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THE KINETICS AND HEPATOTOXICITY OF BENZYL CHLORIDE VAPOR IN RATS

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Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Engineering

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APPROVAL SHEET

Title of Thesis : THE KINETICS AND HEPATOTOXICITY OF BENZYL CHLORIDE VAPOR IN RATS

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ABSTRACT

Title of Thesis : THE KINETICS AND HEPATOTOXICITY OF BENZYL CHLORIDE VAPOR IN RATS

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Male Sprague Dawley rats (250g) were exposed to benzyl chloride vapor at a concentration of 220 ppm for 6 hours. During inhalation, the blood benzyl chloride concentration increased to reach a steady state level of 9-10 ug/ml between 4 and 6 hours of exposure. A small concentration of benzyl chloride (0.86 ug/ml) was present in the blood four hours after the termination of exposure. The absorption half life was 0.53 hours (0.39-0.81) and the elimination half life was 1.12 hour (0.93-1.40).

The absorbed benzyl chloride was largely retained in the blood and some of it (0.22 ug/g) was sequestered into adipose tissue. Benzyl chloride was not detected in the brain, liver, lungs, pancreas and testis. A new HPLC method was used to separate benzylmercapturic acid in the rat blood. In the first hour of exposure, an amount equal to the parent compound was metabolized into benzylmercapturic acid. By 4 hours post exposure, the concentration of benzylmercapturic acid was 25% of the parent compound detected in blood. Liver and kidney glutathione levels in the treated rats were depleted to 13.5 and 46% of the control values, respectively. Liver appears to be the major source of the thiol group in benzylmercapturic acid, while kidney is the secondary source. Benzyl chloride is heptatoxic and causes microvesicular fatty change, inflammation and glycogen depletion in the liver, in the absence of lipid peroxidation in vivo.

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INTRODUCTION

A. Background Information on Benzyl Chloride

Benzyl chloride, molecular weight 126.58, is a colorless liquid with a pungent and somewhat unpleasant and irritating odor. It is a powerful lacrimator and has an irritating effect on the mucus membrane. It is insoluble in water (49.3 ug/100 ml), but readily soluble in alcohol, chloroform and ether. It is regarded as a moderately volatile liquid with a vapor pressure of 1 mm Hg at 22^{o}C. It is a refractive liquid and is volatile with steam. It is hydrolyzed slowly by water to benzyl alcohol and hydrochloric acid, and the half life for the hydrolysis of benzyl chloride at pH 7 and 25^{o}C is 15 hours (Mabey and Mill, 1976).

Benzyl chloride is a commercially important alkylating agent that reacts readily with both organic and inorganic nucleophilic agents; and so may be expected to react with amines, carbohydrates, lipids, proteins and nucleic acids in the living systems. Benzyl chloride reacts rapidly with sulfhydryl groups (Hirade and Ninomiya, 1950), and binds to the epsilon group of lysine in collagen (Gratacos, 1969).

The principle method of commercial production is still the controlled chlorination of toluene. The excess toluene is distilled off and recycled. Hydrogen chloride gas formed in the reaction is used to produce a 30% aqueous solution that can be sold as muriatic acid. The crude product contains 90% to 95% benzyl chloride, 5% to 10% benzal chloride and benzotrichloride. Further distillation results in a product of about 99% purity (Freeman, 1957).

Benzyl chloride is available in the U.S. as technical and refined grades (Sidi, 1964). It undergoes Friedel-Crafts condensation reaction in the presence of metals, such as iron, aluminum, and tin and moisture and/or heat favors such reactions (Grassie and Meldrum, 1971). A stabilizer, such as sodium carbonate, triethylamine, dioctylamine and propylene oxide is often used to inhibit these condensation and decomposition reactions (Hawley, 1971). The effective storage life of benzyl chloride at normal temperature is 2-3 months.

As regards to production and use of benzyl chloride, there are four major manufacturers of this compound in the U.S., who reported a total production of 90 million pounds in 1976. Approximately 60-70% of the total benzyl chloride produced was used captively by its manufacturers mainly for the preparation of n-butyl benzyl phthalate, a plasticizer

for the flexible vinyl polymers used in coatings, and floor coverings. The remaining 30-35% was used as an intermediate in the production of benzyl alcohol, quaternary ammonium compounds and other chemical products, such as benzyl acetate, benzyl cyanide, benzyl salicylate and benzyl cinnamate. Benzyl chloride is reported to be effective as an extreme pressure lubricant (Buckley, 1973 and Davey, 1945).

B. Benzyl Chloride Toxicity

National Institute for Occupational Safety and Health estimated that 3,000 workers in the U.S. are potentially exposed to benzyl chloride. The occupations with potential exposure to benzyl chloride are algicide, butyl benzyl phthalate, drug, dye, pharmaceutical, perfume, resin and rubber workers.

Back et al (1972) reported the oral LD_{50} of benzyl chloride for rats as 1231 mg/kg body weight and for mice, 1624 mg/kg body weight. They further noted that rats and mice survived a 1-hour exposure to benzyl chloride at 2000 mg/cu m. Mikhailova (1964) studied the lethal effects of benzyl chloride in rats and mice that were exposed to vapors of this compound for 2 hours in a 100 liter static chamber. The LC_{50} values for 2 hours of exposure were 390 mg/cu m for mice and 740 mg/cu m for rats. The author observed, at

test concentration greater than 100 mg/cu m, the animals showed marked hyperemia of the tails, paws, and ears, irritation of the eyes and slowed respiration. The histopathological examination of the animals revealed inflammation of the respiratory system, albuminoid and fatty degeneration of the liver cells and degeneration of the convoluted tubular epithelium.

Wolf (1912) conducted acute inhalation toxicity studies in cats and rabbits and exposed them for 7-8 hours to benzyl chloride concentration ranging from 160-17,700 mg/cu m. All animals were observed during and after exposure till recovery or death. At all the concentrations, intense local inflammation of the eyes, nose, mouth, and the air passages to the alveoli of the lungs, was the main effect observed and the severity of the effect varied with the concentration and duration of exposure. Schutte (1915) reported his observations on 12 cats, 5 rabbits, and 1 dog that had been exposed to benzyl chloride at 800-23,600 mg/cu m of air for 0.5-8 hours. His observations agreed with those by Wolf (1912). The severe irritation of the eyes, corneal turbidity and passive behavior were some of the changes seen in the animals after inhalation exposure.

Landsteiner and Jacobs (1936) conducted sensitization tests on guinea pigs that had been previously injected intracutaneously .01 mg benzyl chloride/animal, twice weekly for 12 weeks and noted positive effects such as erythema and

swelling at the treated site. The authors concluded that benzyl chloride had sensitizing capacity.

Holmberg and Malmfors (1974) discussed the cytotoxic effects of benzyl chloride on ascites tumor cells in vitro. At 100 ppm benzyl chloride, the proportion of dead cells increased from 5.0% at zero time to 14.5% after 5 hours of incubation, which the authors regarded was a moderate cytotoxic effect.

Stekol (1947), in his studies on the mechanism of inhibition of growth of rats by benzyl chloride, reported that all animals lost weight while on benzyl chloride diet. The weight loss was alleviated when the diet was supplemented with L-cystine, DL-methionine and DL-homocystine. The author suggested that the weight loss, in part, was due to interferences by benzyl chloride with utilization of sulphur containing amino acids needed for growth.

Preussman (1968) published the first evidence of tumor production by benzyl chloride. The test compound was administered subcutaneously once weekly, in oil solution, to BD rats. After an induction time of 500-600 days, tumors were noted as local sarcomas at the site of injection. Druckrey et al (1970) reported that after a mean induction time of 500 days, 3 of 14 animals that had received 40 mg/kg dose had developed sarcomas at the injection site. Six

of the eight animals given 80 mg/kg dose had larger sarcomas, most of which had metastasized to the lungs. The tumors were further reported to be transplantable. Poirier (1975) reported the results of a study of the induction of lung tumors in mice, injected intraperitoneally with benzyl chloride. The average number of lung tumors per mouse injected with 2, 1.5 and 0.6 g/kg dose of benzyl chloride was 0.25, 0.5 and 0.25 respectively. The results after benzyl chloride injection were not statistically significant and the authors hypothesized that the absence of any effects by benzyl chloride may have been due to greater metabolic inactivation of benzyl chloride in the liver by the intraperitoneal route rather than by the subcutaneous route.

McCann et al (1975) tested the mutagenic potential of benzyl chloride in 2 strains of Salmonella typhimurium and reported that benzyl chloride was much more active as a mutagen in strain TA 100 than with parent strain TA 1535. The results from experiments on bacteria and rodents indicate that benzyl chloride is a weak mutagen in microbial systems, and following subcutaneous injection in rats causes neoplastic changes at the injection site. No data is, however, available on mutagenic and carcinogenic risks associated with pulmonary exposure to benzyl chloride.

C. Metabolism of Benzyl Chloride

Stekol (1939) studied the detoxification of benzyl chloride, benzaldehyde and S-benzyl homocystine in rabbit and rat, and reported that the major excretion product, following ingestion of benzyl chloride, was benzylmercapturic acid. In an earlier study, Stekol (1938) reported the detection of benzylmercapturic acid in dog urine, following feeding of benzyl chloride and S-benzyl cysteine; and in 1939, he successfully isolated benzylmercapturic acid from the urine of rats and rabbits that were given benzyl chloride subcutaneously. Also, Bray (1958) examined the fate of orally administered benzyl chloride (200 mg/kg bw) in rabbits. The ether-soluble acid fraction of rabbit urine showed major excretion product benzylmercapturic acid, which accounted for 49% of the administered dose. Knight and Young (1958) described the conversion of benzyl chloride directly into benzylmercapturic acid without the formation of premercapturic acid precursors. Barnes et al (1959) measured the amount and rate of formation of benzylmercapturic acid in rat urine, following the oral administration of benzyl chloride (158 micromoles/100 g bw). Twenty-seven percent of the administered dose was excreted as benzylmercapturic acid at 14.7 mg/kg hour and the excretion rate was found to be constant between hours 2 and 6, following administration of benzyl chloride. The excretion rate began to decline after the 6th hour.

