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EXTRA-HEPATIC GLUTATHIONE CONJUGATION AND THE TOXICITY OF THREE HALOGENATED HYDROCARBONS

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EXTRA-HEPATIC GLUTATHIONE CONJUGATION AND THE TOXICITY OF THREE HALOGENATED HYDROCARBONS

by

Ronald Trevor MacFarland

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In Partial Fulfillment of the Requirements
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STATEMENT BY AUTHOR

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This thesis has been approved on the date shown below:

I. Glenn Sipes

I. GLENN SIPES
Professor, Pharmacology & Toxicology

Dec 8 1982

Date

To my parents,

teachers by example

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ABSTRACT

In contrast to many of the other short-chain halogenated hydrocarbons, the toxicology of the compounds 1,2-dibromoethane (DBE), 1,2-dibromo-3-chloropropane (DBCP), and 1,2-dichloroethane (DCE) has been characterized by a predominantly extra-hepatic response. Strong evidence indicates that a direct, enzymatically-mediated conjugation with glutathione may be at least partially responsible for the toxicity associated with exposure to 1,2-dibromoethane. In this study, the *in vitro* GSH-dependent cytosolic metabolism of DBE, DBCP and DCE was assessed, using cytosolic fractions prepared from the liver, kidney, testes and stomach tissues of Sprague-Dawley rats and Swiss Webster mice. DBCP was the most rapidly metabolized by the rat, in all tissues examined, followed in descending order by DBE and DCE. In the mouse, however, DBE was the most readily metabolized of the three compounds, followed by DBCP and DCE. In every case, hepatic rates of metabolism exceeded those of the extra-hepatic tissues tested.

INTRODUCTION

1,2-Dibromo-3-chloropropane (DBCP), 1,2-dibromoethane (DBE, ethylene dibromide, EDB) and 1,2-dichloroethane (DCE, ethylene dichloride, EDC) represent three members of the class of short-chain halogenated hydrocarbons which have been the subject of an extensive amount of toxicological investigation in recent years. An effort in this area is clearly warranted, as all three of these compounds have been heavily produced and utilized in a number of industrial, agricultural and consumer settings. The likelihood of their environmental dispersion is heightened by their manner of use--often in large quantities as pesticides or solvents, and by the relatively high volatility and lipophilicity of these and other members of this class.

Finally, recent studies based on the suggestion of these compounds as the causative agents in numerous pathological conditions, indicate that they may undergo a novel scheme of metabolic bioactivation to their potentially toxic forms. This latter aspect, concerning the metabolism of DBCP, DBE and DCE, make these compounds unique among their class.

Production, Uses and Occurrence

1,2-Dibromo-3-chloropropane is an amber to brown oily liquid at room temperature, with a molecular weight of 236.3, a boiling point of 196^{o}C, and a vapor pressure of 0.8 mm Hg at 21^{o}C. Used almost exclusively for agricultural purposes as a nematocide, DBCP has been

marketed in a variety of forms, including emulsified concentrates, granular preparations, and as a component in fertilizer mixtures. Its lack of toxicity to mature plants combined with a long residence life in soil, make it a highly effective soil fumigant (IARC, 1979).

Commercial production of DBCP began in the United States in 1955 using the same process as is currently used, in which bromine is added to allyl chloride. The compound was registered for use as a nematocide in 1964, and is currently produced in several European countries and Japan, as well as in the U.S. An estimated 5.4 million kg of DBCP were used in the United States in 1972, with approximately 59% of this total being applied in the Pacific coast states (OSHA, 1978). Use of DBCP has, however, been sharply curtailed since 1977 when an order was issued by the EPA, suspending use of the compound on 19 food crops in which it was believed residues might occur in edible portions (OSHA, 1978). The current Occupational Safety and Health Administration (OSHA) standard for occupational exposure to gaseous DBCP is 1 part per billion (8 hr time weighted average) (IARC, 1979).

1,2-Dibromoethane has largely superceded DBCP as the major soil fumigant in current use in the United States, with an estimated 5 million pounds being employed for this purpose annually (Fishbein, 1980). Its extensive use in the fumigation of produce, as part of the Medfly quarantine program, has further expanded DBE's role as a pesticide (Walsh, 1982).

Both DBE and its chlorinated analog, 1,2-dichloroethane, are heavy, colorless liquids with boiling ranges of 131-132 and 83-84 ^{o}C,

respectively. DBE and DCE have respective vapor pressures of 8.5 and 66 mm Hg at 20°C, making them somewhat more volatile than DBCP (Rannug, 1980).

Despite its current importance as a pesticide, only about 10% of all DBE produced is used in this capacity (Walsh, 1982). DBE's principle use is as a lead scavenger in anti-knock blends, a component of leaded gasolines.

Commercial production of DBE involves the exothermic reaction of ethylene and bromine, representing the single largest use of the latter in the world. DBE production in the United States in 1975 was estimated at 125 million kg. The present federal OSHA standard for occupational exposure to DBE is 20 ppm. The California branch of OSHA (Cal OSHA) has adopted a new standard of 130 parts per billion, in response to concerns posed relating to the compound's use in the Medfly quarantine program (Walsh, 1982).

Like DBCP and DBE, 1,2-dichloroethane has been used in a limited capacity as a pesticide. The major use of this compound, however, is as an intermediate in the production of vinyl chloride, and a number of other organic compounds including 1,1,1-trichloroethane, trichloroethylene, perchloroethylene and vinylidene chloride. Other uses for DCE include its application as a solvent for textile cleaning and metal degreasing, as a component in paint and finish removers, as a dispersant for nylon, rayon and plastics, and as a lead scavenger in the formulations of leaded fuels (Fishbein, 1980). This latter application probably represents the single largest dispersive use of DCE.

Synthesized through the chlorination or oxychlorination of ethylene, DCE is the most heavily produced of the three compounds being considered in this thesis (Rannug, 1980). Commercial production began in 1922, but did not escalate until around 1960, paralleling the increase in the use of plastics. With a current estimated production of approximately 11 billion pounds annually, DCE is the largest volume synthetic organic chemical manufactured in the United States (Gold, 1980). The National Institute for Occupational Safety and Health (NIOSH) estimates as to the number of workers potentially exposed annually to DCE run as high as 1.6 million (NIOSH, 1976). The current OSHA standard for exposure to DCE in ambient air is 50 ppm. Because of its high volatility, most exposure to DCE is likely to occur via the inhalation route.

Although their major uses vary somewhat, exposure to relatively high levels of DBCP, DBE or DCE is most likely to involve some type of occupational setting, whether it be industrial or agricultural in nature. At the same time, however, the patterns of use of these 3 compounds combined with their physical and chemical characteristics may permit their environmental dispersion. Thus, those at risk may be divided into two groups, the general population, and a smaller subgroup potentially exposed to much higher levels in the workplace.

In the case of DBCP, the high exposure subgroup would be represented by both the worker in the manufacturing plants, and the farmer engaged in the application of the material. Distribution of much lower levels to the general population could occur through the presence

of residual DBCP on the produce grown in treated soil (Newsome et al., 1977). The United States Environmental Protection Agency (EPA) has estimated human dietary exposure to DBCP in the range of 2.2×10^{-6} mg/kg/day (OSHA, 1978). A study examining the presence of DBCP in fumigated soil demonstrated the retention of some of the compound 40 weeks post-treatment (Newsome et al., 1977).

Similar potential routes for environmental dispersion can be postulated for both DBE and DCE, such as exposure related to the use of self-serve gasoline stations. DCE has also been shown to be present in the drinking water supplies of several major cities, in the parts per billion range (Simons et al., 1975).

Toxicology

As previously stated, the commerical use of DBCP has sharply declined in the past several years, as a direct result of a 1977 EPA action suspending the use of DBCP-containing pesticides on most food crops. This order closely followed a NIOSH health hazard evaluation at the Oxidental Chemical Company plant in Lathrop, California, when it was learned that a number of workers had shown abnormally low sperm counts (NIOSH, 1978).

Evidence for effects of DBCP on male reproductivity existed prior to the Oxidental Chemical investigation, and have since been elucidated more clearly in a variety of species. Torkelson, Sadek and Rowe (1961) reported the results of two independent toxicologic investigations on DBCP in which the compound was administered to a number of species via several major routes of exposure. Oral LD50s

ranged from 410 and 300 mg/kg in the mouse and rat, respectively, to as low as 60 mg/kg in the chick. Single exposures of male rats to DBCP vapors produced irritation to the eyes and respiratory passages at concentrations of 60 ppm or higher. Slight to moderate depression of the central nervous system, as evidenced by sluggishness and ataxia, and kidney effects were noted in some animals exposed to concentrations as low as 50 ppm. An LC_{50} of 368 ppm for a 1 hour exposure was suggested.

Chronic inhalation studies (7 hrs/day, 5 days/week) in male rats produced gross lesions in the lung, intestinal mucosa, kidneys and testes when DBCP was administered at 20 ppm. In similar experiments using rats, guinea pigs, and rabbits at levels of DBCP in the 12 ppm range, the same authors describe degenerative changes in the testes as the most striking feature present in all species at the time of autopsy. In the rat, these changes were characterized by degeneration of the seminiferous tubules and an increase in the number of Sertoli cells. Centrilobular congestion and dilation of the sinusoids of the liver were noted, as was a cloudy swelling of the epithelial lining of the proximal convoluted tubule in the kidney.

In a more recent study by Kluwe (1981a), the acute toxicity and effect of route of administration on DBCP toxicity were examined. In a dose-response experiment, the authors found the kidney to be susceptible to toxicity at lower doses (40 mg/kg) than produced significant damage to the testes or liver. This disparity between the kidney and other tissues was accentuated when a subcutaneous route of administration was used.

Histological evaluation of the kidneys of F-344 rats administered a single dose of 100 mg/kg DBCP revealed degenerative changes in the proximal convoluted tubule at 1 day post-treatment (Kluwe, 1981b). These morphologic changes were preceded by a prominant polyuria, glucosuria and proteinuria, with a decrease in urine specific gravity, all in accord with the suggestion of the proximal convoluted tubule as the primary site of DBCP-induced toxicity in the kidney. Considerable tubular regeneration was observed 10 days post-treatment, however kidneys examined from rats sacrificed at 30 days contained large areas of fibrotic tissue. The authors suggest DBCP-induced degenerative changes to the kidney may be cumulative, as a single dose of 120 mg/kg produced quantitatively similar changes as 4 consecutive daily treatments of 40 mg/kg (Kluwe, 1981b).

Testicular and hepatotoxicity were apparent at somewhat higher doses of DBCP, the former being characterized by focal seminiferous tubular degeneration, with desquamation of germinal cells at all stages of maturation. Epididymal cells also exhibited degenerative changes, with sloughing off into the tubular lumen. Both the seminiferous tubular and epididymal changes could account for the reduced sperm counts observed in humans (Potashnik, 1979).

DBCP treatment produced small, transient increases in serum GPT and LDH levels, followed by morphological changes in the liver characterized by mild periportal swelling. The histological changes in the liver were reversible, with essentially normal morphology returning within 3 days post-treatment. Higher doses produced centrilobular

necrosis with some vacuolated cells, and increased liver to body weight ratios (Kluwe, 1981a).

Suggestion of DBCP's role as a possible carcinogen began with a report by Olson et al. (1973) on the induction of stomach tumors in rats and mice following chronic oral administration of DBCP and DBE. Gastric tumors described as squamous cell carcinomas were observed in rats receiving DBE as early as 10 weeks. DBCP produced similar tumors in rats, first observed at 40 weeks. In addition, DBCP produced prominent mammary tumors in female rats, but not mice, as early as 14 weeks. Gastric tumors eventually metastasized throughout the abdominal cavity (Ward and Haberman, 1974; Olson et al., 1973). Reported incidences of stomach tumors at the 62 week termination point of the study were greater than 90 and 70% in DBE-treated rats and mice, respectively, and in excess of 60 and 90% in DBCP-treated rats and mice, respectively (Powers et al., 1975).

The carcinogenic potentials of DBCP and DBE when administered via the inhalation route were assessed in a lifetime study using rats and mice exposed to DBCP in the 1-24 ppm range, and DBE in the 1-75 ppm range (Reznik, Stinson and Ward, 1980). Histomorphological alterations produced by both compounds were dose-related, and occurred predominantly in the upper respiratory tract in both species. Necrosis and atrophy of the olfactory epithelium was noted as early as 13 weeks in both species. Squamous cell metaplasia, hyperplasia, and cytomegaly of the respiratory nasal turbinals, as well as of the larynx, trachea, bronchii and bronchioles was observed in both species. The authors also noted

severe toxic alterations in the trachea, lung, kidney, liver and testes of test animals, with tumors occasionally being found in all of these tissues except the testes (Reznik et al., 1980).

In a recent inhalation study attempting to delineate the time course of development of these changes, F-344 rats were exposed to concentrations of DBE in the 0-40 ppm range for a period of 13 weeks (Nitschke et al., 1981). Rats exposed to 40 ppm DBE showed decreased body weight gain throughout the 13 week exposure, with increases in kidney and liver weights noted after 6 and 13 weeks of treatment. Histopathological effects were limited to the anterior sections of the nasal turbinals, and were observed at as early as 6 weeks in the rats receiving 40 ppm DBE. After 13 weeks, rats in this group exhibited multifocal to diffuse hyperplasia, and some individual cell necrosis in the respiratory epithelium. Following an 88 day recovery period, however, a reversal of these changes were observed in all but one rat. The authors speculate, however, that had exposure been continued, the observed histopathological changes could easily have progressed to neoplasia (Nitschke et al., 1981).

Besides the observed similarities in their chronic toxicity when administered both orally and by the inhalation route, DBCP and DBE exhibit similar patterns of acute and sub-chronic toxicity. Like DBCP, DBE toxicity is predominantly extra-hepatic in nature, in striking contrast to most of the other short-chain halogenated hydrocarbons such as carbon tetrachloride (Recknagel, 1967).

Acute human exposure to DBE is reported to cause irritation of mucous membranes, nausea, vomiting and drowsiness (St. George, 1937).

Rats receiving DBE vapors at concentrations of 3000 ppm showed adverse effects within a 7 hr period, including pulmonary edema and hemorrhage, accompanied by inflammatory and early degenerative changes in the kidney (Rowe et al., 1952). The lack of hepatotoxicity of DBE was demonstrated by Plescia (1981) in rats and mice. Doses required to produce an observable hepatotoxic response with significantly elevated serum GPT levels resulted in a high mortality rate in both species. The oral LD_{50} values for DBE in male and female rats, and female mice are 146, 117 and 420 mg/kg, respectively (Rowe et al., 1952).

