKF-525A had no effect on the metabolism of low doses of VC (65 ppm) but did slightly decrease metabolism at VC exposures of the order of 1000 ppm.

Cytochrome P-450 involvement

Liver microsomal fractions have been shown to interact with VC *in vitro* (Salmon, Cancer Lett. 1976, *2*, 109). The addition of VC to rat-liver microsomes and NADPH produced a Type 1 spectral shift, similar

to that seen for phenobarbital, thus indicating direct involvement of a cytochrome *P*-450 species. Other *in vitro* studies have also demonstrated that the oxidation of VC to non-volatile products was dependent both on microsomal enzymes and on NADPH (Kappus *et al.* Toxic. appl. Pharmac. 1976, *37*, 461).

It has been suggested that VC metabolites can destroy cytochrome *P*-450. Ivanetich *et al.* (Biochem. biophys. Res. Commun. 1977, *74*, 1411), for example, showed not only that the binding and metabolism of VC *in vitro* was fully inhibited by SKF-525A, indicating the involvement of the cytochrome *P*-450 system, but that the VC metabolites reduced the level of both cytochrome *P*-450 (reduced to *P*-420) and microsomal haem. The levels of cytochrome b_5 or NADPH-cytochrome *c* reductase were unaffected by exposure to the monomer. Decreases in the level of cytochrome *P*-450 and in the total activity of hepatic microsomal enzymes were observed in rats 24 hr after they had been exposed for 6 hr to a 5% VC atmosphere (Reynolds *et al.* Res. Commun. chem. Path. Pharmac. 1975, *12*, 685). The selective enzymatic deactivation was consistent with a cytochrome *P*-450-centred activation of VC to reactive electrophiles. The destruction of cytochrome *P*-450 was found to be inhibited by glutathione in the study of Ivanetich *et al.* (*loc.cit.*), whereas Guengerich & Strickland (Molec. Pharmacol. 1977, *13*, 993) reported that neither added reduced glutathione nor cysteine offered any protection against the VC-mediated destruction.

The loss of cytochrome *P*-450 activity was attributed by Guengerich & Strickland (*loc.cit.*) to the destruction of haem, and not to lipid peroxidation or the binding of electrophiles to free sulphydryl groups. Since the destruction of *P*-450 required all the components necessary for mixed-function oxidation, it was said that the oxidative metabolism of VC by cytochrome *P*-450 was necessary for the observed destruction. The investigators did not consider, however, that the two commonly proposed VC-metabolites, the epoxide and chloroacetaldehyde, were the agents responsible for the cytochrome *P*-450 loss, as neither compound proved particularly effective at destroying either free or *P*-450-bound haem *in vitro*. Consequently it was postulated that different mechanisms underlie VC's mutagenic activity and its ability to destroy *P*-450. The latter is localized at the activating enzyme, consistent with a highly reactive species, whereas mutagenesis seems to require a metabolite stable enough to be transported from its site of activation to interact with nucleic acids or associated macromolecules.

Comment

The view that the microsomal mixed-function oxidases play an active and possibly the major role in the metabolism of VC monomer has received support from the demonstration that a microsomal-oxidase pathway operates at low VC exposures. The results of *in vitro* studies have also indicated that VC is metabolized by enzymes of the liver microsomes, in particular by a cytochrome *P*-450 species, to give initially chloroethylene oxide and 2-chloroacetaldehyde. Further investigation into VC-mediated destruction of cytochrome *P*-450 is required, but it would seem that here, too, a cytochrome enzyme is involved in the formation of the active metabolite(s).

The metabolic studies conducted by Dow and ICI have confirmed the dose-dependence of the fate of VC in the rat. It would seem, on the evidence now available, that in the rat there is effectively a single saturable metabolic pathway for VC. As the dose of VC increases, an

increased proportion escapes metabolism and is eliminated unchanged in the expired air. The fact that the major urinary metabolites—probably derived from the conjugation of chloroethylene oxide or 2-chloroacetaldehyde with glutathione—appear to be unaffected both qualitatively and quantitatively by dose tends to oppose the hypothesis of alternative pathways varying in importance in relation to dose. If there are a number of important metabolic pathways, then they appear to be leading to the same end products, an unlikely, although not impossible, occurrence.