Stekol (1938) observed that the thiol group of benzylmercapturic acid was attached to the side chain carbon and not to the ring carbon. His findings raised the question regarding the source of the thiol group in benzylmercapturic acid. Barnes et al (1959) suggested that the conjugation might have been with glutathione. They reported that there was a marked drop in the liver glutathione levels in rats that were given benzyl chloride, and this was roughly proportional to the amount of benzylmercapturic acid formed; and further concluded that the rate of glutathione turnover was adequate to account for the quantity of benzylmercapturic acid formed.

The work of Suga et al (1967) supported the above findings of Barnes et al. Suga and coworkers studied the distribution of enzyme catalyzing the conjugation of glutathione with benzyl chloride, in the different organs of the rat. The rat liver had the highest enzyme activity; kidney had 73%, spleen 13%, brain and heart 4% or less, compared with liver.

The findings of Bray et al (1969) further strengthened the importance of liver glutathione as the conjugating agent for benzyl chloride. The authors found a rapid drop in liver glutathione levels after the addition of 53 millimoles benzyl chloride/100 g of liver. They concluded that because the initial drop of glutathione was so rapid,

benzyl chloride, probably conjugated with glutathione rather than tissue proteins. Beck et al (1964) also reported a 50% drop in glutathione levels in 2 hours following intramuscular administration of benzyl chloride in mice.

RATIONALE

Benzyl chloride was chosen for the present inhalation toxicity studies because of its extensive use in industry and potential worker exposure to this chemical. Also, benzyl chloride was one of the chemicals disposed at Love Canal, Niagara Falls, New York by the Hooker Electrochemical Company. According to the company estimates, some 22,000 tons of chemical wastes were buried in Love Canal before the site was closed and sold to Niagara Falls School Board in 1953. Benzyl chloride accounted for 2400 tons of the total chemical wastes. In 1978, state and federal health officials, citing growing evidence of birth defects, miscarriages and liver malfunctions among the area residents, ordered more than 200 families in the canal area to evacuate. The site was declared a disaster area, paving the way for federal aid.

Again, from the industry, Mikhailova (1971) reported that production workers, exposed to benzyl chloride at 10 mg/cu m and above, showed abnormally high bilirubin, adnormally low leucocyte count and non-specific abnormalities in serum proteins. These findings were interpreted by the author as indicative of disturbances of liver function in workers exposed to benzyl chloride vapors.

With the Love Canal toxic disaster and industrial exposure in mind and because of the public interest in the possible health problems as a result of exposure to benzyl chloride vapors, the present investigation was undertaken to determine:

(a) the time course of absorption of benzyl chloride vapors from the lungs into the blood of rat;

(b) the distribution of benzyl chloride following absorption;

(c) the time course of benzylmercapturic acid, a metabolite of benzyl chloride in rat blood;

(d) the source of the thiol group in benzylmercapturic acid;

(e) the hepatotoxicity of benzyl chloride vapors, as revealed by light microscopy; and

(f) the peroxidative changes, if any, as the cause of hepatic injury.

The inhalation route of entry is the most important with respect to acute and chronic toxicity of solvents and vapors (Cornish, 1980). It is, therefore, important that data on toxicity by inhaled vapors become available. As no detailed information is available with respect to massive exposure to benzyl chloride and inhalation route of entry, the present study was undertaken. Also, an understanding of the mechanism by which benzyl chloride produces its toxic response needs to be developed.

That benzyl chloride is a reactive compound, that it can cause disturbances of liver function in humans inhaling it, that it is a direct alkylating agent are important background data on which the present acute toxicity study is based. Although acute benzyl chloride poisoning in humans has not been reported, such acute toxicological data are important to develop. Two kinds of acute toxicity data need to be assembled, via., short and long term effects of such exposure. The present study is limited to the short term effects of acute inhalation exposure to benzyl chloride vapors in male rats.

METHODS AND MATERIALS

A. Animals

Male Sprague Dawley rats (250-290 g) purchased from Taconic Farms, Germantown, New York were used for the acute inhalation study. The animals were housed four to five per cage and were given Purina rodent chow and tap water ad libitum. They were placed on a 12-hour light/dark cycle beginning at 6:00a.m. with a temperature ranging from 68-72^{o}F and relative humidity 50-55%.

B. The Exposure Chamber

The exposure chamber was a spherical unit made of clear acrylic, the overall height of which was 30" and inside diameter 23" (PlasLabs, Lansing, Michigan, XPL-801-MSA). The unit consisted of a white base and a spherical chamber mounted on it. The chamber comprised of two hemispheres with a lattice divider between them. The chamber was made airtight by tightening the clamps between the two hemispheres and a rubber gasket between them sealed off the chamber from the surrounding air. The unit was equipped with an inlet and an outlet, and a monitoring port in the animal breathing zone. The monitoring port was fitted with a rubber stopper and sealed.

The needle of a 1.0 ml syringe was inserted through the stopper and the chamber air was sampled every ½ hour over the test period of 6 hours. The inlet was connected to the tubing conveying vapors of benzyl chloride via air into the test chamber.

The exiting chamber air was first passed through charcoal to absorb the vapors of benzyl chloride and then mixed with the exhaust air. The airflow to the chamber was regulated by means of a flowmeter (LabCrest Scientific, Century Flowmeter Model 100H). Intake air was filtered by charcoal filters prior to entering the chamber. Vapors of benzyl chloride were generated by bubbling clean filtered and regulated volumes of air through liquid benzyl chloride (5 ml) under cold water (50 ml) placed at the bottom of a glass cylinder. The outflowing air carrying vapors of benzyl chloride from the cylinder was let into the chamber by a tubing connected to the inlet. The portable exposure chamber was placed in the laboratory fume hood and all exposures were conducted inside the hood.

C. Time Course Studies

(a) Surgical Procedures:

The ipsilateral carotid artery was cannulated as described by Renwick (1984). The rats were anesthetized with pentobarbital and an incision made to the left of the mid-

ventral line, anterior to the limbs. The subcutaneous tissue was removed by blunt dissection and the common carotid artery lying next to the trachea was exposed. The artery was separated from the parallel nerve tract and brought to the surface by curved forceps. Two cotton ties were placed anterior (1) and posterior (2) to the intended site of incision and tied loosely. The artery was clamped close to the posterior tie near the heart to prevent blood loss during incision. A small incision was made in the artery and a length of cannula tubing, attached to a heparinized saline filled syringe was inserted and passed toward the heart, through the posterior tie. This was tied firmly and the clamp removed. The cannula was then pushed back into the heart and the posterior tie was further tightened. The free end of the cannula was pushed through the skin on the back of the head and held in position by a cotton tie. A needle fitted with a three-way valve was inserted into the cannula. A 3 ml syringe filled with heparinized saline and a 1 ml syringe for blood sampling were attached to the valve. With the valve open to the saline filled syringe, blood was drawn through the cannula till a drop was seen mixing with the saline. The valve was then closed to the saline syringe and opened to the sampling syringe for collecting blood. After sampling, the valve was opened to the saline syringe

and heparinized saline was passed back up the 36" cannula. The valve was then moved such that it closed both the saline and sampling syringes and held the saline in the tubing. Care was taken not to push the heparinized saline into the animal's heart.

(b) Sampling:

Blood samples were obtained at 2,5,10,20,30,60 min and 2,3,4,5 and 6 hours from the beginning of exposure. After the termination of exposure, blood samples were taken every hour for the next four hours (post exposure).

(c) Chamber Benzyl Chloride Concentration:

The chamber operation was a dynamic operation and the chamber atmosphere was continuously being generated in a flow through manner. An equilibrium was set up between material being generated and dilution airflow to yield a steadily flowing constant concentration atmosphere, which was then passed into the exposure chamber. The concentration was maintained at 220$\pm$ 20 ppm over the six-hour exposure period. The flow rate was set at 1.5 L/min. The volume of the chamber was 30 liters and the chamber air was renewed once in 20 min. The chamber benzyl chloride concentration was monitored at regular intervals of 30 min by withdrawing 1 ml chamber air and injecting it into a gas chromatograph. The gas chromatography conditions were:

Varian gas chromatograph 3700, injector 260°C, electron capture detector 290°C, and tenax column 190°C, nitrogen flow 30 ml/min. Standard benzyl chloride for gas chromatographic determination was prepared as follows: 1 ul benzyl chloride was placed in each of the 0.5, 1.0, 2.0 liter volumetric flasks and were quickly sealed. Benzyl chloride was allowed to vaporize in the flask at room temperature. After equilibration, 1.0 ml samples were taken from each container and injected into the gas chromatograph under conditions indicated above. A standard curve was prepared by plotting peak height versus concentration (PPM). The concentration of benzyl chloride in the chamber air sample was determined from the graph.