Like DBCP, DBE has been linked with production of pronounced toxicity to the male reproductive system in a number of species (Amir, 1973; Edwards, Jackson and Jones, 1970). In one study, DBE was administered to calves orally, over a 14 month period, and was shown to produce a decrease in sperm density of the semen (Amir, 1973). Sperm were abnormal in morphology and exhibited decreased motility. When administered by the inhalation route in concentrations ranging from 0-89 ppm for 7 hr/day, 5 days/week over a 10 week period, DBE produced a variety of toxic effects in rats, generally involving the male reproductive system (Short et al., 1979). Male and female rats in the two higher dose groups (39 and 89 ppm) showed reduced body weight gain, and consumed less food compared to controls. At the 10 week termination point of the study, average testes weights for the high dose groups were 0.5 gm, versus an average weight of approximately 3.5 gms in the control and lower dose groups. Rats receiving 89 ppm DBE showed serum testosterone levels of about 50% those of unaffected rats. Mating studies

revealed an inability of these high dose rats to impregnate normal females. Histopathological examination showed atrophy of the testes, epididymis and seminal vesicles. Females exposed to 89 ppm DBE had disrupted estrus cycles, this effect being readily reversible, however, with many subsequently producing normal litters.

The question of whether or not a male anti-fertility effect of DBE may be occurring in humans was addressed in an epidemiological study commissioned by several major producers of the compound (Wong, Utidjian and Karlen, 1978). Examining the inferred fertility of married men at several DBE production facilities, the study revealed a significantly low observed to expected birth ratio at one such plant, prompting the authors to suggest the real existence of an anti-fertility effect in humans.

Tissue distribution studies in mice using 14C-DBE administered intraperitoneally indicated that the compound is readily removed from the circulation within 1 hour, with appreciable portions of the radioactive dose being found in the small intestine, liver, kidney and fat. Only about 5% of the dose had been excreted in the bile at 2.5 hours (Edwards et al., 1970). ^{14}C-DBE administered to rats by the oral route showed a similar pattern of distribution (Short et al., 1979). Plescia (1981) demonstrated a high degree of covalent binding in the stomach of rats following oral administration of ^{14}C-DBE. The author makes the suggestion that a high level of biotransformation may severely limit the amount of parent compound subsequently available to other organs (Plescia, 1981).

Of the three compounds under consideration here, 1,2-dichloroethane appears to be least potent toxin, with an oral LD_{50} of 680 mg/kg in the rat (Maltoni, Valgimigli and Scarnato, 1980). DCE readily enters the body, and at high concentrations has been shown to produce toxic effects on the nervous system, liver, kidney, cardiac and respiratory systems, in addition to being a strong eye and skin irritant (Maltoni et al., 1980). Long-term exposure to inhalatory concentrations of DCE of up to 150 ppm did not produce gross or widespread toxicity in the rat, but toxicity to both the kidney and liver were indicated by a series of clinical chemistry tests, being especially pronounced in somewhat older animals (Speafico et al., 1980). Kidney toxicity was indicated by elevated BUN and uric acid levels, while the implication for liver toxicity came from increases in serum GPT and tendency toward increases in GGT.

Early suggestion for the carcinogenic potential of DCE came from work by Van Duuren et al. (1979) in which repeated skin application of DCE was shown to produce lung tumors in rats. In a long-term carcinogenesis bioassay using DCE administered orally to rats and mice, an increased incidence of sub-cutaneous fibromas, gastric squamous cell carcinomas and, in females, mammary adenocarcinomas were seen (Ward, 1980). Alveolar-bronchiolar adenomas and mammary adenocarcinomas occurred at a higher incidence than controls in mice. The author describes these tumors as being similar to those produced by DBCP and DBE in studies previously described. Interestingly, indication of the carcinogenic potential for DCE was not seen when the compound was administered at concentrations of up to 150 ppm by the inhalation

route (Maltoni et al., 1980). With the exception of mammary tumors in female rats, no significant change in the incidence of tumors was seen in treated groups when compared to controls. This difference in potential carcinogenicity depending upon the route of exposure is difficult to explain, one theory being the requirement of an initial interaction with gut flora in order to produce a carcinogenic metabolite; such a reaction as would not occur when the compound was administered via the inhalation route. A recently completed long-term bioassay in which DCE was administered to mice in their drinking water showed no indication of carcinogenicity or reproductive effects (Lane, Riddle and Borzelleca, 1982).

Distribution studies in which parent DCE was monitored demonstrate a rapid clearance from the blood in a biphasic manner. DCE accumulated rapidly in the liver, peak levels being approached within 10 minutes after i.v. administration, with a slower accumulation in adipose tissue (Speafico et al., 1980).

Mutagenicity

In accord with their observed carcinogenicity in rodents, DBCP, DBE and DCE have all been shown to be mutagenic in a variety of mammalian and non-mammalian test systems (Teramoto et al., 1980; Rannug, 1980). In contrast to compounds such as the epoxides epichlorohydrin and glycidol, which exert a direct mutagenic effect, DBCP, DBE and DCE all show an enhancement of their mutagenic actions in bacterial test systems with the addition of some type of enzymatic activating system (Stolzenberg and Hine, 1979).

In microbial test systems, DBCP was shown to block the growth of *E. coli* Pol A^{-1}, a bacterial strain deficient in DNA polymerase I. This effect was considered indicative of an action on cellular DNA by DBCP (Rosenkranz, 1975). Using tester strains of *S. typhimurium* in which revertence to histidine independence is used as an indicator of mutagenicity, DBCP was found to be positive for strain TA 1530, but not for TA 1538. These findings suggest that the mutations induced by DBCP are of the base-pair substitution, rather than of the frame-shift type, possibly the result of alkylation of DNA. Addition of an S-9 activating system was an absolute requirement for mutagenicity.

Efforts aimed at elucidating DBCP's interaction with DNA using mammalian systems include one study in which single doses of 100 mg/kg were administered to pre-pubertal male mice. Unscheduled DNA synthesis was subsequently observed in the premeiotic germ cells, indicating that the parent compound and/or its metabolites are capable of entering the nucleus, and may cause DNA damage (Lee and Suzuki, 1979). Using a dominant-lethal mutagenicity test system, DBCP was shown to increase the incidence of dead implants in rats, reaching a peak at 4-5 weeks post-treatment (early spermatid stage). No indication for dominant-lethality was seen in mice for DBCP (Teramoto et al., 1980).

In contrast to DBCP in rats, DBE showed no dominant-lethal mutagenic effect in rats or mice (Teramoto et al., 1980). In bacterial test systems, however, DBE appears to be able to exert a greater direct mutagenic effect than DBCP, inducing point mutations of the base-pair substitution type in tester strains of *S. typhimurium* (Rannug, 1980).

DBE mutagenicity is further enhanced with the inclusion of bioactivating systems such as S-9 fractions or the isolated perfused rat liver into bacterial test systems (Kale and Baum, 1979; Ehrenber et al., 1974). Mutagenicity of DBE has also been demonstrated in Drosophila melanogaster and higher plant test systems (Rannug, Sundvall and Ramel, 1978; Rannug and Beije, 1979).

Like DBE, DCE exerts a direct mutagenic effect, causing point mutations of the base-pair substitution type in Salmonella tester strains (Rannug and Beije, 1979). Stronger growth inhibition was seen in a DNA polymerase-deficient strain of E. coli than was seen in the proficient strain, indicating induction of DNA damage (Brem, Stein and Rosekranz, 1974).

DCE's mutagenic effect is enhanced in bacterial systems using an S-9 fraction, however this enhancement is NADPH-independent, and was further increased with the addition of exogenous reduced glutathione (GSH). Pre-treatment of rats with phenobarbital did not significantly increase the mutagenic effect. A similar pattern of mutagenic bioactivation appears to exist for DBE (Van Bladeren et al., 1980).

Metabolism

A positive correlation has been shown between the relative toxicologic potential of a compound, and the degree to which it exhibits enzymatically-mediated covalent binding to cellular macromolecules. In vitro binding studies using microsomal fractions prepared from the livers of control and phenobarbital-pretreated rats have shown the NADPH-dependent enzymatic activity responsible for irreversible

covalent protein binding to be present in this fraction (DiRenzo, Gandolfi and Sipes, 1981; Hill et al., 1978).

Banerjee and Van Duuren (1979) examined the in vitro binding of DBE and DCE to microsomal protein and Salmon sperm DNA using microsomes prepared from the livers and forestomach regions of rats and mice. Considerable irreversible binding, particularly to protein was seen, the reaction being inhibited by the addition of SKF-525A, GSH, or methyl-mercaptoimidazole. Studies using the cytochrome P450 inhibitors metapyrone, SKF-525A and carbon monoxide have shown similar patterns of protein binding inhibition for DCE (Guengerich et al., 1980). The same authors have also demonstrated that addition of alcohol dehydrogenase and aldehyde dehydrogenase to the incubation mixture reduced the levels of irreversible binding, probably by increasing the rate of conversion of the suspected reactive intermediate chloroacetaldehyde, to the less reactive chloroethanol and chloroacetic acid.

Using the corresponding proposed oxidative intermediates of DBE, bromoacetaldehyde and bromoethanol, Banerjee, Van Duuren and Kline (1979) demonstrated high levels of non-enzymatic binding to microsomal protein, particularly for the aldehyde. The authors concluded that bromoacetaldehyde functions as the reactive species responsible for the observed covalent binding of DBE to microsomal protein.

The in vivo metabolites of DBE in rats were examined by Nachtomi, Alumot and Bondi (1966) following single oral doses of 100 mg/kg DBE. Two major urinary metabolites were identified as N-Acetyl-S-(2-hydroxyethyl)cysteine and S-(2-hydroxyethyl)-cysteine.

These same two urinary metabolites were seen following administration of both DCE and bromoethanol, suggesting a conjugation reaction with glutathione in the liver for both DBE and DCE.

This conjugation reaction is further indicated by observed decreases in the levels of hepatic sulfhydryl compounds following DBE administration (Nachtomi et al., 1966). The major in vitro reaction product of DBE and glutathione is S-(2-hydroxyethyl)glutathione, the bis conjugate being found only in trace amounts. Conversion of this primary conjugation product to its mercapturic acid derivative would be expected to occur in the kidney.

Conjugation of xenobiotics with glutathione is classically considered to serve a detoxifying role in the cell, by both preventing the binding of electrophilic intermediates to cellular constituents, and by subsequently aiding in their excretion, this reaction representing the first step in mercapturic acid formation (Chasseaud, 1974). Compounds conjugated with glutathione are anionic, with molecular weights exceeding 300 daltons.

The family of enzymes responsible for the conjugation of GSH with exogenous compounds, the glutathione S-transferases, have been identified in all mammalian species tested to date (Warholm et al., 1981). The transferases are frequently categorized on the basis of their substrate specificities (e.g. alkyl-, aryl-, alkene-, epoxidetransferase). Studies with purified forms of these enzymes, however, have indicated a great deal of overlap in their activities towards certain compounds, such as 1-chloro-2,4-dinitrobenzene (Habig, Pabst and Jakoby, 1974). These findings have prompted the use of a

system of letter designations, based on their order of elution from a carboxymethylcellulose column, a final step in the purification process.

The transferases are basic proteins, with molecular weights of about 50,000, composed of two subunits of 25,000 daltons each. In the rat, 6 forms have been isolated (A, AA, B, C, D and M) from hepatic cytosol, most having been sufficiently purified to allow analysis of their amino acid composition and enzymatic properties with a variety of substrates (Simons and VanderJagt, 1977). Five forms of GSH-transferases have been isolated from human liver, all exhibiting characteristics similar to the rat forms of the enzyme. Interestingly, however, the 5 human forms all possess the same antigenic determinant, while only 2 of the 6 rat forms show immunological cross-reactivity (Kamiska et al., 1975). Although formerly thought to be exclusively cytoplasmic, recent studies involving cell fractionation and immuno-histochemical techniques show the presence of GSH-transferase activity in various hepatic membranes, including the microsomes, mitochondria, and nuclei. In one such study, these particulate forms were estimated to account for approximately one-tenth of the overall transferase activity of the liver (Mukhtar, Baars and Breimer, 1981).

The glutathione S-transferases have been shown to occur in a wide variety of tissues, sometimes in forms distinct from those found in the liver of the same species (Guthenberg and Mannervik, 1979). Human transferases have been purified from erythrocytes and placental tissue, and have been demonstrated in the kidney, lung, adrenal cortex, testes, skin, ovary and gastrointestinal tract, using immunohisto-chemical techniques (Campbell, Bass and Kirsch, 1980). A form of

GSH-transferase unique to the lung has been isolated from both rats and humans, with the human form exhibiting high activity towards polycyclic aromatic hydrocarbons such as benzo(a)pyrene-4,5-oxide (Guthenberg and Mannervik, 1979; Warholm et al., 1981). Warholm and coworkers (1981) noted the presence of the human lung form in only some individuals, possibly due to differences in induction or genetic makeup. They suggest that the presence of this form may be discriminatory in defining the ability of certain individuals to metabolize and detoxify various carcinogenic polyaromatic hydrocarbons.

In general, the presence of significant levels of GSH-transferase activity is associated with those tissues which may be directly exposed to exogenous chemical contaminants (Clifton and Kaplowitz, 1977). Using antibodies prepared against ligandin, a predominantly hepatic transport protein which has been shown to be identical to GSH-transferase B, Campbell and co-workers (1980) examined the presence of those transferase forms which cross-react with this antibody, in a number of human tissues. Significant levels of the enzyme were indicated in the liver, kidney, lung and gastrointestinal tract, with lesser amounts being seen in the testes, adrenal cortex, skin and ovaries. Clifton and Kaplowitz (1977) examined transferase activities in the gastrointestinal tract of the rat, noting significant levels toward aralkyl, alkene and epoxide substrates. These activities were inducible with polycyclic aromatic hydrocarbons and phenobarbital, the highest levels being seen in the proximal regions of the small intestine. The authors suggest that the GSH-transferases may represent the primary system for detoxification of epoxides in the gut, in view of

the low activity of the epoxide hydrases in that organ. Pinkus, Ketley and Jakoby (1977) examined more closely the specific regions of the gastrointestinal tract associated with transferase activity towards the substrate 1-chloro-2,4-dinitrobenzene. Values for specific activities (expressed in nmoles/mg protein/minute) ranged from a low of 12 in the stomach itself, to 410 and 400 in the duodenum and jejunum, respectively. The highest levels of activity were consistently seen in those regions associated with a high degree of absorptive activity.

Clearly, glutathione conjugation as catalyzed by the glutathione S-transferase enzymes has historically represented a pathway of detoxification. Evidence, however, for the role of this GSH-dependent route of metabolism in the development of toxicity following exposure to DBE, DCE and DBCP has come from several areas.

As previously mentioned, both DBE and DCE exhibited their greatest mutagenic effect in bacterial models when both an S-9 bioactivating system and excess reduced glutathione were present (Van Bladeren, Van der Gen and Breimer, 1981). The role of a GSH-dependent reaction in the formation of a mutagenic species was further indicated using a synthetic DCE-glutathione conjugate, which was shown to exert a strong, direct mutagenic effect (Rannug and Beije, 1979). Van Bladeren et al. (1980) demonstrated that the enzymatic activity involved in the GSH-dependent reaction resided in the 100,000 g (cytosolic) liver fraction, and not in the microsomal fraction. The same authors also showed a strong direct mutagenic effect using the synthetic proposed metabolite of DBE, S-(2-bromoethyl)-N-acetylcysteine.

