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Trapping With 3,4-Dichlorobenzenethiol of Reactive Metabolites Formed in vitro From the Carcinogen Vinyl Chloride

Watanabe
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Here it is guys!
Perry

AMBIO Vol. 3 No. 6, pp 234-236 (1974)

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Many mutagenic and carcinogenic compounds are known to be biologically active only after metabolic transformation in the body. In many cases, transformations to reactive metabolites occur by means of the microsomal detoxification system of the liver. In order to facilitate the identification of such metabolites, formed *in vitro* from potentially mutagenic or carcinogenic compounds, the use of suitable thiols as trapping agents is proposed. The thiols must be acidic to ensure a sufficient concentration of negatively charged thiolate anions, highly reactive as nucleophiles. Furthermore, the choice of lipophilic, chlorine-containing aromatic thiols facilitates detection of reaction products by means of gas chromatography and mass spectrometry.

The present work describes the use of 3,4-dichlorobenzenethiol to trap reactive metabolites formed from vinyl chloride (I) (Figure 1), recently reported to be carcinogenic for mice at low doses (50 ppm) in air and to cause liver cancer in persons working in PVC-plants. Vinyl chloride has also been shown mutagenic in *in vitro* systems in the presence of liver preparations.

Cell-free liver microsome preparations were exposed to vinyl chloride in the presence of air. The reactive metabolites were trapped by means of the above thiol, added to the liver preparation or, alternatively, exposed in a separate vessel to the gas mixture leaving the liver preparation. One of the products formed from the trapping agents was identified as 3,4-dichlorophenylthioacetaldehyde by means of combined gas chromatography–mass spectrometry. The results are consistent with the formation of chloroethylene oxide (II) as a reactive metabolite of vinyl chloride, but can also be interpreted as indicating the formation of chloroacetaldehyde (III). This is known to be formed from chloroethylene oxide by a slow intramolecular rearrangement. Its formation along other routes under the *in vitro* conditions used, is considered unlikely but cannot so far be wholly excluded.

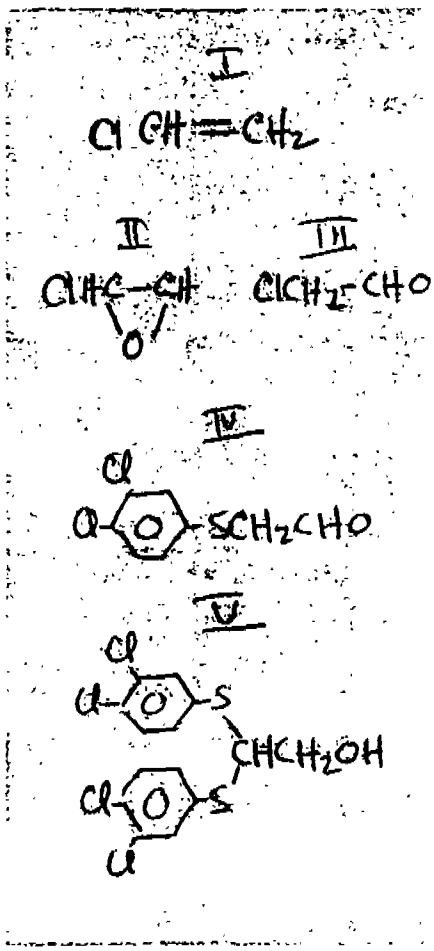

Figure 1. Vinyl chloride (I) is transformed *in vitro* by the microsomal mixed function oxygenase detoxication system to a volatile reactive metabolite, most probably chloroethylene oxide (II), trapped by 3,4-dichlorobenzenethiol as the aldehyde IV, or the as yet not detected derivative V. Alternatively, II rearranges spontaneously to chloroacetaldehyde (III) which with the thiol likewise gives IV.