Calculation:

$5\ mg/m^3 = 1\ ppm$

$5\ mg/L = 1000\ ppm$

1 ul benzyl chloride = 1.1 mg

$1.1\ mg/L = 220\ ppm$

(d) Blood Benzyl Chloride Concentration:

Benzyl chloride concentration in the blood of rat was determined as follows:

0.5 ml blood was mixed with 2.5 ul o-chlorotoluene, the internal standard and 0.5 ml extracting solvent, pentane. The mixture was shaken for 10 minutes and centrifuged at 2800 g for 10 minutes. Two ul of the pentane extract was injected into the gas chromatograph, under

conditions specified above. A standard curve was prepared as follows: rat blood samples spiked with benzyl chloride to obtain a concentration of 5,10 and 20 ug/ml were extracted and injected into the gas chromatograph. A standard curve for benzyl chloride concentration and peak height ratios was constructed and used to determine benzyl chloride concentration in test blood samples.

Six cannulated animals were allowed to recover completely from pentobarbital effect before they were exposed to benzyl chloride vapors. The animals had regained their normal activity and were moving freely in the chamber. The cannula from each animal was passed through an opening at the top of the exposure chamber and held by adhesive tape to the outer wall of the inhalation chamber. The free end of the cannula was fitted with a three-way valve described above. This arrangement facilitated easy blood sampling from live animals at desired intervals of time. Also, the clear acrylic top of the exposure chamber permitted observation on the behavior of the experimental rats.

D. Distribution of Benzyl Chloride

A group of 3 rats that had been exposed to benzyl chloride (220 ± 20ppm) for six hours were sacrificed by decapitation, and the following organs were removed and weighed:

the brain, lungs, liver, pancreas, fat, testis and kidneys. Next, the tissues were homogenized, in cold, with a Potter Elvehjem homogenizer, using a teflon pestle. Three volumes of ice-cold 0.25 M sucrose buffer were used to prepare the homogenates.

The homogenates were spiked with the internal standard (5 ul/ml) and extracted with an equal volume of pentane. The mixture was shaken for 10 minutes and centrifuged at 2800 g for 10 minutes in a Beckman J-6B centrifuge with a JS-3.0 rotor. 2 ul of the pentane extract was analyzed for benzyl chloride by gas chromatography as indicated before.

E. Benzylmercapturic Acid Determination

Three rats were cannulated as described above and exposed to benzyl chloride vapors (220 ± 20 ppm). Blood samples (0.5 ml) were taken every hour during the exposure. After the termination of exposure, blood samples were taken at 30 min and every hour for the next four hours, for the determination of benzylmercapturic acid by High Performance Liquid Chromatography (HPLC). Benzylmercapturic acid was synthesized as follows:

A solution of L-cysteine hydrochloride (8g) in 2N-NaOH(50 ml) and ethylene glycol monomethyl ether (10 ml) was treated with freshly distilled benzyl chloride (8 ml) and the resulting solution was stirred at room temperature for 24 hours. The reaction mixture gradually turned cloudy

and a white precipitate was formed. The product was collected by filtration and washed with ether. For analytical purposes, a small sample was recrystallized from 50% aqueous ethanol, M.P. 218-220°C (Lit 221°C)(Bray,James and Thorpe, 1958).

N-Acetyl S-Benzyl L-Cysteine

To a solution of benzyl cysteine (2g) in 10 ml of IN-sodium hydroxide at 0°C (ice bath) was added 2.5 ml of acetic anhydride with vigorous stirring; followed by 10 ml of IN-NaoH solution and 3 ml of acetic anhydride. The solution made acidic by the addition of sulfuric acid (pH 4) became turbid. After standing for 24 hours in the refrigerator, the white crystals which had separated were collected by filtration and recrystallized from aqueous ethanol. M.P.: 145-147°C (Lit 146°C)(Zbarsky and Young, 1943).

A HPLC procedure was developed for the determination of benzylmercapturic acid, a metabolite of benzyl chloride in rat blood.

Reagents:

1. 0.1 N HCl
2. Methylene Chloride
3. Acetonitrile
4. 1.0 M Phosphoric Acid
5. Benzylmercapturic Acid 50 mg% in Methanol
6. N-Acetyl S-Cyclohexyl L-Cysteine as internal standard 200 mg% in Methanol
7. Benzyl Chloride 100 ug/ml Mobile Phase

The HPLC Conditions were:

Beckman HPLC, Ultrasphere ODS 5 um 4.0 mm x 150 mm Column; the mobile phase was acetonitrile and 1 M Phosphoric

acid in the ratio of 1:1 v/v, flow rate 1 ml/min; the column was at ambient temperature, under a pressure of 2000 psi. The UV detector was set at 210 nm.

Assay Procedure:

1. To 0.5 ml of rat blood, 0.2 ml of 0.1 N HCl was added followed by 10 ul of the internal standard and 3.0 ml of methylene chloride.

2. The mixture was shaken for 10 minutes and centrifuged in a Beckman J-6B centrifuge with a JS-3.0 rotor for 10 minutes at 2800 g.

3. The methylene chloride layer was pipetted out into a fresh tube and evaporated to dryness in a water bath held at 50^{o}C.

4. The residue was reconstituted in 0.5 ml mobile phase and 15 ul injected into HPLC.

Preliminary experiments were conducted with pure compounds to determine the pattern of separation of benzylmercapturic acid, benzyl chloride, and the internal standard.

A standard curve was prepared as follows:

Rat blood samples were spiked with benzylmercapturic acid to give a concentration of 5,10,15 ug/ml; ten ul internal standard were then added to each sample. The samples were extracted with 3.0 ml methylene chloride as mentioned before and evaporated to dryness. The residue was reconstituted in 0.5 ml mobile phase and 10-15 ul were injected into HPLC.

The peak height ratios were plotted against benzylmercapturic acid concentration to obtain the standard curve. Benzylmercapturic acid concentration in unknown samples was determined from the graph.

F. Glutathione Determination

Glutathione concentration in whole blood was determined according to the method of Beutler et al (1963).

Reagents:

1. Phosphate solution 0.3 M Na_2HPO_4
2. Precipitating solution
   1.67 g glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml distilled water
3. DTNB Reagent
   40 mg 5,5' dithiobis - 2 - nitrobenzoic acid in 100 ml 1% sodium citrate

Procedure:

1. 0.2 ml blood was mixed with 1.0 ml distilled water
2. 3 ml precipitating solution was added to the hemolysate and vortexed
3. The mixture was centrifuged at 1000 g for 10 minutes at room temperature
4. 1.0 ml of the supernatant was added to 4.0 ml phosphate solution
5. 0.5 ml DTNB reagent was added and the absorption at 412 nm was measured in a Beckman DU-8 spectrophotometer.

Glutathione concentration in liver, kidney, lung and eye ball tisses was determined as follows:

The organs were removed from the experimental and control animals and sliced. A small piece of the tissue was weighed and homogenized in 2.0 ml ice cold 0.25 M sucrose,

using a Potter Elvehjem Homogenizer and a teflon pestle. 0.5 ml of the homogenate was mixed with 1.5 ml distilled water and assayed for a glutathione as described above. A standard curve was prepared with absolute ug amounts of glutathione and their absorbance values at 412 nm. Tissue glutathione concentration was calculated from the graph and the values expressed as ug/g tissue. The values were statistically analyzed by Student's t test with $p < 0.5$ as the level of significance.

G. Lipoperoxide Determination

A fluorometric assay described by Yagi (1976) was used to determine the lipoperoxide concentration in plasma separated from 0.1 ml rat blood.

Reagents:

1. 0.9% NaCl solution
2. N/12 H_2SO_4
3. 10% phosphotungstic acid
4. Thiobarbituric acid reagent (TBA)
   0.67% TBA aqueous solution and glacial acetic acid 1:1 v/v. TBA was insoluble in water and dissolved in 5% NaOH.
5. Malonaldehyde stock solution
   1 mM/L (24 ul in 100 ml distilled water)
6. Malonaldehyde working solution
   1 ml stock solution was diluted x 1000 with distilled water to a final concentration of 1 nm/ml (Nanomole).

Lipoperoxide studies were conducted simultaneously with time course studies described in section C. 0.1 ml blood samples were taken from 5 animals at 2,5,10,20,30,60,

120,240,300 and 360 minutes from the beginning of exposure. After the termination of exposure, blood samples were taken every hour for the next four hours.

Procedure:

1. 0.1 ml blood was mixed with 1.0 ml 0.9% NaCl solution and shaken gently.
2. The mixture was centrifuged at 5000 g for 10 minutes.
3. 0.5 ml of the supernatant was transferred to a fresh tube and mixed with 4.0 ml N/12 H_2SO_4.
4. Next 0.5 ml 10% phosphotungstic acid was added and mixed.
5. After standing at room temperature for 5 minutes, the mixture was centrifuged at 5000 g for 10 min.
6. The supernatant was discarded and the sediment was mixed with 2.0 ml N/12 H_2SO_4 and 0.3 ml 10% phosphotungstic acid and again centrifuged at 5000 g for 10 minutes.
7. The sediment was suspended in 4.0 ml distilled water and 1.0 ml TBA reagent was added.
8. The reaction mixture was heated at 95°C for 60 minutes in a water bath.
9. After cooling with tap water, 5.0 ml n-butanol were added and the mixture shaken vigorously.
10. After centrifugation at 5000 g for 10 minutes, the butanol layer was taken for fluorometric measurement at 515 nm excitation and 553 nm emission in an Aminco Bowman Spectrofluorometer.
11. One nanomole of the working solution was assayed as before and its fluroescence intensity set at 70 to obtain a standard curve.
12. A control containing 0.5 nanomole was assayed along with the samples.

H. Hepatic Lipid Peroxidation in vivo

For studies on hepatic lipid peroxidation in vivo, livers from three control and three experimental rats

exposed to 220 ppm benzyl chloride for 6 hours were quickly excised, under ether anesthesia. The liver pieces were weighed, coarsely sliced and immersed in ice cold solution of 0.15 N KCl, .003 M EDTA, pH 7.4.