The reactive species thought to be produced through direct conjugation of DBE with glutathione is a highly reactive half-mustard (episulfonium ion) which may undergo a subsequent hydration to form the 2-hydroxyethyl conjugate. Production of a double conjugate or ethylene may subsequently occur through the reaction of the episulfonium ion intermediate with a second molecule of glutathione (Nachtomi, 1970; LIvesey and ANders, 1979). A summary of the possible routes of metabolism of DBE is presented in Figure 1.

Animal studies provide further evidence for the role of the GSH-dependent reaction in the toxicity of DBE. Rats administered concentrations of gaseous DBE in conjunction with dietary disulfiram, an inhibitor of oxidative metabolism in this case, showed greater generalized toxicity than did those receiving DBE alone (Wong et al., 1982). Van Bladeren et al. (1981) attributed a similar enhancement of carcinogenicity using disulfiram reported in a previous study by Plotnick (1978) to the inhibition of the oxidative route of metabolism, permitting a greater production of the more toxic cytosolic intermediate. Pretreatment of rats with phenobarbital, known to potentiate the microsomal oxidative metabolism of DBE and its binding to protein, prevents the early biological alterations associated with cell division following DBE administration (Nachtomi, 1980).

Using the method of alkaline elution, in which DNA damage is assessed by examining the production of single-strand breaks, White et al. (1981) showed a dose-dependent increase in hepatic DNA damage following i.p. administration of DBE to Swiss-Webster mice. In

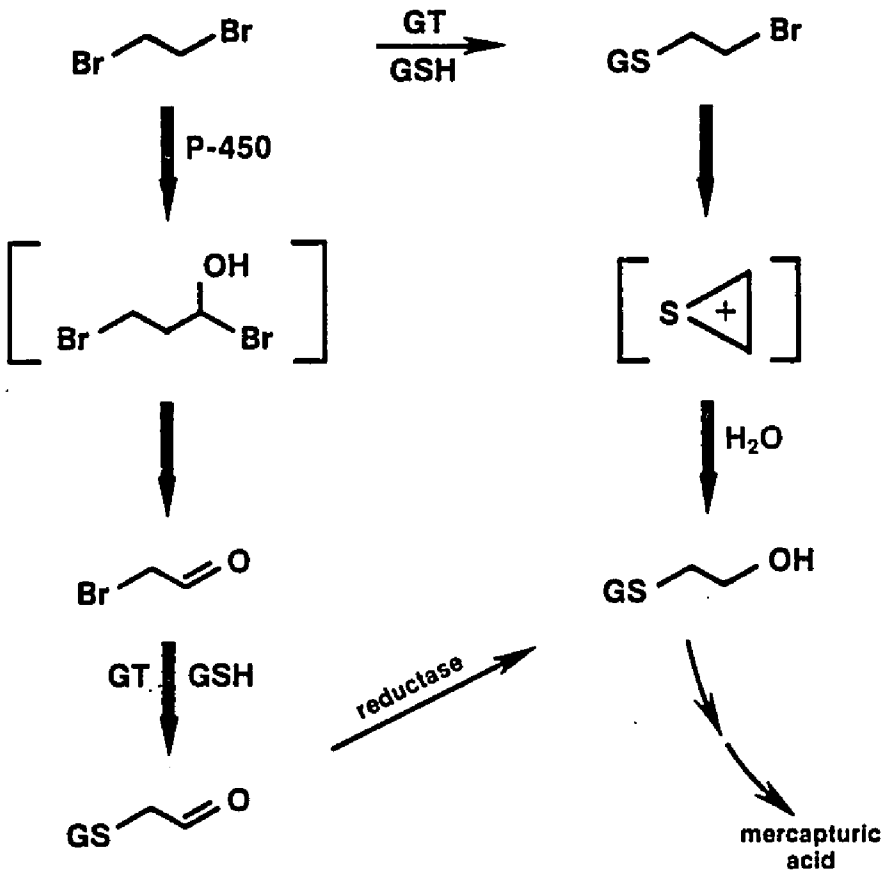

Figure 1. Proposed metabolism of 1,2-dibromoethane (DBE).

subsequent *in vitro* incubations using hepatic nuclei and either a microsomal, NADPH-dependent, or a cytosolic, GSH-dependent bioactivating system, the authors observed a much greater production of single-strand breaks using the latter, GSH-dependent route.

A recent study conducted by Shih and Hill (1981) attempted to demonstrate more clearly the patterns of DBE binding to microsomal protein and DNA. The highest levels of protein binding were achieved using a microsomal incubation system, this effect being NADPH-dependent and inducible with phenobarbital pre-treatment. In contrast, the greatest levels of binding to poly-cytidilic acid (representing DNA) occurred using a cytosolic incubation system, and were GSH-dependent, suggesting this as the pathway responsible for the interaction of DBE with cellular nucleic acids.

Evidence for the toxicity of the product of DBE conjugation with glutathione is also indicated by studies in which the effects of *in vivo* administration of the compound on enzymatic activities were assessed. Di Simplico, Pierini and Segre (1977) measured the specific activities of the GSH-transferases in rat liver supernatant following single doses of carbon tetrachloride. Transient decreases in activity were noted, reaching a maximum of about 40% of controls at 48 hours. Similar observations were noted for DBE.

Statement of the Problem

A great deal of evidence indicates the central role of a GSH-dependent route of metabolism in the development of toxicity following exposure to 1,2-dibromoethane. Its chlorinated analog,

1,2-dichloroethane, although less extensively studied, appears to undergo metabolism along the same pathways, again with the suggestion that the cytosolic enzyme-mediated route may be responsible for the more profound toxicity. 1,2-Dibromo-3-chloropropane has received the least amount of attention concerning its metabolic route of activation. The possible significance of a GSH-dependent route in its toxicity is implied by its structural similarity to 1,2-dibromoethane, as well as by its analogous pattern of predominantly extra-hepatic toxicity. This latter aspect, most notably involving the kidney, testes, and gastrointestinal tract, is shared by both DBE and DCE, and as previously stated, is in sharp contrast to observations made concerning the toxicity of other short-chain halogenated hydrocarbons.

Using cytosolic fractions prepared from kidney, testes, stomach, and liver tissues of both rats and mice, this study will assess the potential for the enzymatically-mediated, GSH-dependent metabolism of DBE, DCE and DBCP within each of these tissues. The premise that metabolism along a GSH-dependent route may be occurring within the extra-hepatic tissues themselves is supported by the observation of relatively high GSH-transferase activities found in these organs, as previously discussed. In addition, the 3 compounds will be ranked as to their relative rates of metabolism via the various tissue enzymes, permitting comparison of these findings with their previously observed levels and patterns of toxicity in the corresponding whole animal models.

METHODS

Chemicals

1,2-Dibromoethane (98% by GLC) was purchased through Matheson, Coleman and Bell, Norwood, OH, or through Eastman Kodak Co., Rochester, NY, and redistilled prior to use (boiling point 125°C). 1,2-Dibromo-3-chloropropane and 1,2-dichloroethane (98% by GLC) were purchased from Pfaltz and Bauer, Inc., Stamford, CN. ^{14}C-1,2-dibromoethane (24 mCi/mMole) and ^{14}C-1,2-dichloroethane were obtained from New England Nuclear Inc., Boston, MA. Reduced glutathione, 1-chloro-2,4-dinitrobenzene, and serum albumin stock solution were obtained from the Sigma Chemical Co., St. Louis, MO. 1-Chloro-2,4-dinitrobenzene was recrystallized from ethanol prior to use. Dimethyl sulfate (Gold Label) was purchased from the Aldrich Chemical Co., Milwaukee, WI. Bromide standard solution was purchased from Orion Research Inc., Cambridge, MA. Silica-gel thin-layer chromatography plates with $CaSO_4$ binder (20 x 20 cm) were purchased from Supelco Inc., Bellefonte, PA.

Animals

Male Sprague-Dawley rats (250-350 g) housed 2-4 per cage were obtained either through the breeding colony, Division of Animal Resources, University of Arizona Health Sciences Center, Tucson, AZ, or through Hilltop Laboratories, Hilltop, PA. Male Swiss Webster mice (25-40 g) were purchased from Charles River Breeding Laboratories Inc. Wilmington, MA, and housed 4-6 per cage. All animals were provided food (Wayne Lab Blox) and water ad libitum prior to sacrifice.

Preparation of Cellular Fractions

Hepatic and extra-hepatic cytosolic fractions were prepared from rats and mice by procedures similar to those described by Sipes, Podolsky and Brown (1977) using liver. Animals were sacrificed either by CO_2 asphyxiation (rats) or by cervical dislocation (mice). Livers, kidneys, testes and stomachs were removed and immediately placed in ice-cold 0.05M sucrose. Like tissues were pooled at each sacrifice. All groups of tissues were weighed and homogenized in 3 volumes of cold 0.05M Tris-HCl, 1.15% KCl, at pH 7.4. Liver and testes were homogenized by hand following removal of excess connective and adipose tissue, using either a Kontes Dounce Tissue Grinder (Vineland, NJ) (40 ml) or a Kontes Duall Tissue Grinder (10 ml), depending upon the volume of tissue. Homogenization of kidney and stomach tissues was carried out using an electric grinding apparatus consisting of a Black and Decker 1/4" drill (Towson, MD) mounted on a Craftsman drill press (Sears Roebuck & Co., Chicago, IL). Using 20 ml grinding tubes with Teflon pestles (A. H. Thomas, Philadelphia, PA) kidneys and stomachs were homogenized at low speed for 30-60 seconds.

Cytosolic fractions were prepared by differential centrifugation. Following centrifugation at $4^{\circ}C$ in a Sorvall RC-1 centriguge, at 3000, 10,000 and 15,000 rpm (10 min at each speed, from lowest to highest), tissue supernatants were transferred to a Beckman ultra-centrifuge, and spun at 100,000X g for 45 min. Tissue supernatants were filtered using glass wool, prior to dialysis. Cytosolic fractions from the 4 sets of tissues were dialyzed in separate containers under refrigerated conditions for 18 hr, using one change of 0.05M sucrose,

pH 7.4, and 2 final changes of Tris-KCl pH 7.4 (described above). Cytosols were stored at -70°C prior to use.

Cytosolic protein content determinations were made using the biuret procedure (Gornall, Bardwill and David, 1949). Duplicate cytosol samples of 0.025-0.25 ml were adjusted to a final volume of 0.5 ml with deionized water, and mixed with 2.5 ml of biuret reagent. Color blanks, in which no cytosol was added, and turbidity blanks in which cytosol was added to a solution of NaK tartrate were run , these values being subtracted from those obtained for the samples, to insure that absorbance changes were the result of the presence of protein. Samples were incubated at room temperature for 30 min, and read at 540 nm against water, on a Gilford Stasar II spectrophotometer. Standard curves for each determination were prepared using the Sigma albumin stock solution (10 g/100 ml).

Transferase Activities in Cytosolic Fractions

A colorimetric assay based upon a method by Habig and co-workers (1974) was used to compare levels of transferase activity in the various cytosolic fractions. This method determines the rate of formation of the product of enzymatic reaction between GSH and the substrate 1-chloro-2,4-dinitrobenzene, by absorbance at 340 nm. The reaction mixture consisted of the following: 1 mM glutathione, 0.001-0.1 mg/ml cytosolic protein, 1 mM chlorodinitrobenzene, in a 3 ml final volume of 0.1 M potassium phosphate buffer, pH 6.5. Reference cuvettes (quartz) were identically prepared, except the GSH was omitted. Readings were taken every 20 sec over a period of 5 min, using a Beckman ACTA CS III

double-beam spectrophotometer, and the rate of change determined from the region of linear response. Specific activities were calculated using an extinction coefficient value of 9.6 $mM^{-1}cm^{-1}$, as determined by Habig for this substrate.

In Vitro Metabolism of DBE, DCE, and DBCP

Cytosolic incubations were carried out in 12 ml screw-top culture tubes. A typical reaction mixture consisted of the following: 1 mM substrate (DBE, DCE or DBCP dissolved in 100% ethanol), 1 mg/ml cytosolic protein, 5 mM GSH, in a 2 ml total volume containing 0.05 M Tris-HCl 1.15% KCl, pH 7.4. Cytosolic protein concentrations were varied over the range of 0.5 to 5.0 mg/ml, and GSH concentrations of 1 mM were used in some instances. Experimental blanks were identical except the GSH was omitted. Samples were incubated in a 37°C water bath for the appropriate periods of time, with the reaction being subsequently terminated using one of three means: 1. freezing in a methanol:dry ice bath, 2. addition of 3 volumes of petroleum ether, 3. precipitation of the protein by immersion of the tubes in boiling water.

Samples from cytosolic incubations to be analyzed for the presence of free inorganic bromide were frozen at -70°C prior to lyophilization, to remove unreacted parent compound. Samples were rehydrated with distilled water, and the bromide ion concentration determined using the method of Maiorino, Gandolfi and Sipes (1980). In this procedure, aliquots of the reconstituted sample are reacted with the methylating agent, dimethyl sulfate in concentrated sulfuric acid, at 60°C for 20 min. This reaction was carried out in 2 ml reaction vials

with gas-tight septum caps. The resulting methyl bromide is present in the headspace at 37°C, and was quantitated by injecting 1 ml of the headspace vapor onto a Varian 3700 gas chromatograph equipped with a Porapak Q column. Either flame-ionization or electron-capture detectors were used, depending on the range of bromide ion concentration in the sample (ECD being used for those samples containing lower amounts). Standards were prepared using the Orion bromide standard solution. Peaks were quantitated using a Varian CS-111 electronic integrator, or in some cases by peak height. Bromide concentrations as low as 10 μM were detectable using electron capture.

Samples from cytosolic incubations in which the formation of ^{14}C-labelled product was used as an indicator of metabolism were exhaustively extracted three times with 3 volumes of petroleum ether (reagent grade) in order to remove residual, unreacted parent compound (^{14}C-labelled). The remaining non-extractable ^{14}C-labelled product was quantitated by scintillation counting, using 500 μl aliquots of the extracted sample mixed with 5 ml Betaphase scintillation cocktail. Using a Beckman LS8100 scintillation counter, the relationship between counting efficiency and H# was determined using known amounts of the radioactive standard ^{14}C-toluene with varying amounts of the quenching agent nitromethane. Samples were counted using a library program in which both ^{3}H (CH1) and ^{14}C (CH2) were counted for 10 min. Higher than normal quenching in the sample was indicated by excessively high counts in CH1.

Analysis of extracted <u>in vitro</u> samples was carried out using a thin-layer chromatography method. Aliquots of the extracted samples

(25-200 µl) were placed on silica gel plates, using solvent systems consisting of either butanol:ammonia:water (4:1:1), or propanol: ammonia:water (4:1:1). Plates were analyzed in the case of those samples containing ^{14}C-labelled product, by scraping 1 x 3 cm sections. Scintillation counting was performed by addition of 500 µl of water to the silica scrapings, followed by 5 ml of Betaphase, resulting in the formation of a gel. The extracted aqueous phases of both ^{14}C-labelled and non-labelled samples were also analyzed using the spray indicator described by Knight and Young (1958). Using this method, "premercapturic acids" appear as dark brown spots against an orange background, with inorganic halogen ions appearing as white spots against an orange background.