[J. Hopkins]

GETTING RID OF PENTACHLOROPHENOL

As a bactericide, fungicide and herbicide, pentachlorophenol (PCP) finds use in a wide range of applications; in particular, it has become an important wood preservative. Its potential significance as a major environmental contaminant must therefore be recognized.

PCP appears to be readily absorbed through the skin and has been held responsible for aplastic anaemia, muscular paralysis and sensory loss in factory workers who failed to use adequate protective clothing and who worked in poorly ventilated areas (BIBRA Bull. 1965, *4*, 314). The toxicity of PCP to rats was shown to be greater when the compound was inhaled than after oral or subcutaneous administration (*ibid* 1977, *16*, 204). The question of PCP toxicity is complicated, however, by the fact that technical-grade PCP is frequently contaminated with varying amounts of toxic by-products, including other chlorinated phenols and, in some cases, chlorodibenzo-*p*-dioxins and chlorodibenzofurans. Thus Kimbrough & Linder (Toxic. appl. Pharmac. 1978, *46*, 151) found that whereas technical PCP fed for 8 months at a level of 500 or 100 ppm in the diet caused pronounced morphological changes in the livers of rats and some change was noted even with 20 ppm, no change was detected in rats fed 20 or 100 ppm purified PCP and only slight changes were apparent in those given 500 ppm in the diet.

Nevertheless, PCP itself has been shown to be an extremely efficient uncoupler of oxidative phosphorylation in mitochondria (Weinbach, J. biol. Chem. 1954, *210*, 545; Weinbach & Garbus, Nature, Lond. 1969, *221*, 1016) and to be capable of disturbing microsomal detoxication mechanisms in the liver (Arrhenius *et al.* Chemico-Biol. Interactions 1977, *18*, 35). By gas-chromatographic analysis of the sub-cellular fractions of the livers of rats treated orally with 0·15 mmol (*c.* 40 mg) PCP/kg, the latter group demonstrated that, compared with the cytosol, the mitochondria contained markedly lower concentrations of PCP and the microsomes showed a high accumulation (*idem, ibid* 1977, *18*, 23), suggesting that the effects of PCP on the detoxication functions of the endoplasmic reticulum are at least as relevant physiologically as the compound's capacity for mitochondrial uncoupling.

The fate of a single oral dose (10 or 100 mg/kg) of ^{14}C-labelled pure PCP was studied in rats for 9 days, with a final examination of tissues for residual activity (Braun *et al.* Toxic. appl. Pharmac. 1977, *41*, 395). The primary route of excretion of PCP was through the kidneys, with most of the remaining fraction of the dose appearing in the

evidence of direct mutagenic activity, its activity was doubled by the addition of the S-9 fraction from a PCB-treated rat. The results of a similar study by Andrews *et al.* (Mutation Res. 1976, *40*, 273) seem to be anomalous, in that VC at levels of up to 15% in air were shown to be directly mutagenic in TA1535 and an S-9 fraction from PCB-treated rats produced only a slight enhancement. However, the differences between these two sets of results may partly be explained by the different incubation times involved.

Vinyl chloride is not thought to be mutagenic *per se*. The increase in the number of histidine revertants seen when VC is incubated with strains TA1530, TA1535 or TA100 in the absence of any mammalian metabolizing system is thought to be a result of the non-enzymic breakdown products of VC or of compounds formed by the bacterial enzyme system (Bartsch *et al. loc.cit.*).

Aqueous solutions of VC (initial concentration 0·083 M) gave no evidence of mutagenicity when incubated with *S. typhimurium* strains TA1530, TA1535 or G46 together with a fortified 9000-*g* liver supernatant from phenobarbital-treated mice (Bartsch *et al. loc.cit.*). A similar lack of activity of VC in solution (at an initial concentration of 0·022 M) was reported by Elmore *et al.* (Biochim. biophys. Acta 1976, *442*, 405) in TA100. It was thought that the inactivity of VC in these systems may have been caused by rapid diffusion of monomer from the liquid phase into the atmosphere.

S. typhimurium strains TA1530, TA1535, TA100 and G46 respond with varying sensitivity to monofunctional alkylating agents (base-pair substitutions or deletions). Strains TA1536, TA1537 and TA1538, which are specifically reverted by frameshift mutagens, were unaffected by 20% VC atmospheres even in the presence of liver fractions from rats or mice (Bartsch *et al. loc.cit.*; Rannug *et al. loc.cit.*).