About 1 g liver was used to make a 5% (w/v) whole liver homogenate. All subsequent steps were carried out in the cold. The tissues were homogenized with a Potter Elvehjem homogenizer using a teflon pestle.

The homogenate was centrifuged at 5000 g for 10 minutes (0-4^{o}C) in a Beckman J-6B centrifuge with a JS-3.0 rotor. The post mitochondrial supernatant fraction was removed and spun at 100,000 g for 60 minutes in a Beckman L5-65 ultracentrifuge with a type 30 rotor to obtain the microsomal fraction.

The sedimented microsmal fractions from treated and control rats were assayed for the presence of lipid conjugated dienes, according to the method of Recknagel and Ghoshal (1956). The lipids were extracted from the microsomal pellets into a chloroform/methanol mixture (2:1 v/v) according to the method described by Folch et al (1957). The chloroform layer was evaporated to dryness under nitrogen. The lipid residues were weighed and dissolved in an appropriate volume of cyclohexane to give a final concentration of 1 mg/ml cyclohexane. The absorbance from 220 to 275 nm was recorded against a cyclohexane blank in a Beckman dual beam spectrophotometer.

Absorbence values at 230 nm per mg lipid were recorded. Estimation of lipid peroxidation depended on the determination of the mean difference in absorbence at 230 nm per mg lipid of the treated animals compared with the controls.

I. Glucose-6-Phosphatase Determination

Glucose-6-phosphatase activity was determined by the method of Harper (1965).

Reagents:

1. Citrate buffer 0.1 M, pH 6.5
2. Glucose-6-phosphate 0.08 M
3. Trichloroacetic acid 10% v/v
4. Ammonium molybdate $2X10^{-3}M$
5. Reducing agent $4.2X10^{-2}M$
   1-amino-2naphthol-4sulfonic acid; 0.56 M SO_3^{-2}
6. Phosphate standard solution $5X10^{-4}M$

Procedure:

1. Liver was chilled in an ice bath immediately after removal from the animal and 250 mg tissue was homogenized, as before, with 9.75 ml citrate buffer and then filtered; the homogenate contained 2.5 mg tissue/0.1 ml.

2. 0.1 ml aliquots of the homogenate were pipetted into tubes marked experimental and control (1). Control (2) received 0.1 ml citrate buffer solution.

3. The tubes were placed in a water bath at $37^{o}C$ and after 5 minutes, the following additions were completed: 0.1 ml aliquots of glucose-6-phosphate solution to the experimental and control (2) tubes and 0.1 ml buffer to control tube (1).

4. The time of addition was noted and after incubation at $37^{o}C$ for exactly 15 minutes, 2 ml trichloroacetic acid were added to each tube. The mixture was centrifuged at 2800 g for 10 minutes and the clear supernatant used for phosphate determination.

The phosphate content of the supernatant was determined colorimeterically at 660 mu by the method of Fiske and Subba Row (1925).

Procedure:

1. 5 ml aliquots of molybdate solution were added to tubes containing 1 ml supernatant from the experimental and control tubes.
2. A standard was prepared by mixing 5 ml molybdate solution with 1 ml phosphate standard solution.
3. When all tubes were prepared, 1 ml reducing agent was added to each tube and time noted.
4. The tubes were allowed to stand for 15 minutes and the optical density was read at 660 mu in a Beckman DU 8 spectrophotometer.
5. The instrument was zeroed with the tube prepared from control (2).

Calculation:

$$\frac{E_E - E_{C_1}}{E_S} \times (P) \times 2.2 = \text{umoles phosphate liberated in the enzymatic reaction}$$

where

E_E = O.D. of the experimental tube

E_{C_1} = O.D. of the control (1)

E_S = O.D. of the standard tube

(P) = umoles of phosphate in the standard (0.5 umoles)

2.2 = volume of the reaction mixture after the addition of trichloroacetic acid

To convert to umoles phosphate/min/g/tissue, multiply by 1000/15 x 2.5.

where

15 = period of enzymatic reaction
2.5 = mg tissue in the reaction tube
1000 = to convert mg to g

J. Light Microscopy

Liver, kidney, heart, lung and brain tissues were removed from treated and control rats and preserved in buffered formalin (10%). The tissues from treated rats were obtained 2, 21, 44, 66 and 88 hour post exposure. They were dehydrated with graded series of ethyl alcohol and embedded in paraffin. Six um sections were prepared and stained with hematoxylin and eosin for histological study.

RESULTS

During the entire period of study, the concentration of benzyl chloride in the exposure chamber was maintained at 220 $\pm$ 20 ppm. Gas chromatographic analysis was used to monitor the benzyl chloride concentration in the chamber.

A feature of this study was the generation of time course data for benzyl chloride in rat blood, following acute inhalation exposure (Table 1). The concentrations of benzyl chloride after 2 and 4 hours of exposure were 4.68 and 9.50 ug/ml, respectively, and a maximum concentration of 10.01 ug/ml was reached by the fifth hour of exposure. During inhalation, the blood benzyl chloride level increased to reach a steady state concentration between 4 and 6 hours of exposure. One, 2 and 4 hours after the cessation of exposure, the concentration of benzyl chloride in blood decreased to 4.74, 2.42 and 0.86 ug/ml, respectively.

The data presented in Table 1 was analyzed by the method of residuals to calculate the absorption rate constant for a one-compartment system (Gibaldi and Perrier, 1975). Based on this model, $t_{\frac{1}{2}}$ absorption was 0.53 hour (0.39 - 0.81) corresponding to a rate constant of 1.301 $hour^{-1}$ and $t_{\frac{1}{2}}$ elimination was 1.12 hour (0.93-1.40) corresponding to a rate constant of 0.6199 $hour^{-1}$.

TABLE 1

TIME COURSE OF BENZYL CHLORIDE IN RAT BLOOD FOLLOWING ACUTE INHALATION EXPOSURE

Time (hr)	Concentration of Benzyl Chloride ug/ml
a. Exposure	
1	1.10 ± 0.10
2	4.68 ± 0.86
3	6.27 ± 1.37
4	9.50 ± 2.02
5	10.01 ± 1.65
6	9.13 ± 1.97
b. Post Exposure	
1	4.74 ± 0.55
2	2.42 ± 0.22
3	2.21 ± 0.42
4	0.86 ± 0.40

Values represent the mean and SD from five rats. Based on one compartment model, $t_{\frac{1}{2}}$ absorption was 0.53-hour corresponding to a rate constant of 1.301 hour and $t_{\frac{1}{2}}$ elimination was 1.12-hour corresponding to a rate constant of 0.6199 hour.

The distribution of benzyl chloride in rat following 6 hours of acute exposure to 220 ppm is given in Table 2. The concentration of the chemical in the blood was determined and found to be 9.13 ug/ml. The adipose tissue in the region of the kidney was found to contain 0.22 ug/g. Benzyl chloride was not detected in the other organs such as brain, lung, liver, pancreas and testis. Apparently benzyl chloride seems to persist and remain in the blood. Being lipid soluble, the chemical enters the adipose tissue and is stored in fat.

One of the major excretion products following inhalation of benzyl chloride is benzylmercapturic acid (a cysteine conjugate). The results reported in Table 3 show the time course of benzylmercapturic acid in rat blood following acute inhalation of benzyl chloride. As early as 1 hour after exposure, benzylmercapturic acid was detected in rat blood at a concentration of 1.01 ug/ml. The concentration of this conjugate doubled to 2.25 ug/ml by the fourth hour of exposure. The maximum concentration of 2.58 ug/ml was reached 30 minutes after the termination of exposure. The concentration of benzylmercapturic acid started to decline after 2 hours from post exposure and reached 0.28 ug/ml by 4 hour post exposure.

Benzylmercapturic acid in rat blood was extracted and separated by High Performance Liquid Chromatography (HPLC). Figure 1 shows the separation of benzylmercapturic acid and N-acetyl S-cyclohexyl L-cysteine, the internal standard.

TABLE 2

DISTRIBUTION OF BENZYL CHLORIDE IN RAT FOLLOWING ACUTE EXPOSURE

Tissue	Concentration of Benzyl Chloride ug/ml or ug/g
Whole blood	9.13 ± 1.97
Fat	0.22 ± 0.01
Brain	ND
Lung	ND
Liver	ND
Pancreas	ND
Kidney	ND
Testis	ND

ND = none detected

Values represent mean and SD from 4 rats exposed to 220 ppm for 6 hours.

TABLE 3

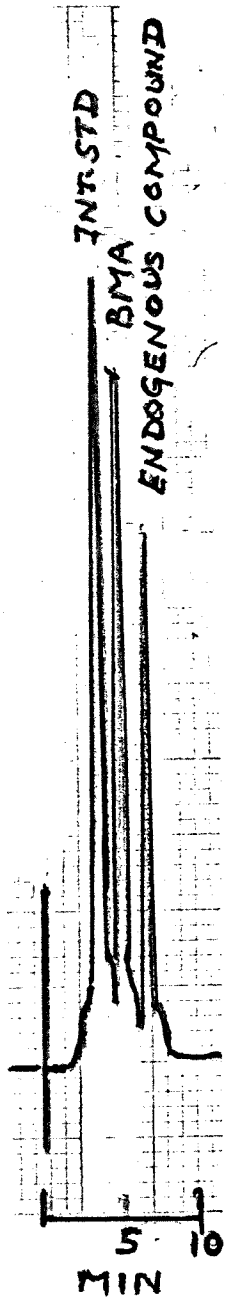
TIME COURSE OF BENZYLMERCAPTURIC ACID IN RAT BLOOD FOLLOWING ACUTE EXPOSURE TO BENZYL CHLORIDE

Time (hr)	Concentration of Benzylmercapturic Acid (ug / ml)
a. Exposure	
1	1.01 ± 0.29
2	1.52 ± 0.24
3	1.70 ± 0.82
4	2.25 ± 0.25
b. Post Exposure	
0.5	2.58 ± 0.62
1	2.37 ± 0.41
2	0.89 ± 0.32
3	0.62 ± 0.06
4	0.28 ± 0.09

Values represent mean and SD from 3 rats exposed to 220 ppm.