Statistics

Statistical analysis was carried out using one of two methods. Where only two groups were being compared, an unpaired Student's t-test was used to evaluate differences in their respective means. Where 3 or more groups were being examined, one-way analysis of variance, in conjunction with the least significance difference (LSD) test was employed. Groups were judged significantly different when a p value of less than 0.05 was obtained.

RESULTS

Cytosolic Glutathione S-transferase Activities in the Rat and Mouse

A colorimetric assay was used to determine the levels of glutathione S-transferase activity in cytosolic fractions, prepared from the liver, kidney, testes and stomachs of rats and mice. Among the most comonly used compounds to assay for overall transferase activity, 1-chloro-2,4-dinitrobenzene has been shown to be an excellent substrate for most of the GSH-transferases, when tested with the purified forms of the enzymes (Habig et al., 1974). This feature of broad specificity, combined with a relatively low rate of non-enzymatic reaction with reduced glutathione, make it a suitable substrate for estimation of overall transferase activities in tissue preparations.

The dependence of the GSH-dependent enzymatic metabolism of 1-chloro-2,4-dinitrobenzene on time and protein concentration was verified within individual determinations. In general, the reaction was linear with time for at least 3-5 min, and with protein concentrations of up to 0.1 mg/ml.

Table 1 shows the various rates of glutathione S-transferase activity among hepatic and extra-hepatic tissues of the rat and mouse. Overall activities are within the same range for both species, with the highest rates being obtained using hepatic cytosolic fractions.

Table 1. Glutathione S-transferase activities in rat and mouse cytosolic fractions. -- Data expressed as mean ± standard deviation obtained from multiple determinations using 2-6 sets of pooled cytosolic fractions. Determined using the substrate 1-chloro-2,4-dinitrobenzene.

Tissue cytosol	Specific Activity[1] Rat	Mouse
Liver	815±100	1891±401
Kidney	151±45	573±87
Testes	615±72	406±109
Stomach	79±29	281±97

[1] Specific activities expressed in nmoles product/mg protein/min.

Optimization of in vitro Conditions

In order to permit inter-species comparison in the rates of GSH-dependent metabolism of1,2-dibromoethane, 1,2-dichloroethane, and 1,2-dibromo-3-chloropropane, effort was made to optimize the in vitro enzymatic conditions. Metabolism for each of the 3 compounds was assessed using at least one of two methods. Rates of hepatic and extra-hepatic GSH-dependent metabolism of DBE and DBCP were examined by analysis of released free bromide ion, as would be produced during the conjugation reaction with reduced glutathione. Using the ^{14}C-labelled forms of DBE and DCE, a second assay for overall rates of GSH-dependent metabolism was employed. Following exhaustive organic extraction of the incubation mixture, to remove unreacted parent compound, aliquots of the aqueous fraction were assayed for the presence of the non-extractable ^{14}C-labelled product resulting from enzymatic glutathione conjugation. Because of the use of these two different methods of analysis, comparison in the rates of enzymatically-mediated glutathione conjugation for the three compounds is somewhat limited.

Formation of ^{14}C-labelled water-soluble product and release of bromide ion were determined to be linear with respect to time for at least 20 min in all cases, except where otherwise noted (Figure 2, see also Appendix A). Using hepatic cytosolic fractions, reactions were determined to be linear with respect to protein concentration over the experimental ranges (Figure 3, see also Appendix A). The levels of 5 mM GSH and 1 mM substrate were selected as being sufficient to achieve saturating conditions without producing significant non-enzymatic product formation or bromide ion release. The effect of GSH

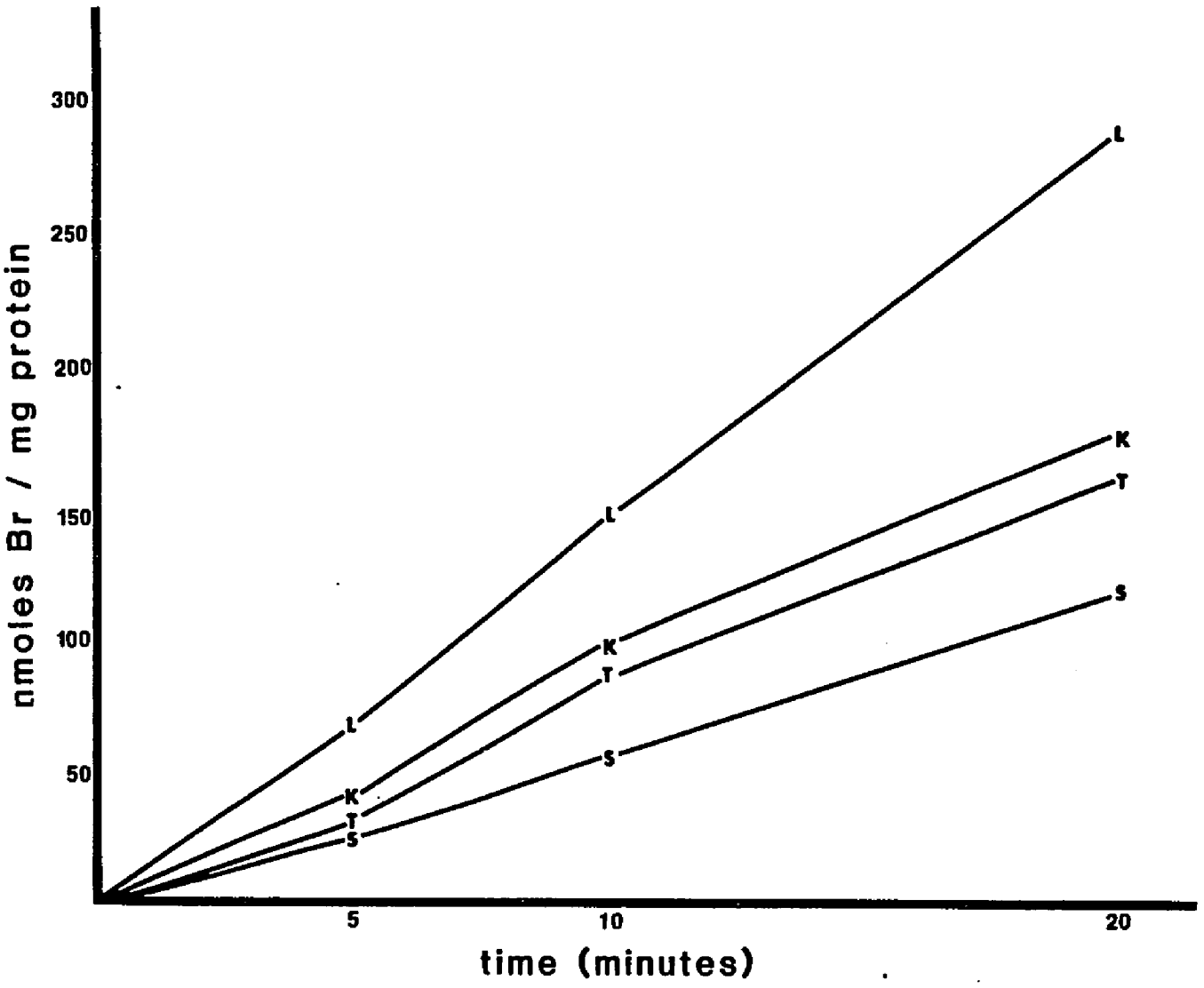

Figure 2. GSH-dependent debromination of DBCP by rat liver cytosol with time. -- In vitro conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM DBCP.

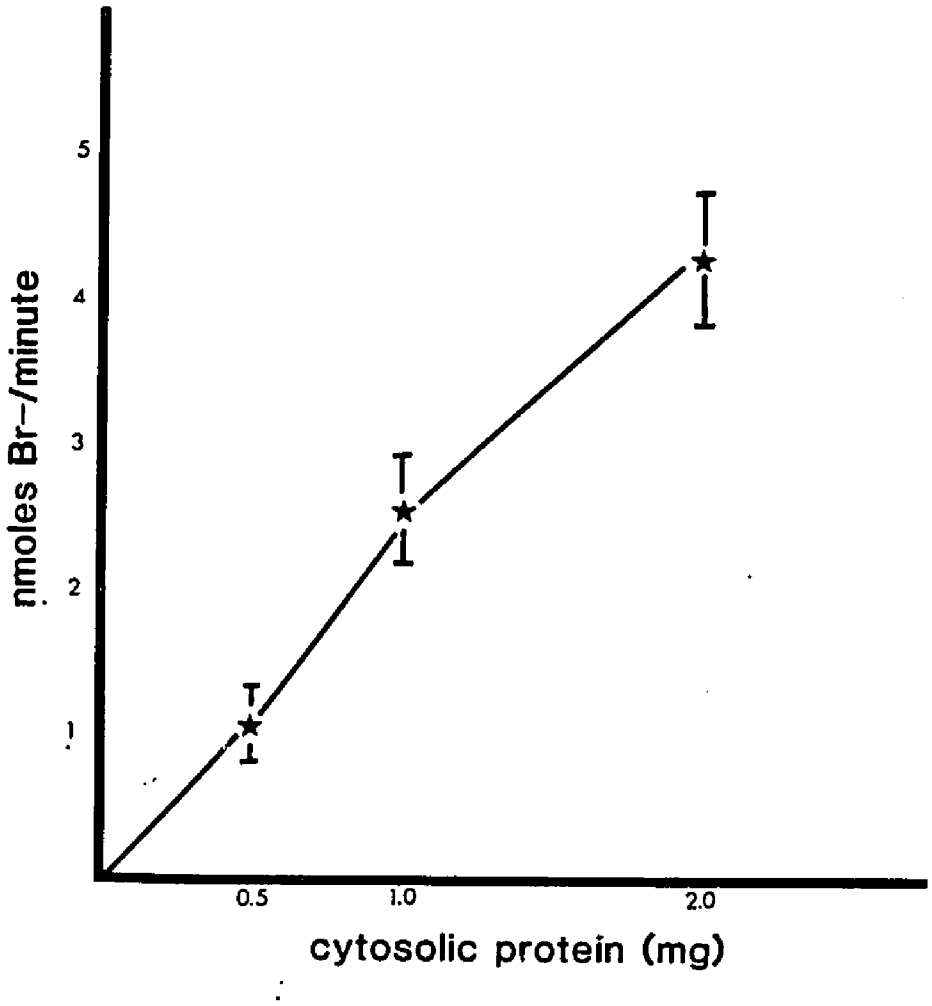

Figure 3. In vitro GSH-dependent metabolism of DBE by rat liver cytosol, as a function of protein concentration. -- In vitro conditions: 0.5, 1.0 and 2.0 mg/ml cytosolic protein, 5 mM GSH, 1 mM DBE.

concentration on the metabolism of 1,2-dibromoethane by mouse liver cytosol is presented in Figure 4. The level of 5 mM GSH did not produce a marked increase in the rate of GSH-dependent metabolism of DBE compared to a level of 1 mM GSH, at either of two protein concentrations.

Hepatic and Extra-hepatic GSH-dependent Debromination of 1,2-dibromoethane and 1,2-dibromo-3-chloropropane

Figure 5 presents a comparison in the rates of bromide release using DBCP and DBE and hepatic cytosols from rats and mice. Highest rates of bromide release were obtained with DBE using mouse liver cytosol, representing roughly a three-fold increase over the rates obtained using the rat enzymes. In contrast, the reverse situation is observed for the metabolism of DBCP, where the rat cytosol exhibits greater rates of GSH-dependent bromide release than the mouse.

Differences in the relative affinities of the rat and mouse enzymes towards the metabolism of DBE and DBCP are further reflected in the rates of extra-hepatic GSH-dependent metabolism (Figures 6 & 7). While hepatic rates of metabolism are significantly higher than those seen with the extra-hepatic cytosols, species differences analogous to those observed for DBE and DBCP using hepatic enzymes are seen with the 3 extra-hepatic tissue cytosols. Generally, the rates of extra-hepatic debromination follow the order kidney, testes, stomach, for both compounds. The sole exception to this pattern is seen in the metabolism of DBCP by the mouse, where the testicular rate appears to exceed that of the kidney. However, with the exception of the metabolism of DBE by the rat, where the kidney exhibits a significantly higher rate than

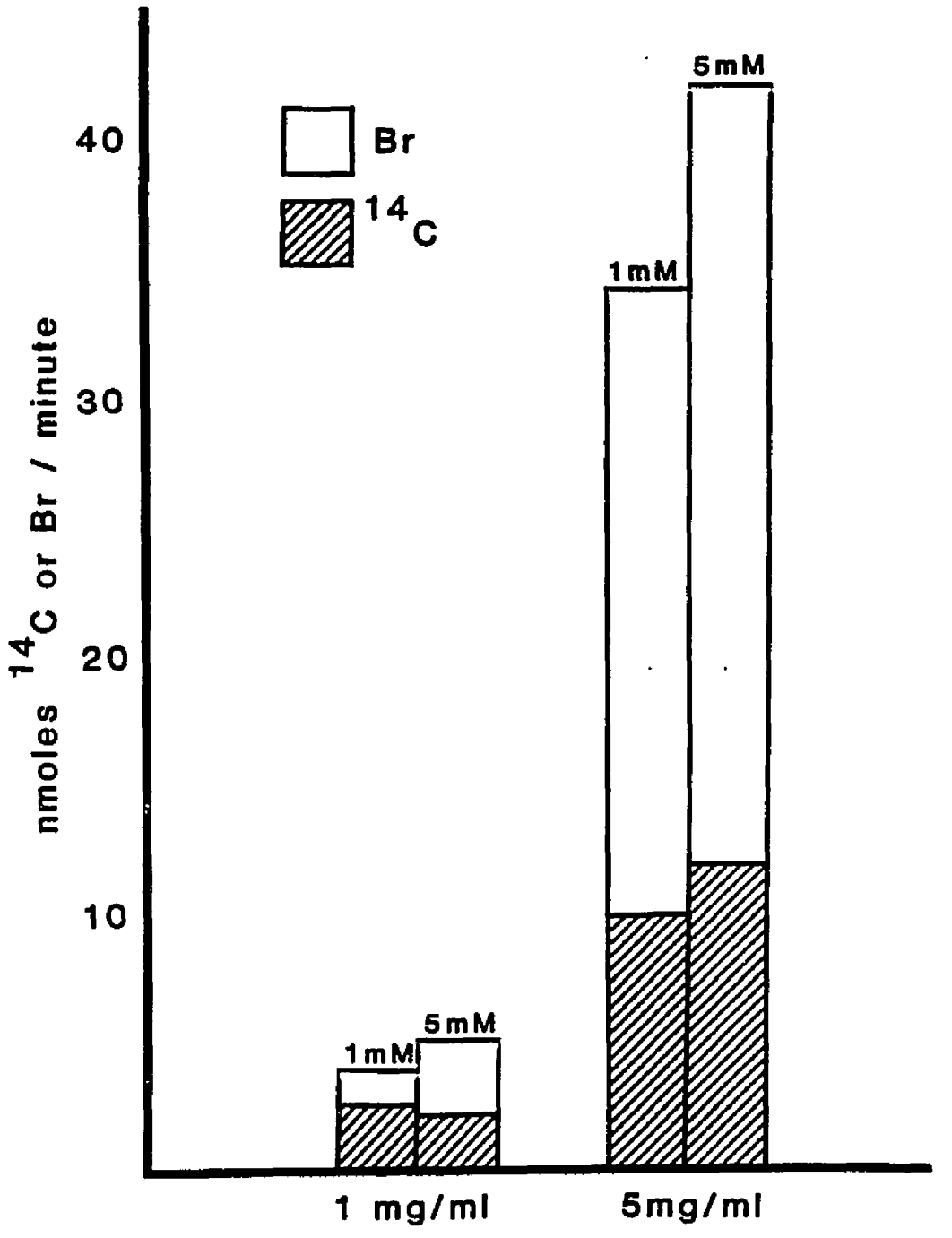

Figure 4. The effect of protein and GSH concentration on the <u>in vitro</u> GSH-dependent metabolism of DBE using rat liver cytosol.