The effects of the various liver fractions seen in the studies reviewed by Bartsch & Montesano (Mutation Res. 1975, *32*, 93) indicate that the mixed-function oxidases are responsible for the conversion of VC to mutagenic metabolites in mammals. Garro and his colleagues (*ibid* 1976, *38*, 81), however, did not believe that the hepatic fractions were exerting their effect by an enzymatic mechanism. In contrast to the results of Bartsch *et al.* (*loc.cit.*) who reported that 20% VC in air induced a mutagenic response in TA1530 only when an NADPH-generating system was present, Garro *et al.* (*loc.cit.*), testing 75% VC in air in the same Salmonella strain, found that the activating effect of the S-9 microsomal fraction on VC mutagenesis was independent of the NADPH-generating system (NADPH being a necessary co-factor for the mixed-function oxidases) and was largely unaffected by a thermal treatment that would be sufficient to inactivate all enzyme activity. The increases in VC-induced mutagenicity observed when the liver fractions were obtained from either untreated or PCB-treated animals were similar, although the PCB would be expected to increase the mixed function oxidase content of the liver. Garro *et al.* (*loc.cit.*) considered, therefore, that the liver fraction might have been increasing the mutagenicity of VC non-enzymatically, perhaps by promoting the formation of free radicals. A free-radical generating system, riboflavin irradiated with UV light, doubled the number of VC-induced revertants in th *S. typhimurium*

of the carbonic anhydrase activity in the testis was contributed by the erythrocytes in the circulating blood and how much was derived from the local tissues. From the findings, it was concluded that alterations in the carbonic anhydrase activity of the testes following Cd administration reflected changes in the blood content and therefore in erythrocyte carbonic anhydrase in the organ.

The exact role played by these and possibly other factors in the damaging effects exerted on the testis by Cd remains to be established. The problem continues to attract wide interest, however, and it may be that the placing of a few more key pieces will enable the rest of the jigsaw to be fitted into place.

[P. Cooper]

VINYL CHLORIDE—PART 2: MUTAGENICITY

Last month, we reviewed the more important metabolic studies on vinyl chloride (VC). Now we turn to mutagenicity. This aspect of VC's biological profile has been extensively investigated, particularly in bacteria.

Mutagenicity in Salmonella typhimurium

The preliminary studies of mutagenicity involved the Ames test. When VC, at a concentration of 20% (v/v) in air, was incubated with *Salmonella typhimurium* strain TA1535 and a mammalian metabolizing system, namely a fortified post-mitochondrial rat-liver supernatant, the number of histidine revertants was increased to three times the spontaneous mutation rate (Rannug *et al.* AMBIO 1974, *3*, 194). An increase over the spontaneous mutation rate also occurred in strains TA1535, TA1530 and G46 when they were exposed to a similar VC atmosphere in the presence of a 9000-*g* mouse-liver supernatant (S-9 fraction) fortified with an NADPH-generating system (Bartsch *et al.* Int. J. Cancer 1975, *15*, 429). The greatest response was seen with TA1530, VC treatment (for 48 hr) being associated in this case with a 28-fold increase in the number of histidine revertants. The VC concentration of the incubation medium below the 20% atmosphere was found to be 0·004 M at equilibrium. Although exposure to 20% VC in air proved to be mutagenic towards TA1530 in the absence of metabolic activation, mutagenic activity was doubled by the addition of liver fractions from phenobarbital-treated mice. The highest mutagenic responses occurred in the presence of a 9000-*g* supernatant or with the recombined microsomal and soluble protein fraction in the presence of an NADPH-generating system. Purified microsomal fractions were less efficient activators, and soluble liver proteins (100,000-*g* supernatant) alone were almost without effect. The addition of alcohol dehydrogenase and NAD^+ to either the fortified post-mitochondrial or the 100,000-*g* liver supernatant did not increase the mutation rate (Bartsch *et al. loc.cit.*).