FIGURE 1

DETERMINATION OF BENZYLMERCAPTURIC ACID (BMA) IN RAT BLOOD



HPLC record of blood sample using an ultrasphere ODS column and UV detection at 210 nm. The assay is sensitive to 0.1 ug BMA. N-acetyl S-cyclohexyl L-cysteine, the internal standard eluted at 3 minutes and BMA at 5 minutes.

The retention time for benzylmercapturic acid was 5 min; the internal standard eluted at 3 minutes. The detection limit for benzylmercapturic acid was 0.1 ug; the extraction efficiency for this compound was 70%. Figure 2 depicts the standard curve for benzylmercapturic acid and it was found to be linear from 0.1 to 15.0 ug/ml.

The source of the thiol group in benzylmercapturic acid was investigated by measuring the levels of glutathione (GSH) in liver, kidneys, lungs, eyeball and blood (Table 4). The GSH level in the liver was significantly lower in the rats exposed to benzyl chloride compared with controls ($p < 0.5$). GSH values were 65.81 and 488.2 ug/g for the benzyl chloride dosed and control groups respectively.

The GSH concentration in the kidney of treated animals decreased to 111.5 ug/g compared with the control value of 239.1 ug/g and the differences were statistically significant at $p < .05$ level. No significant changes were observed in the lung and the eyeball of the treated group compared to the control. When blood GSH was measured in the treated group, no significant change was noted, compared to the control (38 versus 37 mg%).

FIGURE 2

THE STANDARD CURVE FOR
BENZYLMERCAPTURIC ACID

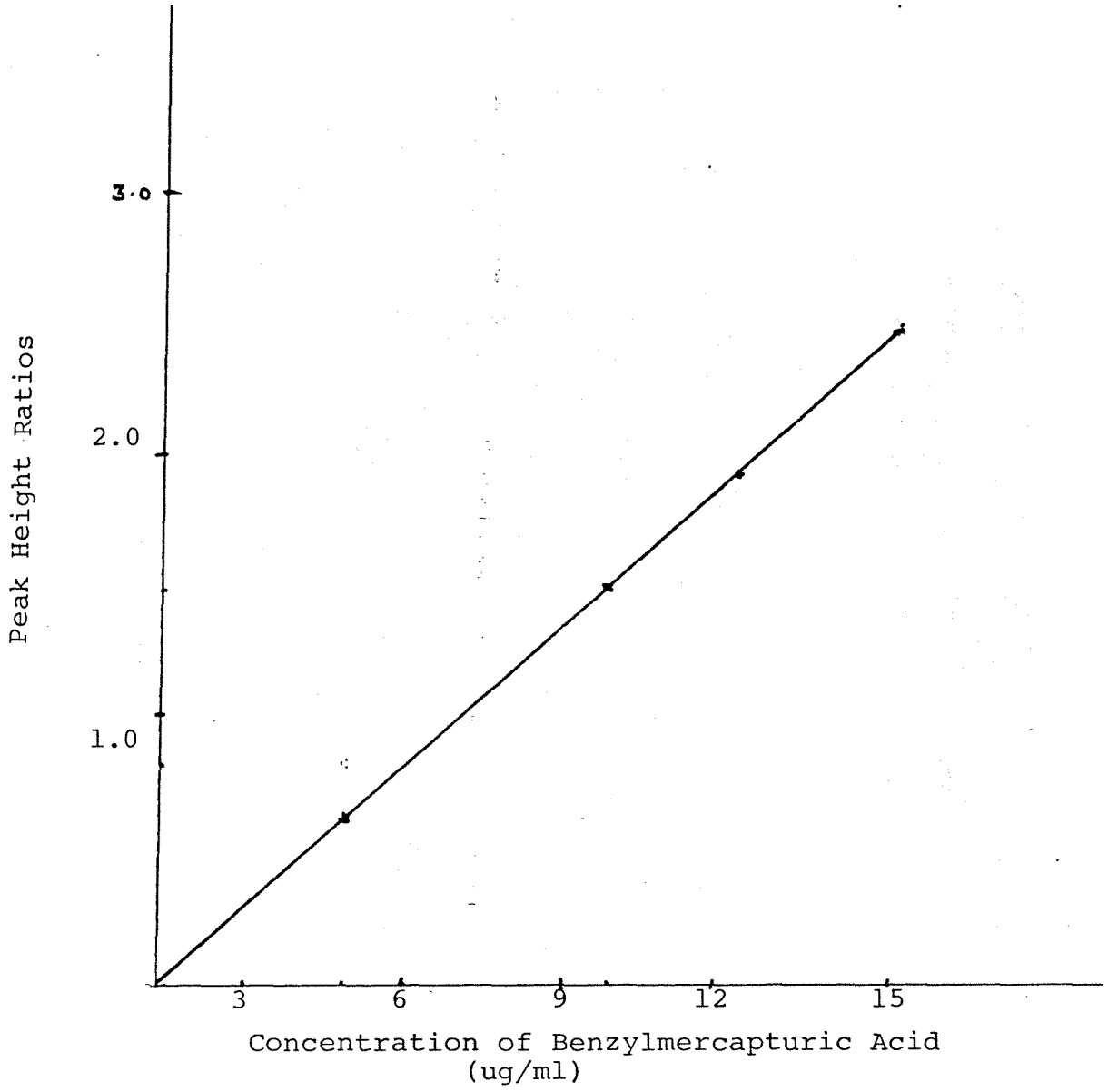


TABLE 4

EFFECT OF BENZYL CHLORIDE ON GLUTATHIONE IN RAT ORGANS[a]

Tissue	Control (ug/g)	Benzyl Chloride Treatment (ug/g)
Liver	488.2 ± 67.2	65.81 ± 9.5[b]
Kidney	239.1 ± 61.5	111.5 ± 16.4[b]
Lung	116.7 ± 32.4	76.8 ± 11.5
Eyeball	129.9 ± 12.1	130.6 ± 12.6

[a]Values represent mean and SD from 4 rats exposed to 220 ppm for 6 hours

[b]Differences are significant ($p < 0.5$) by a paired t-test.

No lipid peroxides were detected in rat blood either during the 6 hour exposure period or 4 hours after the termination of exposure. When the hepatic microsomal lipid changes at 230 nm were measured in the treated group, no significant difference was noted, compared to the control (Table 5).

TABLE 5

EFFECT OF BENZYL CHLORIDE ON
HEPATIC MICROSOMAL LIPID CHANGES

	Control	Benzyl Chloride Treatment
Absorbance at 230 nm	0.29 ± .007	0.30 ± .007

Values represent mean and ± SD from 3 rats exposed to 220 ppm benzyl chloride for 6 hours.

Table 6 includes data on hepatic glucose-6-phosphatase activity in control and benzyl chloride exposed groups. No loss in glucose-6-phosphatase activity was detected between these two groups, either after 6 hours exposure or at 24 hour post exposure time. The differences of 0.5 and 0.1 umoles/min/g observed at the times indicated below were not statistically significant.

TABLE 6

GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE RAT LIVER EXPOSED TO BENZYL CHLORIDE

Time (hr)	Control	Benzyl Chloride Treatment
a. Exposure		
0	9.0 ± 0.4	-
6	8.8 ± 0.4	8.3 ± 0.3
b. Post Exposure		
24	8.3 ± 0.4	8.2 ± 0.3

Values represent the mean and SD as umoles/min/g liver of glucose-6-phosphatase, from 3-4 animals exposed to 220 ppm benzyl chloride.

Histologic effects of exposure to vapors of benzyl chloride are listed in Table 7. Six um sections stained with hematoxylin and eosin (H&E) were examined by light microscopy. The organs examined were liver, lungs, brain, heart and testis. Figure 3 illustrates the effects of benzyl chloride on liver cells. The control section (A) showed cells with distinct nuclei and clear cytoplasm, indicative of glycogen. Section B showed evidence for the production of microvesicular steatosis by the experimental toxin. In this form of fatty liver, the liver cells are filled with many tiny droplets of fat that do not displace the nucleus. Section C showed central focal inflammation, accumulation of cells and glycogen depletion. The three effects, viz., microvesicular fatty deposit, focal inflammation and gylcogen depletion indicate that benzyl chloride vapors are hepatotoxic. The lungs showed congestion and peribronchiolar inflammation. Brain, heart and testis were normal in appearance, like the controls.

The behavior of the rats exposed to vapors of benzyl chloride (220 ppm) was observed during the test period of 6 hours. The immediate responses were eyeblinking, eyelid closing and tearing, indicative of severe irritation. The rats became less active and remained quiescent. They spent less time on grooming and as the exposure progressed, no grooming activity was seen. The rats progressed to inactivity and slowed respiration.