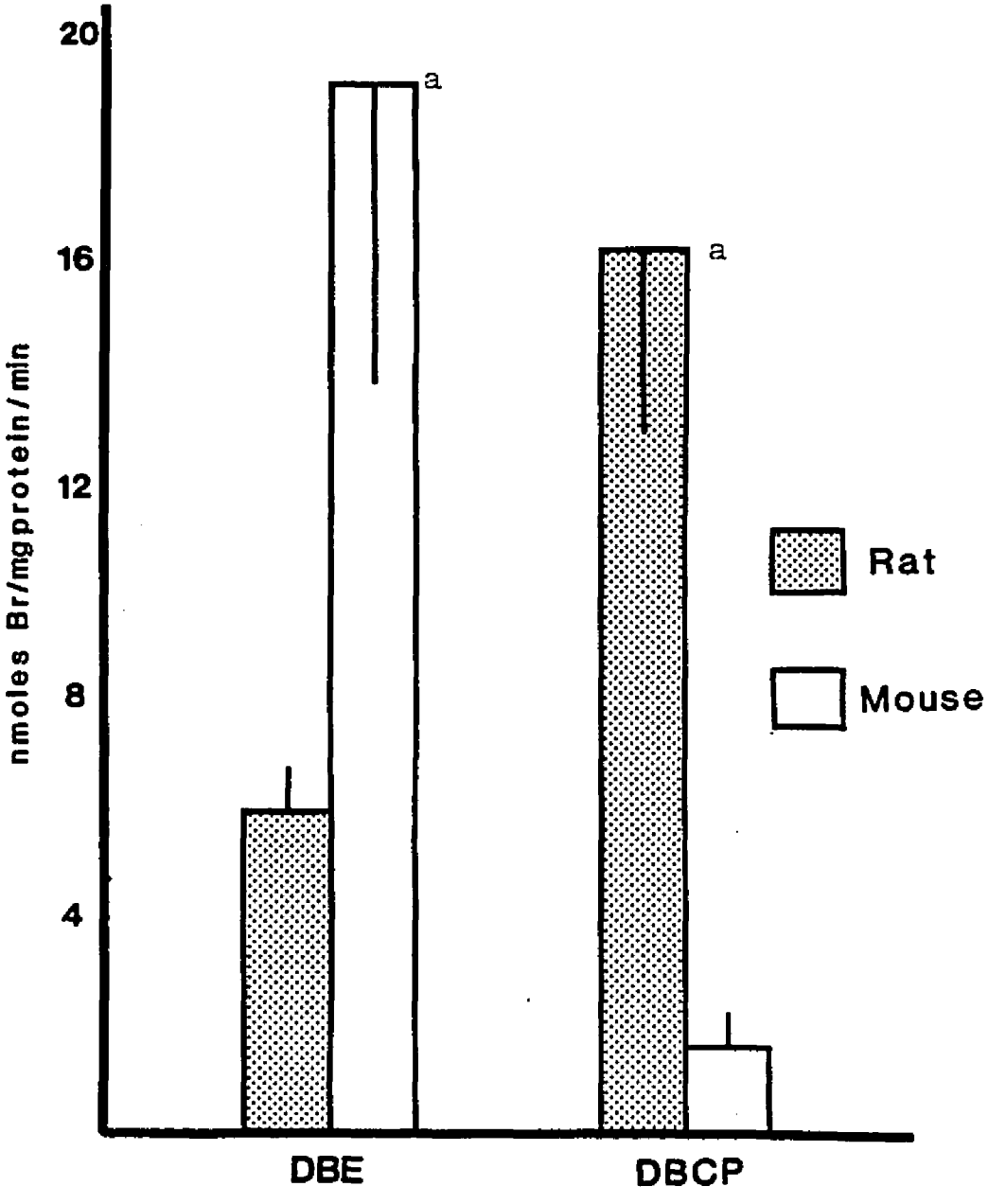

Figure 5. GSH-dependent metabolism of DBE and DBCP using rat and mouse liver cytosols. -- In vitro conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM substrate (DBE or DBCP). Data expressed as mean ± standard deviation obtained from multiple determinations using 2-6 sets of pooled cytosol.

[a]significant species difference between rates ($p < .05$).

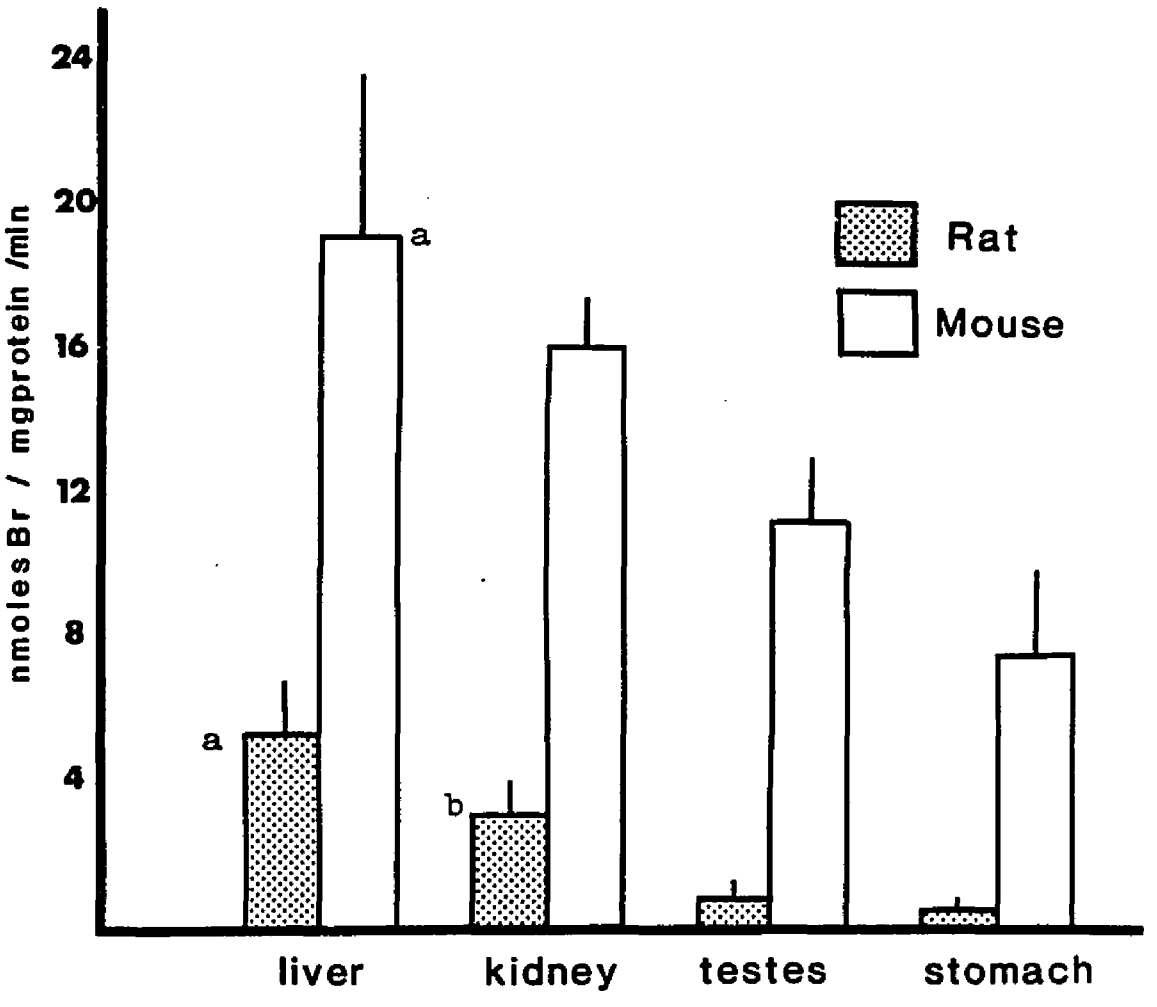

Figure 6. GSH-dependent metabolism of DBE by hepatic and extra-hepatic cytosols of the rat and mouse. -- In vitro conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM DBE.

[a] Significant differences between hepatic and extra-hepatic rates ($p < .05$).

[b] Significant differences between kidney and other extra-hepatic rates ($p < .05$).

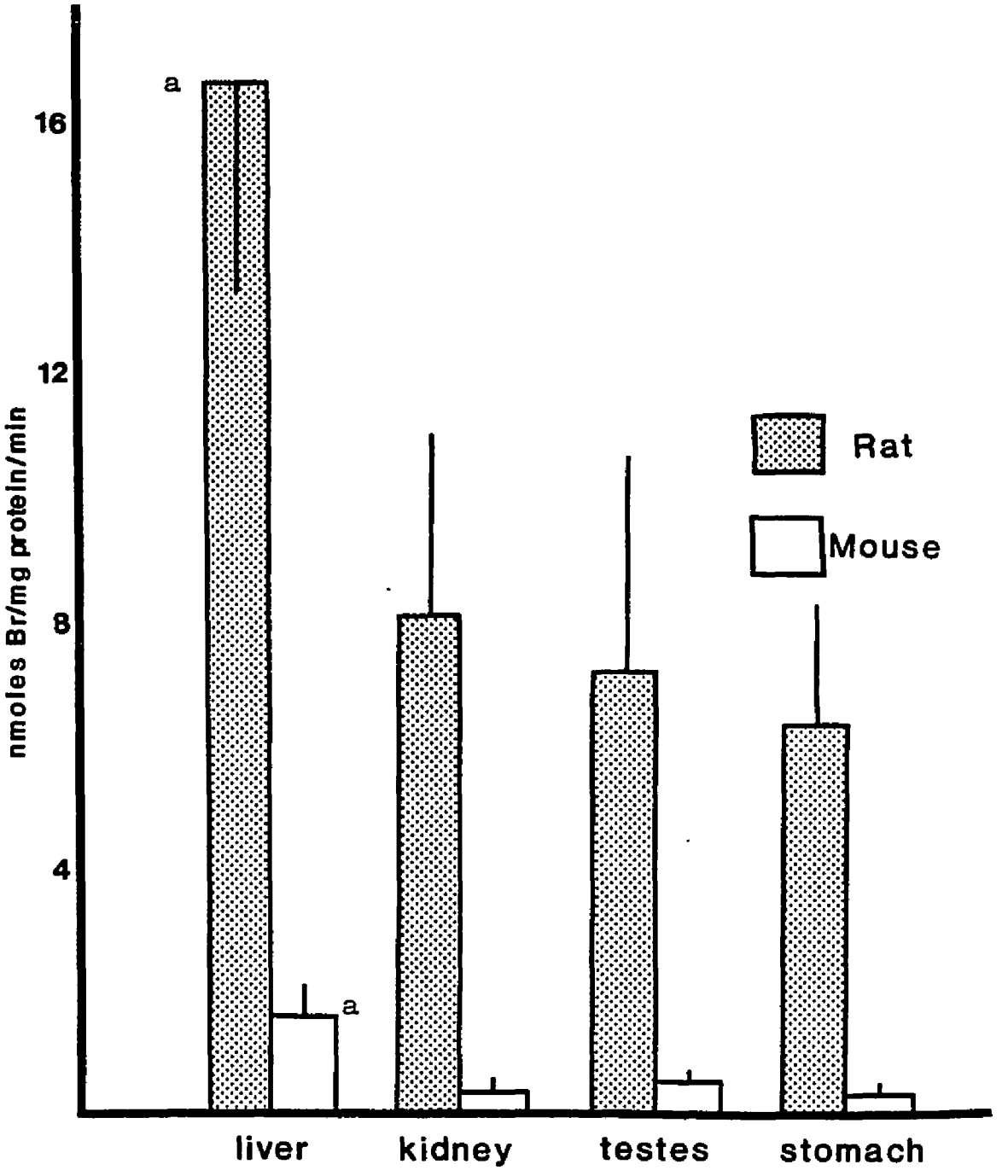

Figure 7. GSH-dependent metabolism of DBCP by hepatic and extra-hepatic cytosols of the rat and mouse. -- In vitro conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM DBCP.

[a] Significant difference between hepatic and extra-hepatic rates ($p < .05$).

either the testes or the stomach, no statistical differences exist between the rates of debromination between the three extra-hepatic tissues with either compound.

Generally, the extra-hepatic rates of DBE metabolism, as indicated by bromide release, appear higher than those seen for DBCP when compared to the corresponding liver rates (Tables 2 and 3). The highest extra-hepatic rate of metabolism of DBE, seen using mouse kidney cytosol, represents roughly 84% of the rate determined using the hepatic enzymes. In contrast, the highest extra-hepatic rate obtained with DBCP, seen for the rat testes cytosol, was only 49% that of the hepatic rate.

Tables 2 and 3 express the hepatic and extra-hepatic GSH-dependent debromination of DBE and DBCP as percentages of the transferase activity in a given tissue, as estimated using the substrate 1-chloro-2,4-dinitrobenzene. The GSH-dependent metabolism of short-chain halogenated hydrocarbons may be assumed to be predominantly the result of the enzymatic action of one or two specific transferase forms, as indicated by kinetic determinations using purified forms of the various transferases on a spectrum of structurally diverse substrates (Habig et al., 1974). As a consequence, the rates of metabolism of the compounds being examined here may represent an indirect means of comparing the relative prominance of these key transferase types within a given tissue and species, when these rates are expressed as a fraction of the overall transferase activity in that tissue.

Examination of the rates of GSH-dependent debromination of DBE by the various tissues of the rat and mouse, expressed as a percentage

Table 2. Hepatic and extra-hepatic metabolism of 1,2-dibromo-3-chloropropane expressed as percentages of activities determined using 1-chloro-2,4-dinitrobenzene. -- Rates expressed in nmoles product/mg protein/min.

Rat Tissue	[1]Rate	[2]% CDNB rate
Liver	16.6	2.05
Kidney	8.1	5.36
Testes	7.2	1.17
Stomach	6.3	7.97
Mouse Tissue	**[1]Rate**	**[2]% CDNB rate**
Liver	1.6	0.085
Kidney	0.36	0.063
Testes	0.51	0.126
Stomach	0.27	0.096

[1]Determined from in vitro experiments

[2]Rate expressed as a percentage of the activity for a given cytosol determined using the substrate 1-chloro-2,4-dinitrobenzene (Table 1).