In the TA1535 and TA100 strains, McCann *et al.* (Proc. natn. Acad. Sci. U.S.A. 1975, 72, 3190) found that although a VC atmosphere (20% v/v) gave

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inactive in this system (Elmore *et al. loc.cit.*). Chl roethylene xide, at concentrations of up to 25 µM, and 2-chloroacetaldehyde, at concentrations up to 12·8 µM, also produced a dose-dependent induction of 8-azaguanine- or ouabain-resistant mutants in Chinese hamster V79-4 cells *in vitro*, but 2-chloroethanol or chloroacetic acid were inactive at concentrations nearly 100 times higher (Huberman *et al.* Int. J. Cancer 1975, *16*, 639).

In the presence of mouse-liver microsomes, vinyl chloride atmospheres (up to 50% v/v) significantly increased the forward mutation frequency of *Schizosaccharomyces pombe* and induced gene conversion in *Saccharomyces cerevisiae* (Loprieno *et al.* Mutation Res. 1976, *40*, 85). No mutagenic activity was apparent in the absence of the microsomal fraction. Chloroethylene oxide was directly and highly mutagenic in these test systems (*idem*, Cancer Res. 1977, *36*, 253). In either the presence or absence of microsomes, a commercial aqueous solution of 2-chloroacetaldehyde exhibited only feeble genetic activity, whilst 2-chloroethanol was totally inactive. Since a concentration of 0·05 mM chloroethylene oxide induced the same mutagenic frequency as 50 mM VC, it seems likely that all the activity of the monomer in this system could be ascribed to the oxide.

In a host-mediated assay in the mouse, VC given at 700 mg/kg by gavage significantly increased the mutation frequency of *Sch. pombe*, which had been injected into the peritoneal cavity during 12 hr of treatment (Loprieno *et al.* 1976, *loc.cit.*). Methyl methanesulphonate, used as a positive control, proved to be almost 200 times as active as VC. No significant increases in forward mutation were seen 3 or 6 hr after an ip injection of 250 mg 2-chloroacetaldehyde/kg (Loprieno *et al.* 1977, *loc. cit.*).

Either in ethanolic solution or in the gaseous state, VC induced no detectable mutagenic change in two strains of *Neurospora crassa*, a eukaryotic micro-organism (Drozdowicz & Huang, Mutation Res. 1977, *48*, 43). The addition of an S-9 liver fraction from either control or phenobarbitone-treated rats had no effect. The apparent lack of sensitivity of this system may have been due to the inability of VC and its metabolites to penetrate the conidia.

A significant increase in the frequency of recessive lethals over that in controls was observed in male *Drosophila melanogaster* exposed to VC at 850 ppm for 2 days and there was a slight increase in mutation rate on exposure to 30 ppm for 17 days (Verburgt & Vogel, *ibid* 1977, *48*, 327). No further enhancement in the recessive lethal incidence was seen after 2-day exposures to VC levels in excess of 10,000 ppm. It was suggested that above this concentration the Drosophila enzymes were no longer capable of metabolizing (activating) additional VC. In contrast to the recessive lethal assay, negative results were obtained in tests on dominant lethals, translocations and entire and partial sex-chromosome loss in Drosophila exposed to VC at 30,000 ppm for 2 days (Verburgt & Vogel, *loc.cit.*).

VC has failed to produce dominant lethals when tested in a mammalian system. Male CD-1 mice, exposed to 3000, 10,000 or 30,000 ppm VC for 6 hr/day for 5 days, were housed for 5 days in each of the following 8 wk

(TA1530) system, and this stimulation was further enhanced by the presence of a photopolymerization accelerator—*N,N,N',N'*-tetramethylethylenediamine.

Mutagenicity of VC metabolites

The two suspected VC metabolites, chloroethylene oxide and 2-chloroacetaldehyde each directly increased the number of histidine revertants in strain TA1530 of *S. typhimurium*. At initial concentrations of 0·4 μmol/ml the oxide was about twice as active as the aldehyde with respect to mutagenicity, but chloroethylene oxide exhibited a lower toxicity; 11% of a cell population survived an oxide concentration of 0·4 μmol/ml while less than 0·004% of the Salmonella cells exposed to the same concentration of the aldehyde survived (Malaveille *et al.* Biochem. biophys. Res. Commun. 1975, *63*, 363).