TABLE 7

HISTOLOGICAL CHANGES IN THE RAT LIVER AND LUNG AFTER BENZYL CHLORIDE EXPOSURE

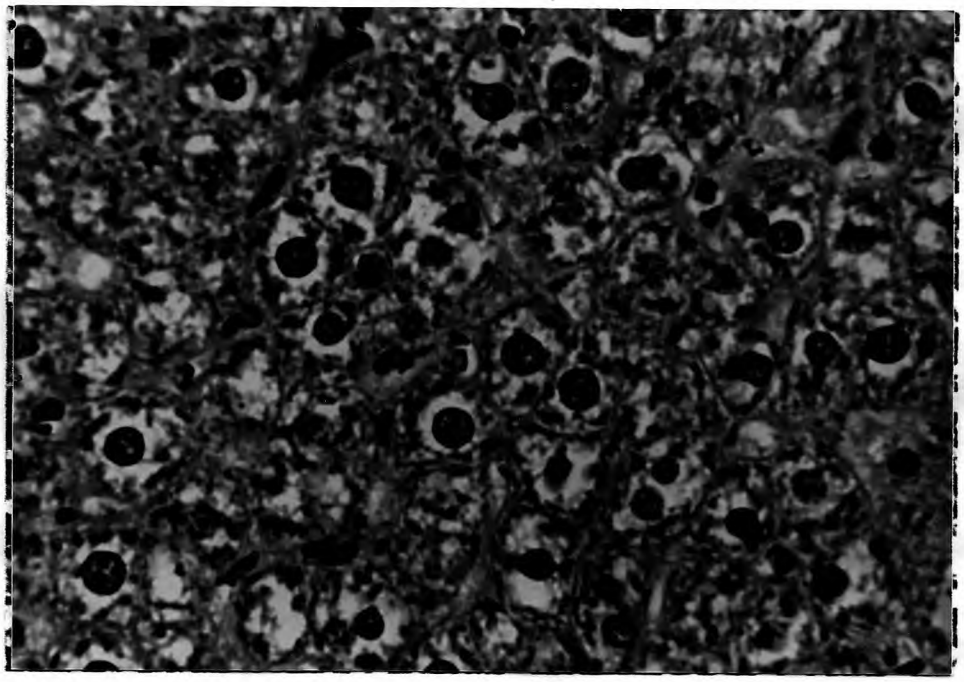
Time Post Exposure (hr)	Organs[a]	
	Liver	Lung
2	Severe congestion Diffuse micro-vesicular Fatty change	Congested bron-chioles Peribronchiolar inflammation
21	Microvesicular fatty change	Interstitial bronchial and vascular in-flammation
44	Microvesicular fatty change focal inflam-mation and glycogen depletion	Peribronchiolar Acute inflam-mation
66	Glycogen depletion Microvesicular fatty change (minimal)	Bronchiolar inflammation
88	Microvesicular fatty change (minimal)	Bronchiolar inflammation

[a]Brain, heart and testis histology was normal, like the controls.

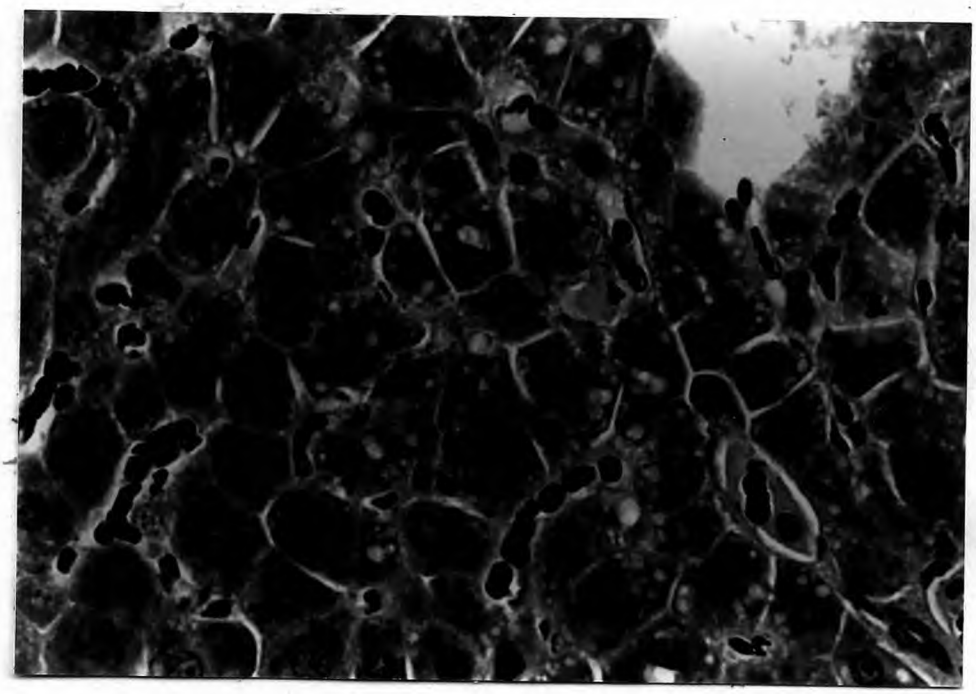
FIGURE 3

HISTOLOGY OF THE LIVER REMOVED FROM RATS
EXPOSED TO 220 PPM BENZYL CHLORIDE FOR 6 HOUR

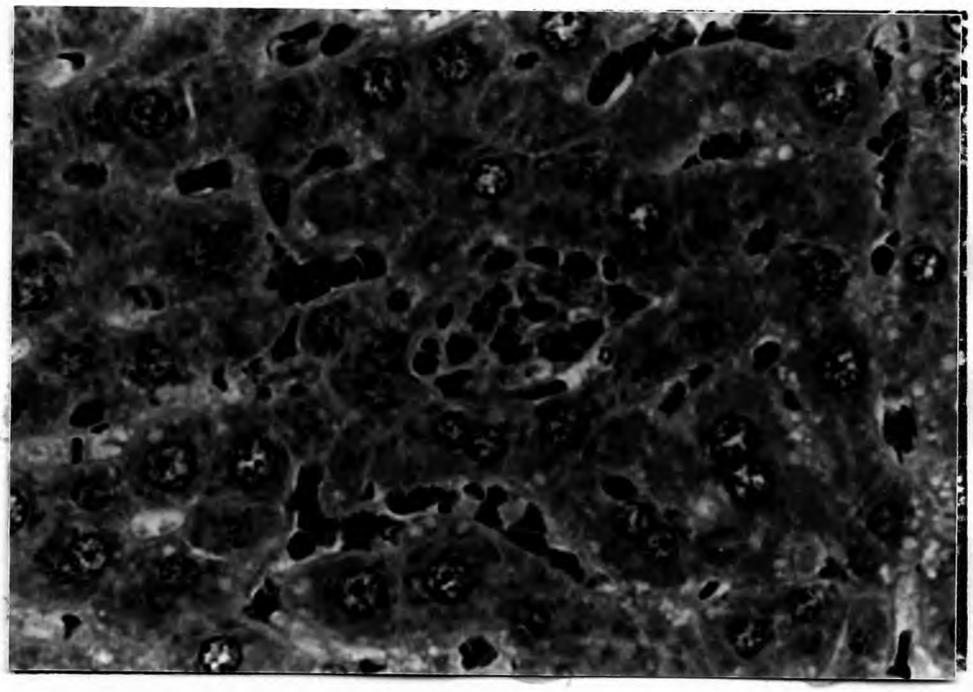
A. Control showing liver cells with clear cytoplasm, indicative of glycogen H&E x 480



B. Showing cells from liver removed 2 hours after the termination of exposure. The hepatcytes show steatosis of the microvesicular type H&E x 480



C. From liver obtained 66 hours after the termination of exposure. Cells show central focal inflammation H&E x 480



A marked hyperemia of the ears, paws and the tail of the animals was observed throughout the exposure period. These signs disappeared, when the animals were removed from the test chamber after 6 hours and allowed to recover.

Apparently, benzyl chloride vapor seem to exert a narcotic effect, as indicated by the animal's passive behavior.

DISCUSSION

Estimation of the dose of the toxic agent administered is most difficult and least accurate for inhalation studies (Kennedy and Trochimowicz,1982). In the present study, the rats were exposed to benzyl chloride vapors at a concentration of 220 ppm for 6 hours. However, this concentration was not given as a discrete measured dose of benzyl chloride. Thus, the actual dose the animals received was related to the exposure chamber benzyl chloride concentration, the animals' respiratory physiology and the duration of exposure. Jemski and Phillips (1965) used the following formula to calculate the inhaled dose.

$$\text{Inhaled Dose} = \text{concentration of the chemical per liter} \times \text{breathing rate} \times \text{exposure duration}$$

The authors calculated the breathing rate, using Guyton's formula:

$$\text{Breathing Rate (ml / min)} = 2.10 \times (\text{body wt in grams})^{3/4}$$

This approach was found adequate for animals up to 3-4 kg in weight. The inhaled dose of benzyl chloride was estimated from the above formula and found to be 8.7, 17.4, 34.8 and 52.2 mg at 1,2,4 and 6 hour exposure respectively. In the absence of data on the actual breathing rates during exposure, the dose estimates derived by the above formula

may not provide accurate estimation of the dose inhaled. Notwithstanding this limitation, a comparison of the estimated inhaled doses indicated above, with blood benzyl chloride concentrations of 1.10, 4.68, 9.50 and 9.13 ug/ml at 1,2,4 and 6 hours exposure respectively, shows that benzyl chloride vapors were retained partially in the blood. The remaining fraction was eliminated by exhalation.