Table 3. Hepatic and extra-hepatic metabolism of 1,2-dibromoethane expressed as percentages of activities determined using 1-chloro-2,4-dinitrobenzene. -- Rates expressed in nmoles product/mg protein/min.

Rat				
Tissue	Rate[1]		[2]% CDNB rate	
	^{14}C	Br	^{14}C	Br
Liver	2.58	5.76	0.316	0.707
Kidney	2.34	3.14	1.55	2.08
Testes	1.24	0.84	0.202	0.137
Stomach	0.33	0.56	0.418	0.709
Mouse				
Tissue	Rate[1]		[2]% CDNB rate	
	^{14}C	Br	^{14}C	Br
Liver	4.84	19.14	0.256	1.01
Kidney	1.29	16.07	0.225	2.80
Testes	0.86	11.80	0.212	2.91
Stomach	0.54	7.80	0.192	0.17

[1]Determined from in vitro experiments

[2]Rate expressed as a percentage of the activity for a given cytosol determined using the substrate 1-chloro-2,4-dinitrobenzene (Table 1).

of the overall transferase activity in that tissue, reveals a somewhat different pattern than was previously observed. As predicted, the rates of metabolism of DBE (as well as DBCP) are significantly reduced when compared to the rates obtained for the colorimetric substrate 1-chloro-2,4-dinitrobenzene. Among the 4 tissues of the rat, the kidney appears to exhibit the greatest relative ability towards GSH-dependent metabolism of DBE, compared to its overall rate of glutathione S-transferase activity. Similarly, it is the kidney, along with the testes, that show the highest activity in the mouse when expressed in this manner. Transferase activity towards DBE appears to represent a somewhat lower proportion of the overall activity in the liver and stomach tissues of both species.

Transferase forms with significant levels of activity towards DBCP appear to predominate in the stomach and kidney of the rat, whereas in the mouse, it is the testicular cytosol which shows the highest relative rate when expressed in this manner. Despite the high rate of debromination of both DBE and DBCP observed using the hepatic cytosols, in no instance are these rates the highest among the 4 tissues, when expressed as a fraction of the overall activity in the liver.

Hepatic and Extra-hepatic GSH-dependent Metabolism of 1,2-dibromoethane and 1,2-dichloroethane as indicated by the Formation of ^{14}C-labelled Aqueous-Soluble Product

Using the formation of non-extractable aqueous-soluble ^{14}C-labelled product as an indicator, the rates of hepatic GSH-dependent metabolism of DBE and DCE were compared (Figure 8). Results of the thin-layer chromatographic analysis of these extracted aqueous layers

Figure 8. Hepatic GSH-dependent metabolism of DBE and DCE using rat and mouse cytosols. -- In vitro conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM substrate (DBE or DCE). Data expressed as mean ± standard deviation obtained from multiple determinations using 2-6 sets of pooled cytosol. [a]Significant species difference between rates.

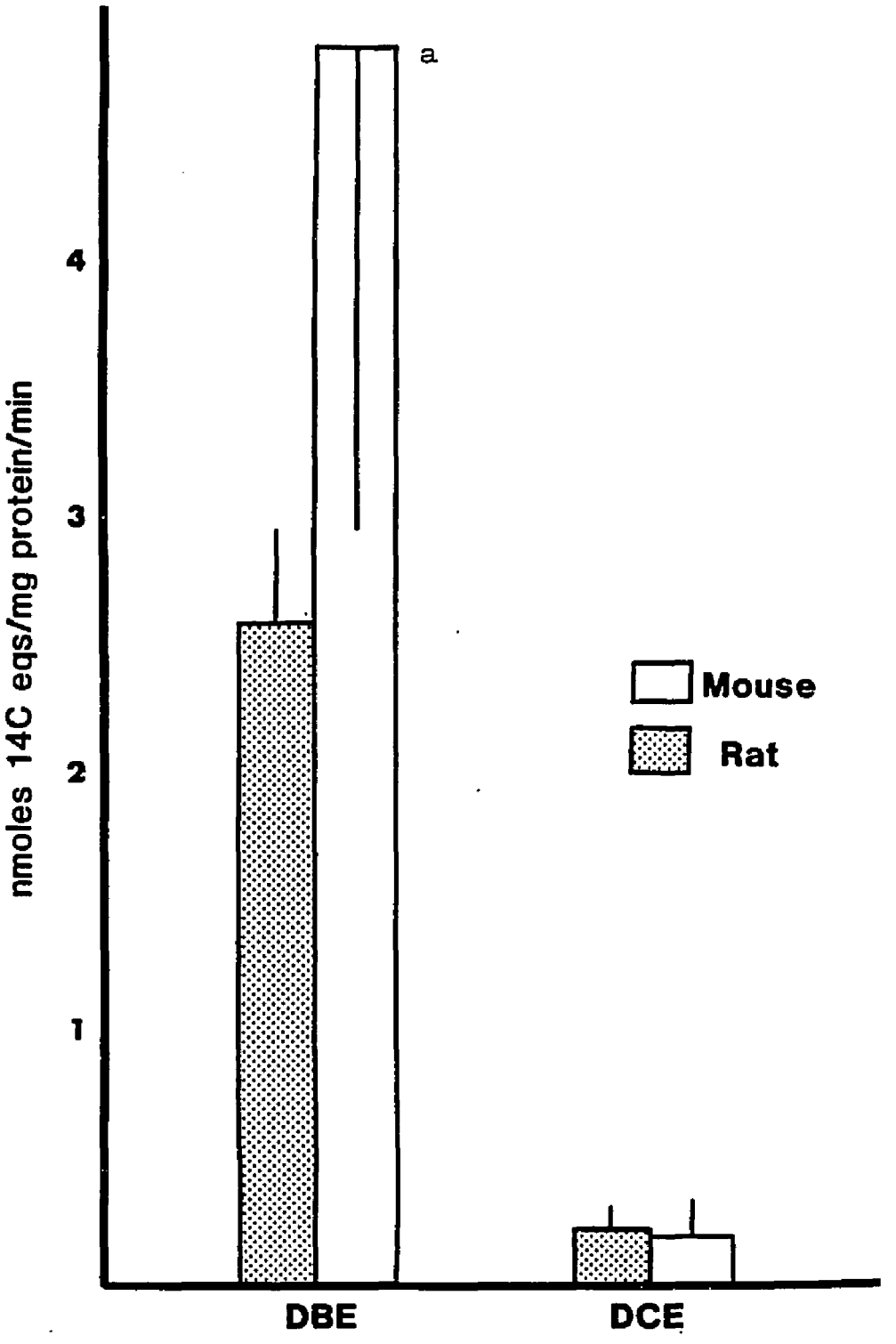

Figure 8. Hepatic GSH-dependent metabolism of DBE and DCE using rat and mouse cytosols.

are presented in Figure 9. Presence of ^{14}C radioactivity was correlated with that region of the plate showing the typical staining reaction associated with the presence of premercapturic acids (Knight and Young, 1958).

As previously observed for DBE using release of inorganic bromide as an indicator of metabolism, the mouse enzymes exhibited greater rates of metabolism as compared to the rat. Levels of GSH-dependent metabolism of DCE were substantially lower than those for DBE, with similar rates of DCE metabolism being seen in both species.

The higher rates of DBE metabolism seen for the mouse versus the rat using hepatic cytosol were not observed with cytosolic fractions from the kidney and testes, using the formation of ^{14}C-labelled product as an indicator of metabolism (Figure 10). Rates of extra-hepatic metabolism of DCE show correspondingly low levels; no significant species differences between the rat and mouse occur, with the exception of the kidney cytosol, where the rat exhibited higher enzymatic rates.

As previously observed for DBE using debromination as an indicator of metabolism, the extra-hepatic cytosols show reduced levels of activity compared to liver when the formation of ^{14}C-labelled product is quantitated. Tables 3 and 4 present the extra-hepatic rates for a given species and compound. Again, the apparent proficiency of the extra-hepatic cytosols may be ranked in the order kidney, testes, stomach in every case, however, in general, rate differences among the extra-hepatic tissues were not statistically different.

Expressed as a percentage of the overall transferase activity for a given tissue, a similar pattern as previously observed emerges.

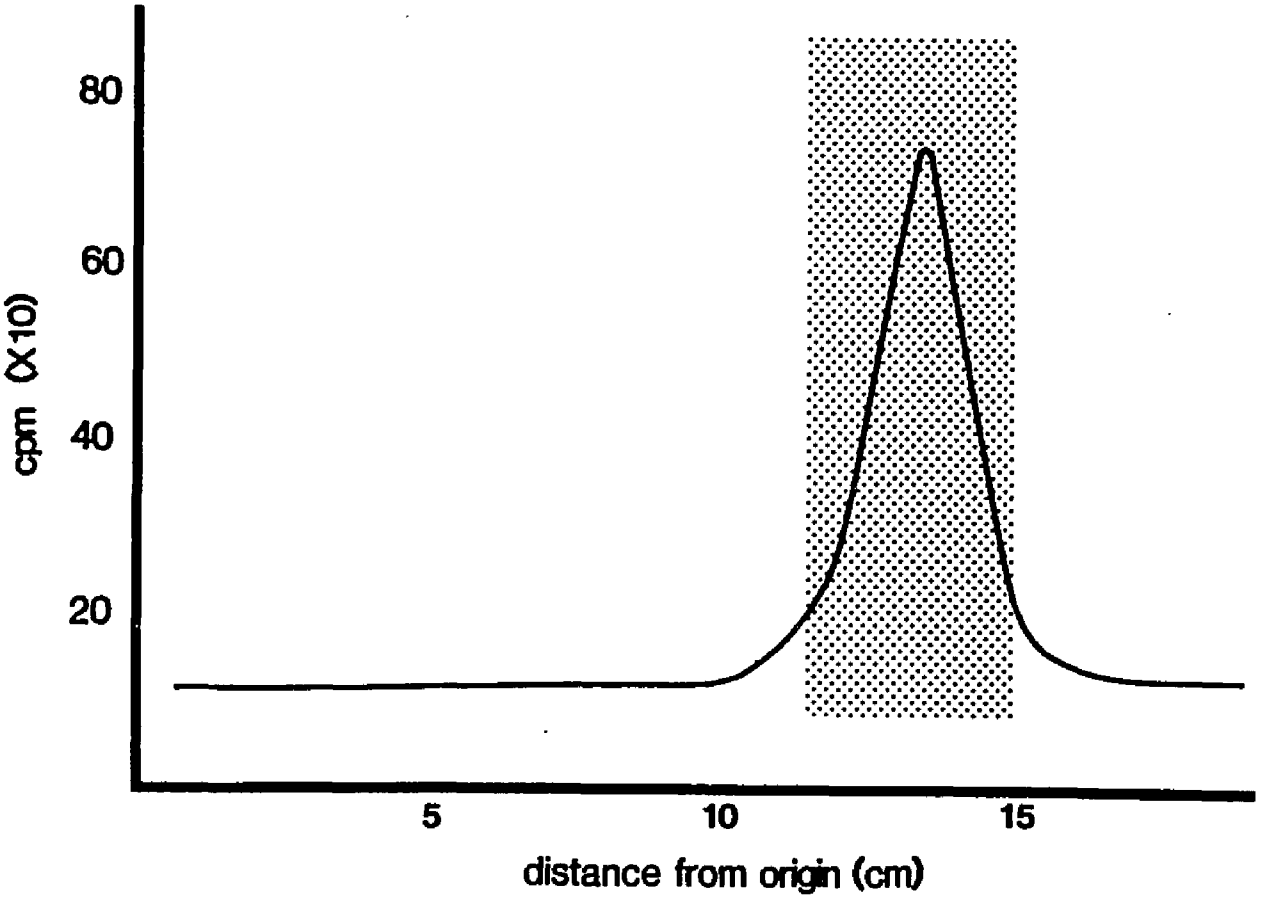

Figure 9. Thin-layer chromatographic separation of extracted aqueous layers from in vitro incubations. -- Shaded region corresponds to region of plate which reacted with spray indicator.

Figure 10. GSH-dependent metabolism of ^{14}C-DBE by hepatic and extra-hepatic cytosols of the rat and mouse. -- *In vitro* conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM ^{14}C-DBE.

[a]Significant difference between hepatic and extra-hepatic rates ($p < .05$).

[b]Significant difference between hepatic rate and those obtained for testes and stomach ($p < .05$)

[c]Significant difference between kidney and other extra-hepatic rates ($p < .05$).

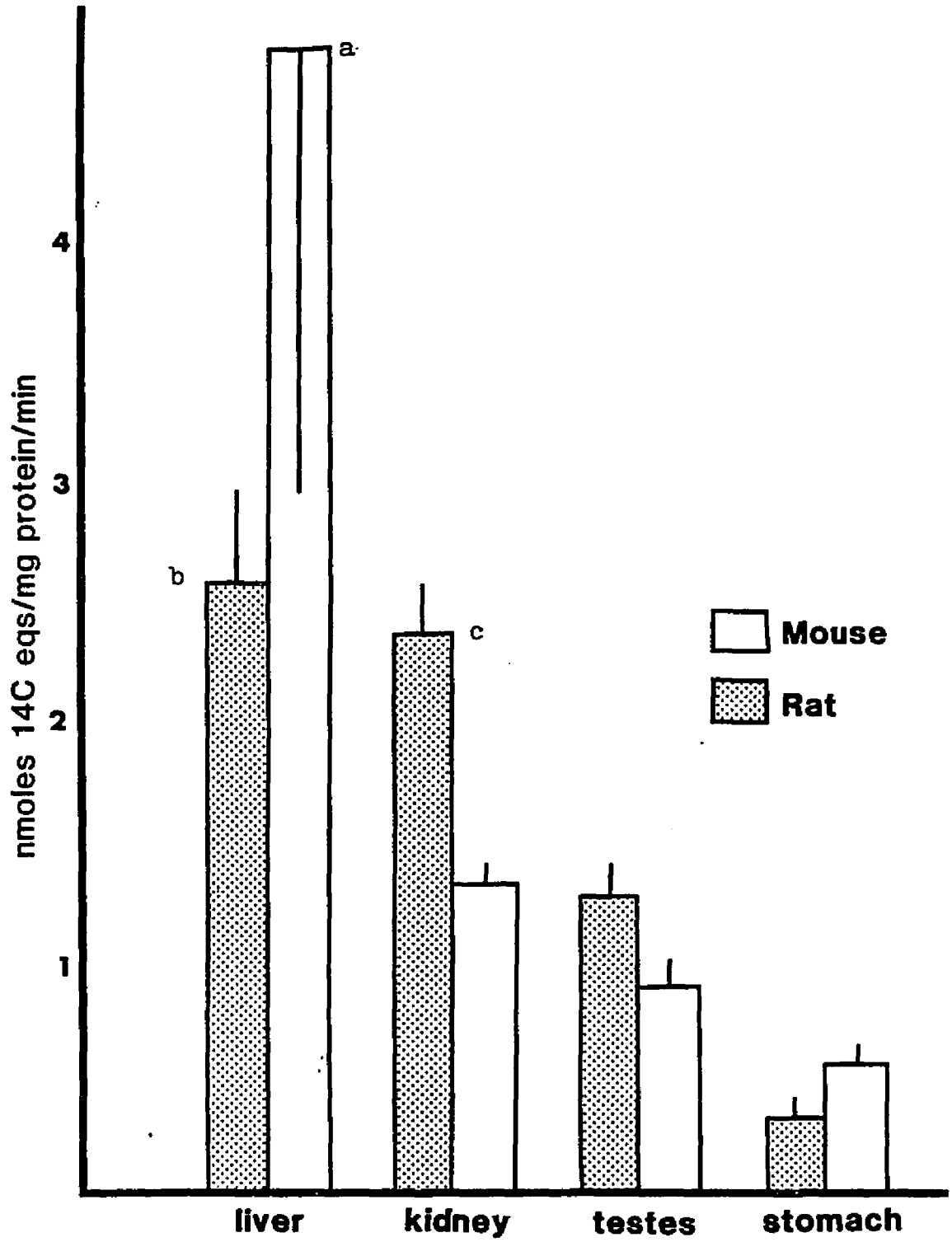

Figure 10. GSH-dependent metabolism of ^{14}C-DBE by hepatic and extra-hepatic cytosols of the rat and mouse.