It has recently been shown in this laboratory (1) that the documented carcinogenic effects in man (2, 3) of long-term exposure to relatively low concentrations in air of vinyl chloride (I) is reflected also in its mutagenic action at higher concentration in *in vitro* systems on *Salmonella* responding to simple alkylating agents. Under the conditions used, its mutagenic activity was clearly demonstrable only after metabolic activation with liver homogenates, prepared according to Ames *et al* (4). An obvious interpretation of these results involves the assumption that vinyl chloride, like aromatic hydrocarbons (5) is subject to epoxidation through the microsomal detoxification enzymes with formation in this case of chloroethylene oxide (II) as a reactive metabolite (1, 6). This highly reactive epoxide has been synthesized from ethylene oxide by several routes (7, 8). In a pure state it is slowly rearranged to chloroacetaldehyde (III) and it hydrolyzes in water to glycol aldehyde (8).

The present investigations were performed in order to verify the above hypothesis by using a simple nucleophile, 3,4-dichlorobenzenethiol as a trapping agent for reactive metabolites. Such a thiol should be acidic enough to ensure a sufficient concentration of thiolate anions already under neutral conditions, a necessary requisite for a rapid reaction with alkylating species. The dichlorophenyl group also gives pronounced lipophilic properties to the expected reaction products and facilitates their detection by gas chromatography (GC) and by combined gas chromatography–mass spectrometry (GC-MS) techniques.

In a typical experiment, an air - vinyl chloride mixture was passed through a tube containing the supernatant of a rat liver homogenate freed from nuclei, mitochondria and other large particles by centrifugation and fortified with all necessary cofactors for the mixed function oxygenase system according to Ames *et al* (4), and in addition fortified with 3,4-dichlorobenzenethiol. The amount of thiol used did not inhibit the microsomal activity, as indicated by the capacity of the system to convert p-nitroanisol to yellow-colored p-nitrophenolate (9). In some experiments, rat liver microsomes freed from cytosol by further centrifugation were substituted for the microsome cytosol mixture (10).

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After two hours of exposure to the gas stream, the reaction mixture was extracted with hexane. The acid fraction, soluble in bicarbonate solution, and the neutral fraction of the hexane extract, respectively, were fractionated by thin-layer chromatography (TLC). Authentic samples of possible reaction products from the postulated metabolite (II) with the thiol were prepared from chloroacetaldehyde dimethyl acetal and chloroacetic acid methyl ester, respectively, and were chromatographed simultaneously as reference substances. The appropriate zones were collected and the extracts were analyzed by GC-MS. The neutral fraction among several peaks gave a peak of the same retention time on GC as 3,4-dichlorophenylthioacetaldehyde (IV), characterized by its mass spectrum which was identical with the mass spectrum of the reference sample concerning molecular ion (M^+, m/e = 220), isotope distribution pattern, and base peak. In addition, a second, strong peak was observed, the mass spectrum (M^+, m/e = 248) of which likewise indicated a compound containing the dichlorophenylthio-group. The question of structure of this compound is so far unsettled and will be discussed in another context.

The bicarbonate soluble fraction of the hexane extract was methylated and then worked up analogously. By means of the GC-MS technique, the presence of 3,4-dichlorophenylthioacetic acid methyl ester was established.

In experiments of a modified type, no thiol was added to the liver homogenate tube. Instead, the air - vinyl chloride mixture was passed through a second tube containing 3,4-dichlorobenzenethiol dissolved in methanol to trap exclusively the volatile metabolites of vinyl chloride. Also in this case, the appropriate TLC fractions from the thiol tube were shown by GC-MS analysis to contain the sulfide IV and the unknown product of molecular weight 248.

In a final control experiment an air - vinyl chloride mixture was blown directly through a tube containing the trapping agent in methanol. After two hours, the reagent solution was worked up as above and analyzed by GC-MS. The ion m/e = 220 was not detectable in the mass chromatogram, confirming absence of the sulfide IV. The ions m/e = 248 and 191, however, were present, indicating that the compound of molecular weight 248 mentioned above is formed directly from vinyl chloride and the thiol under the present experimental conditions.

The results presented here are in accordance with a formation of either or both of the compounds chloroethylene oxide (II) and chloroacetaldehyde (III) as metabolites of vinyl chloride under the *in vitro* conditions used.

The reaction of chloroethylene oxide (II) with thiolate should give the compounds IV and V as major products. Only the compound IV, 3,4-dichlorophenylthioacetaldehyde, can be detected by the present method.