Chloroethylene oxide was strongly mutagenic towards TA1535. At an initial concentration of 0·45 mM, the spontaneous mutation rate of thre mutants/10^8 cells was increased to 96/10^8 surviving cells. Chloroacetaldehyde at an initial concentration of 0·5 mM only increased the spontaneous mutation rate approximately three-fold (Rannug *et al.* Chemico-Biol. Interactions 1976, *12*, 251). It was calculated that chloroethylene oxide, with a half-life in solution of only a few minutes at 37°C, was some 450 times as mutagenic as the aldehyde on a mol:mol basis. The oxide (tested at 0·26 M) and 2-chloroacetaldehyde (0·1 M) were also shown to be mutagenic in Salmonella strain TA100 (Elmore *et al. loc.cit.*). Because of its instability, an increased number of histidine revertants were observed when the oxide was pre-incubated with the test micro-organism at 3°C. All of the four possible forms of 2-chloroacetaldehyde exhibited a mutagenic response against TA100; the pure compound was more active than the monomer hydrate which in turn was more active than the dimer hydrate and trimer, probably demonstrating the importance of the intact electrophilic carboxyl group in eliciting a mutagenic response (Elmore *et al. loc.cit.*).

In the presence of a post-mitochondrial mouse-liver fraction, 2-chloroethanol (108 μmol/plate) increased the number of histidine revertants in the TA1530 strain of *S. typhimurium* about ten times; distinct mutagenic activity, but of a lower order, was also observed in the absence of metabolic activation (Bartsch *et al. loc.cit.*). Chloroacetic acid induced neither a direct nor a tissue-mediated response in this strain. In the study by McCann *et al.* (*loc.cit.*) 2-chloroethanol was weakly mutagenic against TA100, inducing 0·6 revertant colonies/μmol, and demonstrated a trace of activity in TA1535. Microsomes significantly increased the number of compound-induced histidine revertants in TA100 whilst horse-liver alcohol dehydrogenase together with NADH had no analogous effect. Elmore *et al.* (*loc.cit.*) observed no mutagenic response when 2-chloroethanol was incubated at 1 mM with strain TA100. When tested by Rannug *et al.* (1976, *loc.cit.*) 2-chloroethanol and chloroacetic acid were without effect on TA1535 at concentrations up to 1·5 mM; much higher concentrations of 2-chloroethanol (1 M) proved to be weakly mutagenic.

Mutagenicity in other systems

In *Bacillus subtilis*, chloroethylene oxide and 2-chloroacetaldehyde selectively inhibited strain MC-1, a mutant lacking recombination repair of DNA; chloroethanol, chloroacetic acid and VC itself (in solution) were

with successive pairs f untreated females (Anderson *et al.* Mutation Res. 1976, *40*, 359). Using the female mice as the indicator organism there was no evidence that VC had any mutagenic effects on any maturation stage of spermatogenesis. There was no significant increase in the number of post-implantation early foetal deaths, no evidence of pre-implantation egg loss (except at the highest VC dose, where the loss showed a low level of significance but could largely be attributed to a single female), nor any reduction in fertility. A dominant lethal effect was clearly demonstrated in the positive controls given a single ip injection of 200 mg cyclophosphamide/kg or five oral doses of 200 mg ethyl methanesulphonate/kg.

In most of the Salmonella studies, VC demonstrated activity only in the presence of a microsomal liver fraction. The Ames test has given conflicting evidence on metabolism; whilst the studies of Bartsch *et al.* (*loc.cit.*), in line with the other *in vitro* studies, suggested that the microsomal fraction of the liver was acting enzymatically, the experiments of Garro *et al.* (*loc.cit.*) indicated, so far uniquely, that a free-radical mechanism was responsible for the conversion of VC to its active metabolites. VC elicited a mutagenic response in a simple eukaryotic system, demonstrating activity in *Sch. pombe* and *Saccharomyces cerevisiae* both *in vitro* in the presence of liver microsomes and in a host-mediated assay. The incidence of recessive lethal mutation was increased in Drosophila treated with 30 ppm VC for 17 days. Chloroethylene oxide and 2-chloroacetaldehyde have proved to be mutagens—the oxide particularly so—in prokaryotic micro-organisms, namely the strains of Salmonella susceptible to frameshift mutagens and *B. subtilis*. This evidence further implicates chloroethylene oxide as the active VC metabolite.

No evidence of activity was seen in the dominant lethal assay in mice. However, further investigations of mutagenicity in mammals are required before an assessment of the genetic hazards presented by VC to man will be possible.