Figure 11. GSH-dependent metabolism of ^{14}C-DCE by hepatic and extrahepatic cytosols of the rat and mouse. -- *In vitro* conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM ^{14}C-DCE.

[a]Significant differences between hepatic and extrahepatic rates ($p < .05$).

[b]Significant difference between kidney and other extrahepatic rates ($p < .05$).

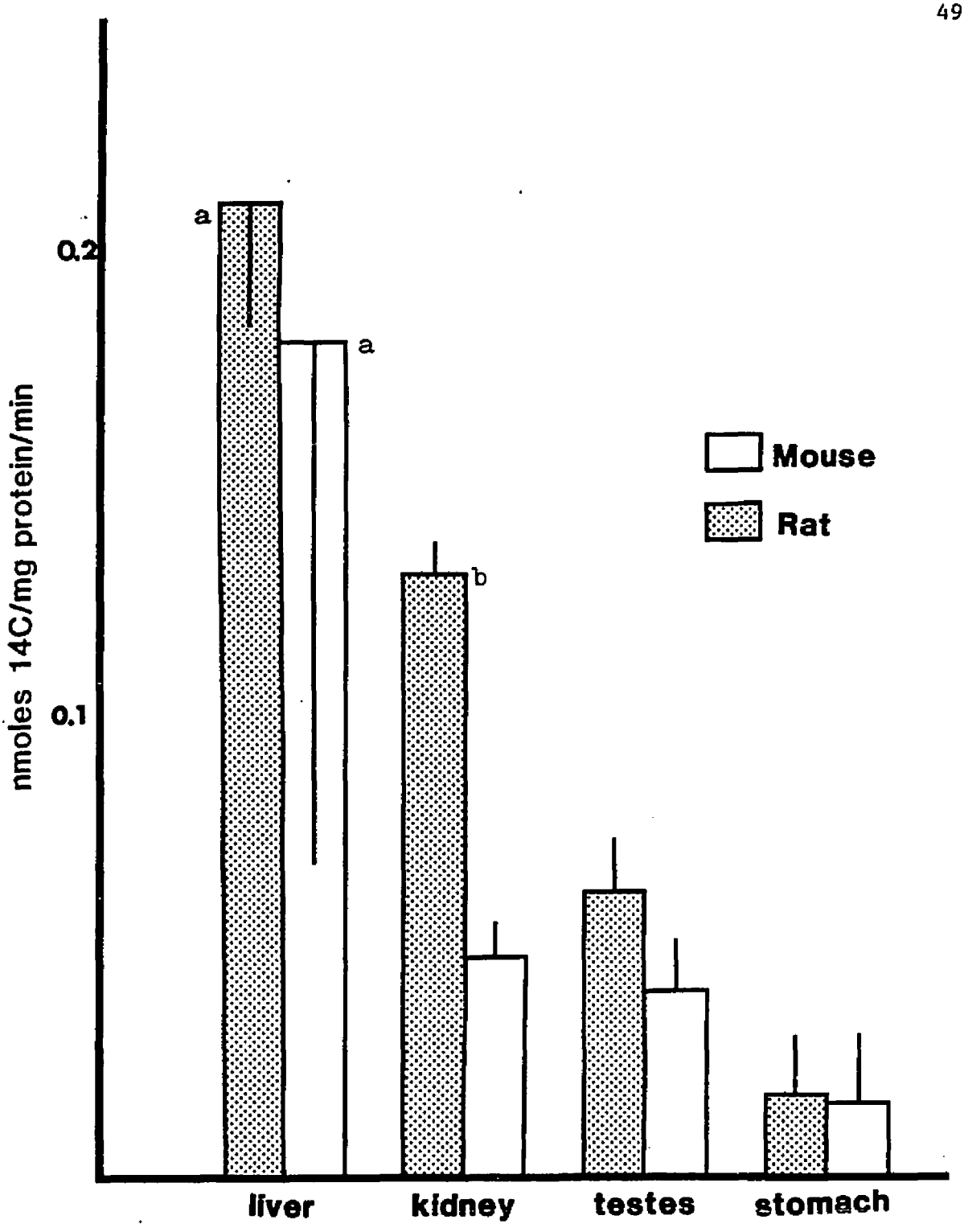

Figure 11. GSH-dependent metabolism of ^{14}C-DCE by hepatic and extra-hepatic cytosols of the rat and mouse.

Table 4. Hepatic and extra-hepatic metabolism of 1,2-dichloroethane expressed as percentages of activities determined using 1-chloro-2,4-dinitrobenzene. -- Rates expressed in nmoles product/mg protein/min.

Rat Tissue	[1]Rate	2% CDNB rate
Liver	0.029	0.026
Kidney	0.103	0.068
Testes	0.062	0.010
Stomach	0.019	0.024
Mouse Tissue	**[1]Rate**	**2% CDNB rate**
Liver	0.182	0.0096
Kidney	0.049	0.0086
Testes	0.041	0.0100
Stomach	0.017	0.0060

[1]Determined from in vitro experiments

[2]Rate expressed as a percentage of the activity for a given cytosol determined using the substrate 1-chloro-2,4-dinitrobenzene (Table 1).

In general, extra-hepatic rates of metabolism of DBE and DCE, when measured by the formation of ^{14}C-labelled product, represent a greater proportion of the overall tissue transferase activity than observed for the liver. A singular exception to this trend is noted in the mouse for the metaboilsm of DBE, where the liver rate appears to account for the greatest percentage of the overall transferase activity among the 4 tissues.

Decreased Rate of GSH-Dependent Metabolism of 1,2-Dibromoethane over Time Using Mouse Liver Cytosol

A lack of linearity in the GSH-dependent metabolism of DBE was noted using cytosolic fractions prepared from mouse liver. This decrease in the overall rate usually became apparent between 2 and 5 minutes, using both the ^{14}C-labelled product assay and debromination as indicators of metabolism (Figure 12). A lack of linearity was not evident in experiments using the extra-hepatic cytosolic enzymes from the mouse with DBE. Rates of GSH-dependent metabolism for DBE by mouse enzymes presented in Figures 3, 4, 5, 6, 8 and 10 represent rates determined from regions of linear enzyme activity (i.e. early time points).

The mechanism responsible for this rapid reduction in rate has yet to be fully elucidated. Preliminary experiments do not appear to indicate an increased rate of destruction of either the substrate (DBE), or the cofactor (GSH), in the presence of the hepatic mouse cytosols. No experiments were carried out to more specifically examine the possibility of inactivation of the enzymes involved, by parent DBE or a potential metabolite.

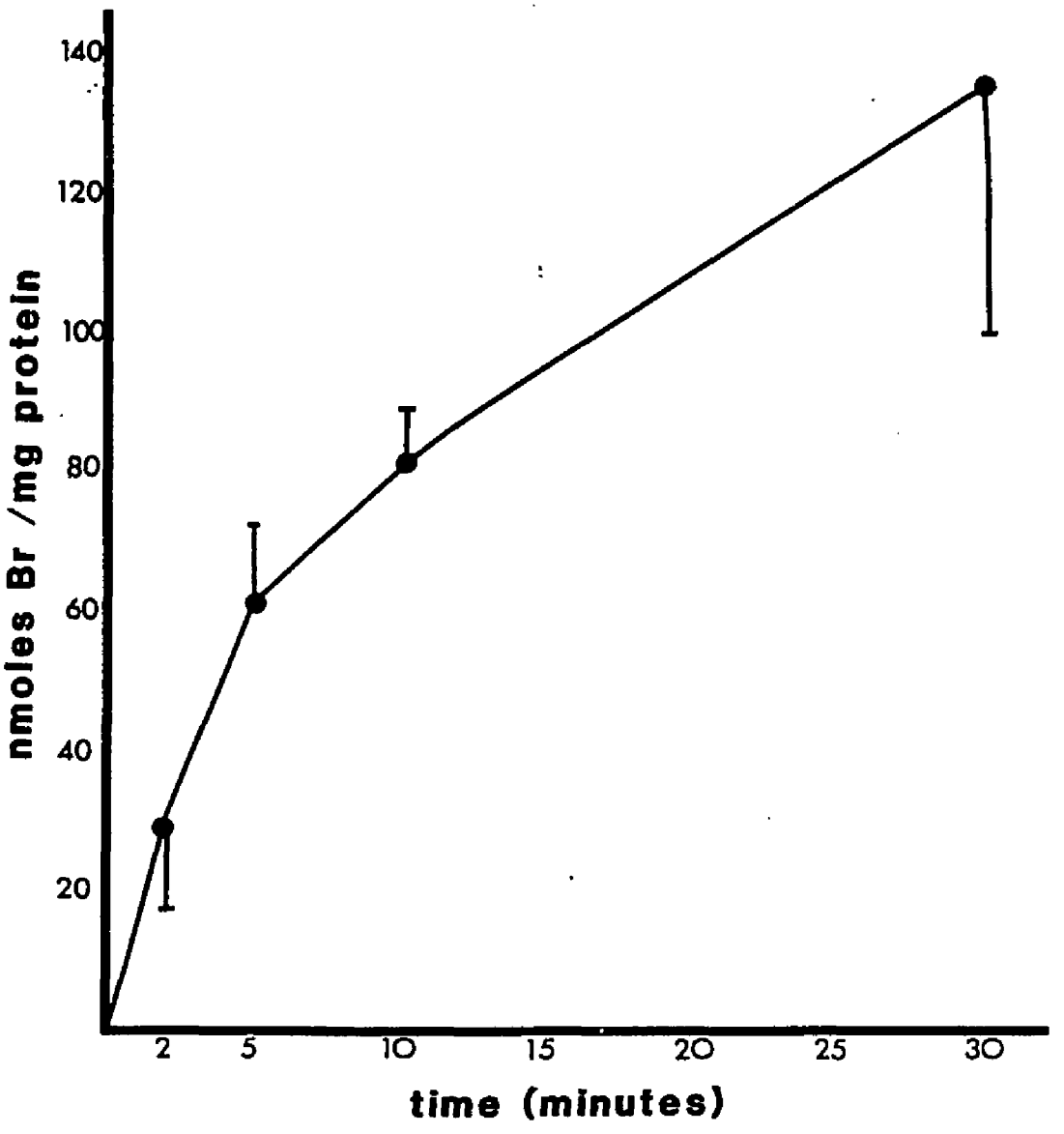

Figure 12. GSH-dependent debromination of DBE using mouse liver cytosol. -- In vitro conditions: 1 mg/ml cytosolic protein, 5 mM GSH, 1 mM DBE.

DISCUSSION

The role of glutathione conjugation in the development of toxicity following exposure to 1,2-dibromoethane is supported by a number of lines of evidence. The goal of this study was to assess the potential for a GSH-dependent mode of bioactivation for DBE, and the structurally similar 1,2-dichloroethane and 1,2-dibromo-3-chloropropane. Evidence in support of, or contradictory to such a mechanism of toxicity can only be indirectly inferred from the data presented here, through correlation of the experimentally determined rates of metabolism with the observed patterns of *in vivo* toxicity.

As previously described, comparison in the rates of enzymatically-mediated glutathione conjugation between 1,2-dibromoethane, 1,2-dichloroethane and 1,2-dibromo-3-chloropropane using hepatic and extra-hepatic cytosols involves the use of two distinct assays of metabolism in this study. Examining the rates of reaction as shown in Table 3 reveals that, only in the case of 1,2-dibromoethane was it possible to use both the release of inorganic bromide and the formation of ^{14}C-labelled water soluble product in the estimation of metabolism. Where direct comparison between rates of debromination and formation of ^{14}C-labelled product are permitted, the rates as determined by bromide release are generally higher, with this difference in most cases not occurring in a stoichiometric ratio.

Theories to explain this greater release of bromide compared to the formation of water soluble product include the possibility of a

non-enzymatic release of inorganic bromide not associated with the binding of glutathione. The occurrence of such a phenomenon is, however, easily controlled for with the use of the appropriate blanks. The formation of a bis conjugate in which two molecules of reduced glutathione displace 2 bromide atoms from a single molecule of the parent compound has been shown to occur under certain reaction conditions using 1,2-dibromoethane, with the corresponding metabolite, S,S'-ethylene bis glutathione having been identified in the livers of rats fed DBE (Nachtomi, 1970). The subsequent urinary metabolite, S,S'-ethylene bis cysteine, has been found following administration of a single dose of 1,2-dichloroethane to mice (Yllner, 1971).

This reaction involving conjugation of a second molecule of glutathione with the mono-conjugate is, however, kinetically unfavorable, and would be likely to occur at a significant rate only after a high ratio of the mono-conjugate to parent compound existed (Nachtomi, 1970). Thin-layer chromatography of fully extracted aqueous fractions revealed migration of a single band identifiable as a reaction product by both the presence of ^{14}C-label, and through the use of the spray reagent indicator (Figure 9). Had a significant amount of the bis conjugate been formed in the reaction, its co-elution with the mono-glutathione conjugate would not be expected, due to large differences in molecular weights.

Livesey and Anders (1979) have demonstrated the occurrence of an alternate reaction, the first rate-limiting step of which involves the GSH S-transferase mediated conjugation of reduced glutathione with a dihaloethane. The resulting S-2 haloethylglutathione may then undergo a

direct, non-enzymatic reaction with a second molecule of GSH, resulting in the foirmation of oxidized glutathione and ethylene. This reaction is favored under somewhat basic conditions in which the ionization of the sulfhydryl group of glutathione is increased. The authors demonstrated the formation of ethylene under in vitro conditions using 1,2-dichloroethane and cytosolic fractions prepared from rat liver and kidney, and found it to be linear with time and protein concentration. The formation of ^{14}C-ethylene, which would be lost during the processing of the samples in this study, could explain a greater than two-fold ratio in some instances, of bromide release compared to formation of ^{14}C-labelled water-soluble product.