Benzyl chloride is distributed largely in blood at a concentration of 9.13 ug/ml. It is also distributed into the slowly perfused adipose tissue at a concentration of 0.22 ug/g. It has been said that the extent to which drugs and chemicals leave the blood and enter the tissues depends on their relative affinities for each tissue. Thus, compounds highly bound to plasma protein but not to tissue will show a relatively high concentration in the plasma, while chemicals with a high affinity for tissue components will have a low plasma concentration. Benzyl chloride appears to have greater affinity for blood components. Benzyl chloride was not detected in the other organs, such as liver, lung, kidney, brain, pancreas and testis, perhaps because the concentration in these organs was below the detection limit of the method used (10 ng).

Benzyl chloride is lipophylic and gets distributed in the adipose tissue, as has been shown for a number of chemicals such as DDT, polychlorinated biphenyls and polybrominated

biphenyls. Toxicants appear to be stored in fat by simple physical dissolution in neutral fat (Klaassen, 1980). The solubility of benzyl chloride in corn oil, rich in neutral fat points to such a possibility of its dissolving in triglycerides in adipose tissue. However, only 2.2% of blood benzyl chloride gets distributed into the fat and most of it persists in blood.

Benzyl chloride is a highly reactive compound that can alkylate in vivo, as confirmed by its rapid conjugation with glutathione in many animal species (Barnes et al, 1959, Suga et al, 1966,1967). Conjugation essentially terminates the alkylating capability of benzyl chloride and in mammals leads to the excretion of the compound, benzylmercapturic acid (Stekol, 1938,1939, Whitter, 1944). Inspection of the data on the time course presented in Tables 1 and 3 indicates that in the first hour of exposure, an amount equal to the parent compound in blood was metabolized into benzylmercapturic acid. By the fourth hour of exposure, about 20% of the concentration of the parent compound detected in blood has been changed to benzylmercapturic acid. Four hours following the termination of exposure, the concentration of benzylmercapturic acid was 25% of the parent compound detected in blood at the same period.

It is relevant to point out that well-fed rats were used in experiments conducted to assess the detoxifying role of glutathione. Furthermore, all exposures in this and other

experiments were conducted between 8:00 a.m. and 2:00 p.m. These experimental conditions were observed in view of the report that hepatic glutathione concentrations have a diurnal rhythm in fed rats with maximum glutathione concentration occurring between 7:00 a.m. and 1:00 p.m., and minimum concentration between 7:00 p.m. and 1:00 p.m. Fasted animals had a decreased concentration of glutathione and a diurnal rhythm, greatly reduced in magnitude (Jaeger et al, 1973).

Glutathione levels in the liver were significantly lower in the rats exposed to benzyl chloride vapors than in the control rats. The hepatic glutathione levels in benzyl chloride dosed rats were depleted to 13.5% of the control values over the 6 hour exposure period. There is considerable evidence in literature implicating liver glutathione as the source of the thiol group for mercapturic acid formation. Barnes et al (1959) noted a marked drop in liver glutathione levels of rats orally administered benzyl chloride and the reduction was roughly proportional to the amount of benzylmercapturic acid formed. The authors concluded that the rate of glutathione turnover (49 mg/100g liver/1 hour) was adequate to account for the amount of benzylmercapturic acid formed.

Enzyme studies by various workers point to the existence of a group of S-glutathione transferases that catalyze the formation of S-glutathione derivatives from foreign compounds

that have been shown to form mercapturic acids in vivo (Boyland and Chasseaud, 1969). A glutathione S-aralkyl-transferase was reported by these authors and this enzyme catalyzed the conjugation of benzyl chloride with glutathione. The enzyme had a pH optimum of 6.8 and according to Suga et al (1967), the ratio of activities in rat liver and kidney was 100:73. The present study has shown that the liver and kidney glutathione levels were reduced by 87 and 54%, respectively, in 6 hours following acute exposure to benzyl chloride vapors. The reduction in hepatic and renal glutathione levels appears to correlate with the ratios of glutathione-S-aralkyl-transferase activities in these organs, as reported by Suga et al.

A probable function, ascribed to this enzyme is the protection of the cellular constituents from strong alkylating agents such as benzyl chloride, shown to be carcinogenic (Preussman, 1968). Bray et al (1969) assessed the relative importance of glutathione and protein thiol groups as conjugating agents for benzyl chloride in rat liver homogenates from adult female rats. The authors observed a rapid drop in glutathione levels after the addition of 53 millimoles of benzyl chloride/100 g of liver. The glutathione concentration was 0.02 millimole/100 g of liver at 8 minutes after benzyl chloride addition. Control glutathione levels remained

at about 0.88 millimoles/100 g of liver. Also, in the same preparation, nonglutathione thiol levels average 1.68 millimoles/100 g of liver for the 8 minutes. The authors concluded that because the initial drop in glutathione was so rapid, benzyl chloride probably conjugated with liver glutathione rather than with tissue proteins. Beck at al (1964) found hepatic glutathione concentrations reduced by 50% in 2 hours, following intramuscular administration of benzyl chloride in mice. In the present study, the liver glutathione level was found to drop to 13% of the control levels in 6 hours of acute exposure to benzyl chloride. Clearly, the evidence revealed that liver glutathione is the major source of the thiol group for the synthesis of benzylmercapturic acid, while the kidney glutathione is the secondary source.

Boyland and Chasseaud (1969) succeeded in isolating from the human liver (foetus and post mortem specimens) the enzymes which catalyze the conjugation of benzyl chloride with glutathione. Seutter-Berlage et al (1977) assessed the urinary mercapturic acid excretion as a biological parameter of exposure to alkylating agents and confirmed that humans are capable of forming mercapturic acids. Thus, despite the lack of data on the metabolism of benzyl chloride in humans, an inference may be drawn from the aforementioned facts that it seems likely that in humans benzyl chloride is excreted as benzylmercapturic acid. If this is true,

benzylmercapturic acid in urine may be used as a parameter of exposure to benzyl chloride in humans.

Some of the morphologic changes that reflected hepatic injury produced by benzyl chloride were microvesicular fatty change, inflammation and glycogen depletion. The microvesicular form of fatty liver, in which the hepatocytes appear to be packed with small droplets seems to be a unique form of injury induced by benzyl chloride. The hepatic lesion in humans produced by tetracycline and its congeners is the microvesicular form of fatty liver (Hoyumpa et al 1975). The authors reported that this form of steatosis resembled the fatty liver of pregnancy and of Reye's syndrome. This type of lesion is strikingly different from the fatty liver induced by ethanol, which is described as marcovesicular, with a large droplet of fat displacing the nucleus to the periphery (Scheuer, 1973). Although Mikhailova (1977) mentioned liver injury caused by benzyl chloride in humans, it is not known if benzyl chloride vapors induce microvesicular fatty changes in human liver. Also, fatty liver caused by benzyl chloride vapors needs to be chemically defined. No necrosis of the liver cells was observed but there was an inflammatory response that included the neutrophils. The lungs showed peribronchiolar inflammation but the heart, brain, kidneys and testis appeared histologically normal. The available evidence points to the fact that benzyl chloride is a heptatoxin.

Lipoperoxides were not detected in the blood of rat either during or after exposure to benzyl chloride. Also, there was no loss of glucose-6-phosphatase in the liver of rats exposed to benzyl chloride vapors. Further, no increase in diene conjugation was observed in the microsomal fractions of liver exposed to benzyl chloride in vivo. The above facts suggest that benzyl chloride produces the hepatoxic effect in the absence of lipid peroxidation. In this respect, benzyl chloride resembles hepatotoxic substances, such as 1, 1-dichloroethylene, dimethylnitrosamine and thioacetamide which do not seem to produce lipid peroxidation in vivo (Plaa and Witschi, 1976). Reynolds and Yee (1967) reported that carbon tetrachloride and chloroform caused fatty liver and centrilobular necrosis. They found that only after carbon tetrachloride was glucose-6-phosphatase activity depressed in the liver; after chloroform, there was no indication that such a depression occurred. The results obtained with benzyl chloride suggest that this chemical, like chloroform, can cause fatty liver without depressing glucose-6-phosphatase activity. Apparently, the unique microvesicular fatty change in the rat liver exposed to benzyl chloride occurs in the absence of peroxidative damage.

CONCLUSION

1. Male Sprague Dawley rats were exposed to benzyl chloride vapor at a concentration of 220 ppm for 6 hours.
2. Benzyl chloride vapors were absorbed from the lungs into the blood, where most of it was retained.
3. Blood benzyl chloride concentration reached a steady state level between 4 and 6 hours of exposure.
4. Very little benzyl chloride was present in the blood four hours after the termination of exposure.
5. The absorption half life was 0.53 and the elimination half life was 1.12 hour.
6. Next to the blood, benzyl chloride was present in the adipose tissue, and none in the brain, liver, lungs, pancrease and testis.
7. Benzylmercapturic acid was detected as benzyl chloride metabolite in rat blood. In the first hour of exposure, an amount equal to the parent compound in blood was metabolized into benzylmercapturic acid. Four hours following the termination of exposure, the concentration of benzylmercapturic acid was 25% of the parent compound detected in blood at the same period.

8. Liver and kidney glutathione levels in benzyl chloride dosed rats were depleted to 13.5 and 46% of the control values, respectively.
9. Liver is the major source of the thiol group in benzylmercapturic acid; kidney, the secondary source.
10. Benzyl chloride is a hepatotoxin and causes microvesicular fatty change, inflammation and glycogen depletion in the liver, in the absence of lipid peroxidation *in vivo*.

BIBLIOGRAPHY

Back, K.C., Thomas, A.A., and MacEwen, J.D. (1972). Reclassification of Materials Listed as Transportation Health Hazards, Report ISA-20-72-3. Department of Transportation, Office of Hazardous Materials, 24-25, A-264 to A-265.