Chloroacetaldehyde (III), if formed by isomerization of II or via other routes, would react directly with the thiolate to form IV.

The present results hence give support

SOME FACTS ABOUT VINYL CHLORIDE

Vinyl chloride at room temperature is a colorless gas with a sweet odor. It has been used as a propellant for various spray products, but its main use is as a monomer (VCM) for polyvinyl chloride (PVC) resin synthesis. The world production of PVC in 1972 is estimated to have been about 16 000 million pounds, with the USA as a leading PVC producer (4300 million pounds). In Sweden about 110 000 tons of PVC are produced each year, in two plants.

VCM causes acroosteolysis, Raynaud's phenomenon, sclerodermia and disturbances of liver function among VC/PVC workmen. VCM was recently demonstrated to be carcinogenic in rats, mice and hamsters. 50 ppm, the lowest tested dose, is carcinogenic in rats and mice, showing angiosarcoma of liver and other organs, lungadenomas and other tumors. In the winter of 1973—1974 VCM was also strongly associated to angiosarcoma of the liver among workmen in the VC/PVC industry. To date (October 15, 1974), 26 cases of angiosarcoma associated with VCM have been discovered in the world. The mean latency time is about 20 years. Twenty-one of these cases occurred in the PVC synthetizing industry, one case in a VCM production industry, three in PVC converting industries (compounding, moulding and so forth) and one case in a plant where spray cans were filled with VC. In Sweden two cases of angiosarcoma of the liver are known among a total risk population at the plant in question of about 620 men. The exposure levels in the plant in question are believed to have been fairly high in the past, at least occasionally. VCM is thus to be considered as a human and animal carcinogen.

The hygienic standards were temporarily lowered in the spring of 1974 in the USA to 50 ppm ceiling value for a 15-minute period and in Sweden to 20 ppm time-weighted average and 50 ppm ceiling value for a corresponding period. In October both the USA and Sweden set standards in the work environment of one ppm time-weighted average and five ppm ceiling value for a 15-minute period, which will be in effect from January 1, 1975. Respiratory masks will be mandatory for workers exposed above that limit from January 1, 1976. Until that date, respiratory masks should be used when levels exceed 20 ppm in Sweden and 25 ppm in the USA.

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to a metabolization of vinyl chloride to chloroethylene oxide (II) only if other routes to chloroacetaldehyde are not operating. A metabolic pathway for vinyl chloride in the rat has recently been proposed, involving 2-chloroethanol as a primary metabolite, subsequently oxidized via the alcohole dehydrogenase route to chloroacetaldehyde and further via chloroacetic acid (6). Such a route would, however, be improbable under the *in vitro* conditions used here, in some experiments involving isolated liver microsomes.

A final interpretation of the connections between the metabolism of vinyl chloride and its mutagenic and carcinogenic properties must await an unambiguous identification of reactive metabolites and a thorough characterization of their reaction patterns, *in vitro* and *in vivo* (11). Such studies are underway.

EXPERIMENTAL

Reagents 3,4-dichlorophenylthioacetaldehyde dimethyl acetal was prepared from 3.4-dichlorobenzenethiol (Aldrich-Europe) by reaction with chloroacetaldehyde dimethyl acetal (Merck-Schuchardt) in lutidine at 60°C overnight. The product was isolated and characterized by its proton magnetic resonance, infrared and mass spectra. The corresponding aldehyde (IV) was not isolated but obtained in solution after hydrolysis, in the absence of air, of the above acetal with aqueous sulfuric acid (2 M) for 100 hours at 20°C and extraction with hexane. 3.4-dichlorophenylthioacetic acid methyl ester was prepared and characterized analogously from methyl chloroacetate and the thiol.

Mass spectrometry A Hewlett-Packard 5930 A mass spectrometer combined with an HP 5933 A datasystem and connected with an HP gas chromatograph 5700 A, with a split to a flame ionization detector, was used for characterization of reaction products from the *in vitro* experiments described below. The 160×0.19 cm (inner diameter) glass column was filled with 2 percent polyethene on acid washed and silanized Chromosorb W 100/120 mesh. The carrier gas (helium) flow was held at 30 ml/min. The column temperature was 130°C for 4 min and then programmed 8°C/min to 200°C.