[J. Hopkins]

REVIEW ARTICLES

VINYL CHLORIDE—PART 3: MACROMOLECULAR BINDING

Chloroethylene oxide and chloroacetaldehyde are probably the principal metabolites of vinyl chloride (BIBRA Bull. 1979, *18*, 7). Evidence of the binding of these metabolites to cellular macromolecules, particularly the alkylation of the nucleic acids, may explain the mutagenicity of vinyl chloride (*ibid* 1979, *18*, 79).

In vivo *studies*

The irreversible binding of VC metabolites to nucleic acid and protein has been demonstrated *in vivo* (Bolt *et al.* in IARC Scientific Publn no.13; IARC, Lyon, 1976, p.151). In rats exposed to atmospheres containing about 140 ppm [^{14}C]VC, roughly half of the radioactivity found in the liver microsomes immediately after the 5-hr exposure was irreversibly bound to protein, although the major proportion of labelled material in the liver cells was present in the cytosol. Immediately after exposure to 44 ppm [^{14}C]VC, from 10 to 40% of the total activity in the tissues was irreversibly bound, while at 48 hr the proportion of bound activity had increased to 70%, although the amount of irreversibly bound VC metabolites stayed the same. Radioactivity was also found in the liver nucleic acids. The peak incorporation of activity into DNA (0·23% total liver activity) occurred immediately after exposure, whereas the specific labelling of the RNA (0·8% of initial activity of the liver) was at a maximum after 24 hr and decreased more slowly. Bolt *et al.* (*loc.cit.*) calculated that as no more than 1% of the labelled VC would enter the one-carbon pool to be incorporated into protein and nucleic acid, this mechanism could not explain the total high level of binding observed in the study. It was possible that the one-carbon pool could account for the amounts of label found in the nucleic acid of the liver. However, the authors thought this unlikely since the speed by which this activity decreased was faster than normal DNA turnover and the rate of incorporation of label was very different in RNA and DNA derived from the same cellular nucleotide pool.

In further studies at the same institute (Laib & Bolt, Arch. Tox. 1978, *39*, 235), liver RNA isolated from rats exposed to VC atmospheres was shown to contain labelled 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine, the alkylation products of adenine and cytosine by chloroethylene oxide and chloroacetaldehyde, as well as the physiological bases. The time-course of dealkylation was found to differ in the two bases. Ninety-two hours after exposure, the level of labelled ethenoadenosine was only one-fifth of its original value; ethenocytidine proved more resistant to repair and the levels of radioactivity associated with this component remained constant over the same period—possibly an indication of the relative importance of cytidine alkylation.

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Watanabe *et al.* (Toxic. appl. Pharmac. 1978, *44*, 571) exposed Sprague-Dawley rats to 1-5000 ppm ^{14}C-labelled VC for 6 hr. The amount of radioactivity bound to macromolecules in the liver did not increase in proportion to the increase in exposure concentrations. Covalent binding to hepatic macromolecules was related to the amount of VC metabolized. There was no detectable binding of ^{14}C to either DNA or RNA in the liver, in contrast to the results of Bolt *et al.* (*loc.cit.*). The authors thought this difference in results might be explained by the higher specific activity used by Bolt *et al.* They also offered the suggestion that the incorporation observed in the earlier work might have originated from the one-carbon pool, but this would not explain the alkylated bases isolated by Laib & Bolt (*loc.cit.*). Watanabe *et al.* (*loc.cit.*) concluded that covalent binding to hepatic nucleic acids occurred to only a limited degree under their experimental conditions but they pointed out that this did not exclude the possibility of other interactions which might result in the loss of the ability to control replication. Hepatic glutathione content was depressed at exposure concentrations above 100 ppm, and according to these authors reactive metabolites of VC may be detoxified by glutathione, suggesting that the carcinogenicity of VC may be related to a decreased ability to detoxify reactive metabolites.