Barnes, M.M., James, S.P., and Wood, P.B. (1959). The formation of Mercapturic Acids - I. Formation of Mercapturic Acid and the Levels of Glutathione in Tissues. Biochem. J., 71: 680-690.

Beck, L.V., Roberts, N., King, C., and Wilson, J.E. (1964). Effects of Bromobenzene on Mouse Tissue Sulfhydxyl and Insulin^{I-131} Metabolism. Proc. Soc. Exptl. Biol. Med., 116: 283-288.

Beutler, E., Duron, O. and Kelly, B. (1963) Improved Method for the Determination of Blood Glutathione. J. Lab. Clin. Med., 61: 882-888.

Boyland, E. and Chasseaud, L.F. (1969). Glutathione S-aralkyl Transferase. Biochem., J. 115: 985-991.

Bray, H.G., Garret, A.J., and James, S.P. (1969). Some Observations on the Source of Cysteine for Mercapturic Acid Formation. Biochem. Parmacol., 18: 1203-1206.

Bray, H.G., James, S.P. and Thorpe, W.V. (1958). Metabolism of some Omega-Halogenealkyl-Benzenes and Related Alcohols in the Rabbit. Biochem. J., 70:570-579.

Buckley, D.H. (1973). Interaction of Some Extreme-pressure type Lubricating Compounds with an Iorn Surface. Report No: NASA TN D-7528. Cleveland, NASA Lewis Research Center. 15pp.

Cornish, H.H. (1980). Solvents and Vapors. In: Toxicology-The Basic Science of Poisons, edited by Doull, J., Klaassen, C.D., and Amdur, N.O., 489. MacMillan, New York.

Davey, W. (1945). The Extreme Pressure Lubricating Properties of some Chlorinated Compounds as Assessed by the Four-Ball Machine. J. Inst. Pet. (London), 31: 73-88.

Druckrey, H., Kruse, H., Preussmann, R., Ivankovic, S., and Landschutz, C. (1970). Carcinogenic Alkylating Substances - III. Alkyl Halogenides,-Sulfates,-Sulfonates and Strained Heterocyclic Compounds. Z. Krebsforch., 74:241-270.

Folch, J., Lees, M. and Sloane, S.G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem., 226: 497-509.

Freeman, S.K. (1957). Infrared Spectrophotometric Determination of Impurities Occurring in Alpha-Chlorotoluene (benzyl chloride). Anal. Chem., 29: 63-68.

Grassie, N. and Meldrum, I.G. (1971). Friedel-Crafts Polymers - VII Condensation Reactions involving benzyl chloride. Eur.Polym.J., 7:629-643.

Gratacos, E. (1969) Detection of Hydrotropic Effects on Skin Collagen. (Ger) Leder., 20:31-35.

Harper, A.E. (1965) Glucose-6-Phosphatase. In: Methods of Enzymatic Analysis, edited by A.U. Bergmeyer, 788-792. Academic Press, New York.

Hawley, G.G. (1971) The Condensed Chemical Dictionary. Van Nostrand-Reinhold, New York.

Hirade, J. and Ninomiya, A. (1950). Studies on the Mechanism of the Toxic Action of Organic Halogen Compounds. J. Biochem. (Tokyo) 37:19-34.

Holmberg, B., and Malmfors, T. (1974). Cytotoxicity of Some Organic Solvents. Environ. Res., 7:183-192.

Hoyumpa, A.M., Greene, H.L., Dunn, G.D., and Schenker, S. (1975) Fatty Liver: Biochemical and Clinical Considerations. Am.J.Dig. Dis., 20: 1142

Jaeger, R.J., Conolly, R.B., and Murphy, S.D. (1973) Diurnal Variation of Hepatic Glutathione Concentration and Its Correlation with 1,1-Dichloroethylene Inhalation Toxicity in Rats. Res. Comm. Chem. Pathol. Pharmacol., 6: 465-471.

Jemski, J.V., and Phillips, G.B. (1965) Aerosol Challenge of Animals. In: Methods of Animal Experimentation, edited by W.I. Gay, 306-307. Academic Press, New York.

Kennedy, Jr., G.L. and Trochimowicz, H.J. (1982) Inhalation Toxicology. In: Principles and Methods of Toxicology, edited by A.W. Hayes, 196-197. Raven Press, New York.

Klaassen, C. D. (1980) Absorption, Distribution and Excretion of Toxicants. In: Toxicology - The Basic Science of Poisons, edited by Doull, J., Klaassen, C.D., and Amdur, M.O., 41. MacMillan, New York.

Knight, R.H. and Young, L. (1958) Biochemical Studies of Toxic Agents - II. The Occurrence of Premercapturic Acids. Biochem. J., 70: 111-119.

Landsteiner, K. and Jacobs, J. (1936) Studies on the Sensitization of Animals with Simple Chemical Compounds. J. Exp. Med., 64: 625-639.

Mabey, W.R. and Mill, T. (1976) Kinetics of Hydrolysis and Oxidation of Organic Pollutants in the Aquatic Environment, presented at the Symposium on Non-Biological Transport and Transformation of Pollutants. Gaithersburg, Md., National Bureau of Standards, 8 pp.

McCann, J., Spingarn, N. E., Kobori, J. and Ames, B.N. (1975) Detection of Carcinogens as Mutagens-Bacterial Tester Strains with R Factor Plasmids. Proc. National Academy of Science, USA, 72: 979-983.

Mikhailova, T.V. (1964) Comparative Toxicity of Chloride Derivatives of Toluene-Benzyl Chloride, Benzal Chloride and Benzotrachloride. Fed. Proc - (Trans. Suppl.) 24: T877-80.

Mikhailova, T.V. (1971) Benzyl Chloride, In: ILO Encyclopedia of Occupational Health and Safety. Geneva, International Labour Office., 1: 169-170.

Plaa, G. L. and Witschi, H.P. (1976) Chemicals, Drugs and Lipid Peroxidation. Ann. Rev. Pharmacol. Toxicol., 16: 125-141.

Poirier, L. A., Stoner, G. D., and Shimkin, M.B. (1975) Bioassay of Alkylhalides and Nucleotide base Analogs by Pulmonary Tumor Response in Strain A Mice. Cancer Res. 35: 1411-1415.

Preussmann, R. (1968) Direct Alkylating Agents as Carcinogens. Food. Cosmet. Toxicol., 6: 576-577.

Rechnagel, R.O. and Ghoshal, A.K. (1966) Quantitative Estimation of Peroxidative Decomposition of Rat Liver Microsomal and Mitochondrial Lipids after Carbon Tetrachloride Poisoning. Exp. Mol. Pathol., 5: 413-426.

Reynolds, E.S. and Yee, A. G. (1967) Liver Parenchymal Injury - V. Relationships Between Patterns of Chloromethane - C^{14} Inevaporation into Constituents of Liver in vivo and Cellular Injury. Lab. Invest., 16: 591-603.

Scheuer, P. J. (1973) Liver Biopsy Interpretation, 2nd edition., 40-55. Williams and Wilkins, Baltimore.

Schulte, H. (1915) Tests with Benzyl and Benzal Chloride. PhD Thesis. Wurzburg, Royal Bavarian Julius - Maximillians University. 27 pp (Ger).

Seulter-Berlage, F., Van Dorp, H.L., Kosse, H.G.J., and Henderson, P.T. (1977) Urinary Mercapturic Acid Excretion as a Biological Parameter of Exposure to Alkylating Agents. Int. Arch. Occup. Environmental Health, 39: 45-51.

Sidi, H. (1964) Benzyl Chloride, Benzal Chloride and Benzotrichloride. In: Kirk-Othmer Encyclopedia of Chemical Technology, 5: 281-289. InterScience Publishers, New York.

Stekol, J. A. (1938) Studies on the Mercapturic Acid Synthesis in Animals-IX. The Conversion of Benzyl Chloride and S-Benzylcysteine into Benzylmercapturic Acid in the Organism of the Dog, Rabbit and Rat. J. Biol. Chem., 124: 129-133.

Stekol, J. A. (1939) Studies on the Mercapturic Acid Synthesis in Animals - XI. The Detoxification of Benzyl Chloride, Benzyl Alcohol, Benzoaldehyde and S-Benzylhomocysteine in the Rabbit and Rat. J. Biol. Chem., 128: 199-205.

Stekol, J. A. (1947) Studies on the Mercapturic Acid Synthesis in Animals - XV. On the Mechanism of Inhibition of Growth of Rats by Benzyl Chloride. J. Biol. Chem., 167: 637-643.

Suga, T., Ohata, I. and Akagi, M. (1966) Studies on Mercapturic Acids - Effect of Some Aromatic Compounds on the Level of Glutathionase in the Rat. J. BioChem., 59: 209-215.

Suga, T., Ohata, I., Kumaoka, H., and Akagi, M. (1967) Studies on Mercapturic Acids - Investigation of Glutathione Conjugating Enzyme by the Method of Thin-layer Chromatography. Chem. Pharm. Bulletin, 15: 1059-1064.

Wilter, R. F. (1944) The Metabolism of Monobromobenzene, Benzene Benzyl Chloride and Related Compounds in the Rabbit, PhD Thesis. Ann Arbor, University of Michigan.

Wolf, W. (1912) Concerning the Effect of Benzyl Chloride and Benzal Chloride on the Animal Organism. Ph.D. Thesis. Wurzburg, Royal Bavarian Julius-Maximillians University, 25 pp (Ger).

Yagi, K. (1976) A Simple Fluorometric Assay for Lipoperoxide in Blood Plasma. BioChem. Med., 15: 212-216.

Zbarsky, S.H. and Young, L. (1943) Mercapturic Acids. J. Biol. Chem., 151: 211-215.