Vinyl chloride metabolization in vitro in the presence of thiolate A tube containing vinyl chloride (Aerosol Packing Co, Vallentuna, Sweden) was held at —20°C in an ice bath. Air was blown through the tube at a rate of 10 ml/min. The resulting gas mixture was led through a similar tube kept at 37°C and containing magnesium chloride (80 μmoles), glucose-6-phosphate (50 μmoles), nicotinamide adenin dinucleotide phosphate (NADP) (40 μmoles), the 9000 × g supernatant (3 ml) from a liver homogenate according to Ames *et al* (4) from male rates (Sprague-Dawley, Anticimex) and 3,4-dichlorobenzenethiol (1 mg) in a final volume of 10 ml, 0.1 M with respect to a phosphate buffer (pH 7.4).

After 2 hours' exposure to the gas stream, the proteins were denaturated with hexane (50 ml). The hexane phase was centrifuged (10 000 rpm) for 10 min. The supernatant was shaken with sodium hydrogen carbonate solution (8 percent, 50 ml). The carbonate phase was stirred for 24 hours with dichloromethane (0.5 ml), dimethyl sulfate (0.5 ml) and tetrabutyl ammonium hydroxide (25 mg). The reaction mixture was extracted with hexane, the hexane extract was evaporated to a small volume and subjected to purification by TLC (Merck Silicagel HF: hexane-ethyl acetate 9:1), whereby 3,4-dichlorophenylthioacetic acid methyl ester was chromatographed as a reference substance on the same plate, separated from the region containing the unknown sample through a free area. The appropriate zone was collected and extracted with ethyl acetate. The presence of 3,4-dichlorophenylthioacetic acid methyl ester was established by comparison with the authentic reference sample. The mass spectrum from the appropriate GC-peak was identical with that of the reference sulfide with respect to molecular ion (M^+, m/e = 250), isotope distribution and base peak (m/e = 191).

The hexane phase from the initial extraction was further extracted with bicarbonate solution until free from thiol. The neutral extract was subjected to TLC as above, 3,4-dichlorophenylthioacetaldehyde (IV) being applied as a reference. The appropriate zone was extracted with ethyl acetate and injected into the GC-MS unit. The peak with the same retention time (1.5 min) on GC as the reference sulfide IV gave a mass spectrum which displayed the same molecular ion (M^+, m/e = 220; 2 Cl) and base peak (M —CHO, m/e = 191; 2 Cl) as the mass spectrum of IV. Also the lower part of the spectra showed similarities, somewhat obscured by the low signal-to-noise ratio in case of the unknown.

Most remaining peaks of the gas chromatograms were characterized by their mass spectra. Fragmentation patterns and isotope distribution indicative of the 3,4-dichlorophenylthio-group was given by the corresponding disulfide, by a few minor peaks and by a large peak (retention time 8.4 min), which displayed a probable molecular ion M^+, m/e = 248.

Trapping of volatile vinyl chloride metabolites A rat liver microsomal suspension was prepared and washed in 0.2 M potassium phosphate buffer pH 7.5 as described by Arrhenius (10). The microsome incubation system was prepared, with minor modifications, as recommended by the same author: To 10 ml final volume of 0.2 M phosphate buffer: 5 ml microsomal suspension corresponding to 40—70 mg protein, 0.5 μmoles NADP, 50 μmoles glucose-6-phosphate, 600 μmoles nicotinamide, and 1.25 Kornberg Units glucose-6-phosphate dehydrogenase.

An air - vinyl chloride mixture as described above was passed via the bottom of a tube containing the microsome incubation system, kept at 37°C, to a second tube containing 3,4-dichlorobenzenethiol (5 mg) in methanol (5 ml), kept at 20°C. After two hours, the methanol solution was worked up as above. The neutral fraction was subjected to purification by TLC. The presence of the aldehyde IV and the compound, M^+ = 248, in the appropriate fractions was confirmed by GC-MS.

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