The *in vivo* binding of VC metabolites to DNA has also been investigated in mice (Osterman-Golkar *et al.* Biochem. biophys. Res. Commun. 1977, *76*, 259). BALB mice took up radioactivity about twice as fast as the CBA and ATL strains. Levels of *S*-(2-hydroxyethyl)cysteine, N^1- and N^3-hydroxyethylhistidine or N^7-hydroxyethylguanine were measured in the hydrolysates of proteins and nucleic acids taken from mice exposed to 98-302 ppm; these compounds were formed from the chemical (sodium borohydride) reduction of 2-oxoethyl groups introduced into the cell macromolecules as a result of the VC treatment. The histidine and guanine derivatives and small amounts of hydroxyethylcysteine were detected in hydrolysates of haemoglobin and liver DNA. The authors calculated that the absolute and relative amounts of alkylated products supported the hypothesis that the main reactive metabolite of VC is chloroethylene oxide. In the BALB mice, the two hydroxyethylhistidines could be detected in small amounts in the protein from the testes.

In vitro *studies*

The binding of the metabolites of VC to cellular macromolecules has also been studied by incubating rat-liver microsomes with NADPH, polyadenosine and [1,2-^{14}C]VC (Laib & Bolt, Toxicology 1977, *8*, 185). The radioactivity identified in the enzymic hydrolysates of the polyadenosine was irreversibly attached to 1,N^6-ethenoadenosine. An analogous reaction occurred when polycytidylic acid was incubated with VC and the microsomal system, labelled 3,N^4-ethenocytidine moieties being formed (Laib & Bolt 1978, *loc.cit.*).

The reaction of chloroacetaldehyde with calf-thymus DNA at pH 4·5 gave a modified DNA product (Green & Hathway, Chemico-Biol. Interactions 1978, *22*, 211). Enzyme hydrolysis of the DNA produced a mixture of naturally occurring deoxyribonucleosides and chloroacetaldehyde reaction products, predominantly ethenodeoxycytidine and thenodeoxyadenosine.

The same products resulted from hydrolysis of liver DNA prepared from rats that had been exposed to 250 ppm VC in drinking-water for 2 yr.

According to the studies of Kappus *et al.* (Toxic. appl. Pharmac. 1976, *37*, 461), the alkylation of protein and uptake of VC by rat-liver microsomes is dependent on concentration, incubation time, enzymatic activity, NADPH and oxygen, and is almost completely blocked by CO. These authors found that only about 1% of the VC taken up by the microsomes became irreversibly bound, a finding that contrasts with the corresponding figure of around 50% observed in the *in vivo* studies of Bolt *et al.* (*loc.cit.*). Glutathione added to the microsomal incubation mixture decreased the level of irreversible protein binding. When trichloropropene oxide, an inhibitor of epoxide hydrase, was present, the irreversible protein binding increased two-fold, even though VC uptake by the microsomes was unaffected.

Other *in vitro* studies have produced evidence of chloroethylene oxide's participation in the metabolism of VC. The reaction product of chloroacetaldehyde or chloroethylene oxide with adenosine was tentatively characterized by Barbin *et al.* (Biochem. biophys. Res. Commun. 1975, *67*, 596) as 3,β-ribofuranosylimidazo-(2,1-*i*)-purine (1,N^6-ethenoadenosine); a product with the same R_F value and elution characteristics on a Sephadex column was formed when VC was incubated with adenosine in the presenc of a microsomal fraction. Barbin *et al.* (*loc.cit.*) passed a mixture of VC and air or oxygen into a medium containing both liver microsomes from a phenobarbitone-treated mouse and an NADPH-generating system. The volatile metabolite trapped by reaction with 4-(4-nitrobenzyl)pyridine (4-NBP) in ethylene glycol had a UV absorption spectrum identical to the product formed from the reaction of chloroethylene oxide (but not 2-chloroacetaldehyde) with 4-NBP.

The evidence from these studies strongly supports the view that chloroethylene oxide is the major reactive VC metabolite. While it is clear that metabolites of VC do bind to cellular macromolecules in rat and mouse liver, the extent of nucleic acid binding has not been clearly resolved.

[J. Hopkins]

BLACK MARKS FOR MBK

The industrial solvent methyl *n*-butyl ketone (MBK) has been implicated in cases of peripheral neuropathy among spray-painters and other workers (BIBRA Bull. 1976, *15*, 360) and has been shown to induce axonal degeneration of the central nervous system and polyneuropathy in experimental animals (*ibid* 1977, *16*, 147).

The metabolic fate and disposition of MBK in the rat has been studied by DiVincenzo *et al.* (Toxic. appl. Pharmac. 1977, *41*, 547) in male rats dosed by gavage with [1-^{14}C]MBK at 20 r 200 mg/kg. Absorption