The rates of GSH-dependent metabolism of 1,2-dibromoethane determined using cytosolic fractions prepared from the liver, kidney, testes and stomach tissues of rats and mice are much lower than those previously observed for the same tissue cytosols using the colorimetric assay. The activities of the four tissue cytosols relative to one another, however, are similar, with the liver exhibiting the highest rates with all substrates in both species. Extra-hepatic rates appear to follow in the descending order: kidney, testes, stomach. A similar pattern among the liver and extra-hepatic rat tissue cytosols using DBE as a substrate has been reported by Hill et al (1978). A potential role for the extra-hepatic metabolism of DBE in vivo is clearly supported by findings relating to its disposition in the body. DBE's pattern of distribution following a single i.p. injection is characterized by rapid uptake in all tissues, with peak levels being reached within 30 min.

This is followed by a rapid drop, possibly due to significant levels of metabolism, with very low levels being seen by 8 hours (Plescia, 1981).

In comparing the rat to the mouse, generally higher rates of GSH-dependent metabolism are seen in the latter, using the release of inorganic bromide as an indicator of DBE's metabolism. With the use of the ^{14}C-labelled product assay, however, observed rates of metabolism are not significantly different between the species. This apparent conflict in the relative metabolizing ability depending upon which assay was used could be accounted for by a difference in the relative rates of ethylene formation in the two species. This explanation, however, would be more plausible in an *in vivo* situation where differences in the levels of free GSH, and the availability of unreacted parent compound could not be controlled, and might differ between species affecting the rate of non-enzymatic ethylene formation.

Determinations of the rates of GSH-dependent metabolism of 1,2-dichloroethane using hepatic and extra-hepatic tissue cytosols of the rat and mouse show DCE to be a much poorer substrate in both species than its brominated analog, exhibiting rates of approximately one-tenth those seen for DBE with the corresponding cytosols. If indeed, it is the GSH-dependent route which is responsible for the major toxicity of DCE and DBE, this greater rate of metabolism of the latter is reflected in the observed *in vivo* toxicity of the two compounds. The oral LD_{50} in rats for example, is 146 mg/kg for DBE, versus 680 mg/kg for DCE (Rowe et al., 1952; Maltoni et al., 1980). That the observed *in vivo* toxicity of DCE is generally associated with only the liver and kidney

may simply be accounted for by the negligible rates of its metabolism the other extra-hepatic tissues tested here.

Of the three compounds under consideration, 1,2-dibromo-3-chloropropane exhibits the highest rates of metabolism in cytosolic fractions from the rat. Similarly high rates of metabolism are not, however, seen for DBCP in the case of the mouse tissue cytosols. The evidence for the role of a GSH-dependent route of metabolism in the development of toxicity due to DBCP exposure is largely circumstantial, being derived from its structural similarity to compounds such as DBE and DCE, as well as its similar pattern of predominantly extra-hepatic toxicity. The greater rates of metabolism of DBCP observed for the rat tissue compared to those of the mouse are in accord with observed species differences in in vivo toxicity. Oral LD_{50}s of 300 and 410 mg/kg have been reported in the rat and mouse, respectively (Torkelson et al., 1961).

Although the observed rates of GSH-dependent metabolism of all three compounds by cytosolic enzymes of the stomach are relatively low compared to other tissues, it should be noted that regional differences in transferase activity may exist within the stomach itself (Pinkus et al., 1977). Using an immunohistochemical technique involving an antibody prepared against ligandin (glutathione S-transferase B), Campbell et al. (1980) examined the presence of this enzyme within different regions of various human tissues. In accord with its observed pattern of in vivo toxicity, the highest levels of regional transferase activity in the gastro-intestinal tract were in the stomach itself. A similar correlation between the presence of transferase enzymes and the

locus of *in vivo* toxicity were noted in the kidney, where the presence of the enzyme was found to be strictly confined to the proximal convoluted tubule and the thick segment of the loop of Henle.

Tables 2-4 express the rates of hepatic and extra-hepatic GSH-dependent metabolism of DBE, DCE and DBCP as a percentage of the overall level of transferase activity in each tissue. For the reasons previously described, expression of the data in such a manner may indicate the relative predominance of those transferase sub-types most responsible for the metabolism of short-chain halogenated hydrocarbons. In general, these results would seem to indicate a greater relative role for such transferase subtypes in the extra-hepatic tissues than in the liver. If such a situation did indeed exist, it would tend to agree with the predominance of an extra-hepatic pattern of toxicity for compounds bio-activated along a GSH-dependent route. Alternatively, for other compounds, such as acetaminophen for example, for which glutathione conjugation appears central to detoxification, a predominantly hepatotoxic response might be postulated, due to the somewhat limited capacity for GSH-conjugation in the liver (Mitchell et al., 1973).

Studies designed to compare the various transferase types in different tissues seem to indicate that, for a given species, the enzyme forms present in the various tissues are similar, if not identical. In one such study, Kaplowitz et al. (1976) compared the transferase activities present in rat liver and kidney supernatant fractions with respect to their substrate specificities, as well as their Michaelis-Menten and inhibitory kinetics. Km values for 4 of the 5 substrates examined were very similar, with the kidney enzymes showing a somewhat

lower affinity for one of the aralkyl substrates tested. GSH transferases found in the gastro-intestinal tract appear to correspond closely to other forms present in both the liver and kidney, as evidenced by their immunologic cross-reactivity, their similar elution profiles, and susceptibility to induction by various chemical agents (Pinkus et al., 1977; Clifton, 1977). In work concerned with the role of glutathione conjugation in the in vitro genotoxic effects of 1,2-dichloroethane, Rannug (1980) has indicated the central role of rat transferase types A and C in the production of the reactive product. Hill et al. (1978) determined the optimum conditions for the rat liver glutathione S-transferase primarily responsible for the metabolism of DBE, estimating Km and Vmax values at 25 mM and 2.1 μmol/g/min, respectively.

In general, DBE, DCE, and DBCP all show rates of extra-hepatic GSH-dependent metabolism comparable to those observed using liver cytosol from the same species. Yet in every case, the hepatic enzymes exhibit the greatest levels of activity, a situation that would seem to point to the liver as a primary target organ for toxicity. As previously noted, however, such is not the case for these three compounds.

A possible explanation may lie in the relative proportion of the parent compound being metabolized along a GSH-dependent route, versus that amount initially entering into a cytochrome P-450-mediated oxidative pathway. As previously discussed, Van Bladeren and co-workers (1981) estimated the metabolism of d_4-DBE along the GSH-dependent pathway versus the oxidative pathway to occur in a 1:4 ratio. It should

be stressed, however, that this represents the ratio in the whole animal, being derived from amounts of specific urinary metabolites, and may not reflect the relative significance of the two pathways within specific tissues or organs.

Several studies have indicated the liver as having markedly higher cytochrome P-450 levels as compared to other tissues (Bend et al., 1972; Orrenius et al., 1973). In a study in which several phase I and phase II activities were compared in rabbit lung and liver, lung levels of activity towards oxidative substrates were frequently negligible or several orders of magnitude less than the levels seen for the liver (Gram, Litterst and Minnaugh, 1973). Phase II activities were not generally as greatly reduced in the lung compared to the liver, with lung aryltransferase activity representing about one-fifth that observed in the liver. Significant levels of epoxide transferase activity have also been reported for the human lung (Grover, Hewer and Sims, 1973).

Extra-hepatic oxidative activity appears to be predominatly associated with a P-448, aryl-hydrocarbon hydroxylase, rather than a P-450 type of activity (Lake et al., 1973), as evidenced by greater levels of observed induction with 3-methylcholanthrene than with phenobarbital. Generally, phase II activity is believed to play a relatively greater role in extra-hepatic tissue compared to liver (Gram et al., 1973; Plescia , 1981; Short et al., 1979). Following pretreatment with diethyl maleate (known to deplete levels of free GSH), reduced levels of covalent binding by ^{14}C-DBE were observed in the extra-hepatic tissues of rats (Plescia , 1981). In contrast, phenobarbital pretreatment did not result in increased extra-hepatic

binding, implying a greater role for the phase II route in the extra-hepatic mediation of the response.

Although the liver may exhibit high rates of transferase activity towards these compounds, it may show equally high or higher levels of oxidative metabolism with the same compounds. An extra-hepatic tissue such as the testes may exhibit rates of GSH-dependent metabolism at levels of 50% those seen in the liver, but may have a negligible capacity for the oxidative route. The result would be a funnelling of virtually all of the parent compound into the GSH-dependent route.

In general, estimates as to the relative extents to which two metabolic pathways operate within an organ or tissue in the intact animal pose a difficult problem. However, in the case of DBE, it was noted that the intermediates produced in the two alternate routes show different patterns of *in vivo* and *in vitro* binding (Shih and Hill, 1981). Oxidative metabolites have been shown to account for most of the observed protein binding, while intermediates of the GSH-dependent route of metabolism preferentially bind DNA. Short et al. (1979) examined the relative ratios of protein to DNA binding in rats administered ^{14}C-DBE. The highest levels of total binding were seen in the kidney, followed in descending order by the liver, stomach and testes. Highest levels of protein binding were observed in the liver, with the highest total DNA binding being seen in the stomach. These results indicate a relative predominance of the GSH-dependent route of metabolism in the stomach. Conversely, the high degree of protein binding seen in the

liver implies the greater role for the oxidative route of metabolism in that organ.

The relative levels of endogenous glutathione within various tissues may also be significant to the expression of toxicity by this group of compounds. The binding of reactive intermediates to cellular nucleic acids may be greatly reduced in those organs which contain high levels of free GSH. Kluwe et al. (1981) looked at levels of reduced non-protein sulfhydryl compounds (NPS) in various tissues of control, DBCP-, and DBE-pretreated mice. Administration of single doses of DBCP and DBE produced dose-dependent decreases in reduced NPS compounds in all tissues examined, becoming apparent at lower doses in the liver and kidney than elicited a detectable decrease in the stomach and testes. The findings agree with the high rates of metabolism observed for DBE and DBCP by the cytosolic enzymes of the liver and kidney seen in the current study. Lower levels of NPS groups in the rat liver compared to the mouse (2.36 and 3.57 umoles/gm, respectively) may account for the greater toxicity of these compounds in the rat, if correspondingly low reduced NPS levels are assumed to exist in extra-hepatic rat tissues as well. In a separate study by the same author (Kluwe, Harrington and Cooper, 1981), the effects of DBE, DBCP and several other organohalides on renal tubular cells were compared. Similar reductions in GSH levels were seen, and were shown not to be associated with corresponding increases in oxidized glutathione.

Correlations between regional differences in GSH levels in the stomach, and the patterns of carcinogenesis resulting from polycyclic aromatic hydrocarbons have been shown by Boyd, Sasame and Boyd (1979).

GSH levels in the gut were shown to vary diurnally, with levels in the glandular stomach being 2-4 times greater than in other regions. While this region is less susceptible to cancer resulting from exposure to polycyclics, it is the regional target for the compound N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). This latter compound's action as an alkylating agent is enhanced by the presence of reduced glutathione. In examining differences in susceptibility to the testicular effects of DBE in bulls and rams, Amir and Tadmor (1981) have suggested the presence of greater levels of physiologically occurring sulfhydryl compounds in the latter may be critical to the reduced toxicity seen in that species.

In summary, GSH may be central to both the production of the toxic intermediates of DBCP, DBE and DCE, and to their subsequent detoxification. GSH levels may even play a role in determining the actual mechanism of the enzymatic reaction catalyzed by the glutathione S-transferases (Pabst, Habig and Jakoby, 1974). In a study involving a purified form of rat liver glutathione S-transferase A, the authors determined that the order of substrate-cofactor binding was influenced by the concentration of free GSH, resulting in two distinct Km values for the same reaction.

A second theory to explain inconsistencies in the observed rates of GSH-dependent metabolism, and the observed patterns of <u>in vivo</u> toxicity involves the lability of the enzymes involved in biotransformation. Figure 12 illustrates that the GSH-dependent metabolism of DBE as measured by bromide release decreases over time, this loss of linearity first becoming apparent between 2 and 5 minutes. This plateau

phenomenon suggests a greater lability of the mouse enzymes, and may be the result of a direct toxicity towards the transferase enzymes, exerted by products or intermediates generated in the metabolism of DBE.

The net result of such a phenomenon would be a reduction in whole animal toxicity through a decrease in the overall production of the toxic product. A similar pattern of DBE metabolism has not been observed for the extra-hepatic tissues of the mouse, possibly due to lower overall rates of GSH-dependent metabolism in these organs, thereby preventing an excessive build-up of the toxic product.

CONCLUSIONS

Despite recent efforts aimed at elucidating the mechanism through which DBE, DCE, DBCP, and many other short-chain halogenated hydrocarbons exert their toxicity, numerous questions remain unanswered. A great deal of evidence indicates the importance of a GSH-dependent reaction in the bioactivation of DBE and DCE, making these compounds uniquely of interest among their class. Yet to assess the actual contribution such a pathway may make towards the development of toxicity *in vivo* presents a problem.

The data put forth in this thesis appear to support the role of a GSH-dependent mode of bioactivation for DBE, DCE and DBCP. This suggestion comes largely from the relatively high levels of extra-hepatic metabolism seen, and correlations between these rates and the observed patterns of *in vivo* toxicity. Certain findings, however, appear to contradict the relevance of the GSH-dependent route of metabolism in the expression of toxicity. Most notable among these is the high hepatic rate of metabolism observed for each of the three compounds, despite their characteristic lack of *in vivo* liver toxicity.

Such inconsistencies may be explained on the basis of a number of complicating factors present in the whole animal model. Such factors, including the levels of free glutathione in the various tissues, regional differences in transferase activity within a given tissue, and the relative predominance of competing bioactivating or detoxifying pathways, must be addressed in future studies before definite

conclusions can be drawn. A survey of the GSH-dependent metabolism of other structurally similar compounds is also indicated, beginning with those compounds which would be especially likely or unlikely to form the proposed reactive intermediate responsible for toxicity.

The final verdict concerning the bioactivation and detoxification of DBE, DCE and DBCP will surely come not from the findings of a single study. Rather, this conclusion will emerge from the results of work in a variety of areas concerned with the physical, chemical and toxicological properties of these compounds.

APPENDIX A

LINEARITY OF *IN VITRO* METABOLISM WITH TIME AND PROTEIN CONCENTRATION

Table A.1. Hepatic and extra-hepatic GSH-dependent debromination of 1,2-dibromoethane (DBE) using rat and mouse cytosols. -- Data expressed as mean ± standard error based upon multiple determinations using 2-6 sets of pooled cytosols.

Rat	Released inorganic bromide[1]			
Time (min)	liver	kidney	testes	stomach
2	11.5±0.8	4.7±0.4	2.6±0.1	0.8±0.1
5	28.8±7.0	15.2±2.0	6.6±2.6	1.9±0.4
20	117.7±14.1	53.9±13.9	24.6±8.1	15.3±6.5
Mouse				
Time (min)	liver	kidney	testes	stomach
2	39.2±9.0	34.2±6.0	17.8±8.2	11.6±0.9
5	97.6±16.8	80.0±12.8	59.0±12.5	27.0±12.8
20	115.0±42.0	321.0±16.6	207.0±41.0	75.9±13.3

[1] nmoles bromide/mg cytosolic protein

Table A.2. Hepatic and extra-hepatic GSH-dependent debromination of 1,2-dibromo-3-chloropropane (DBCP) using rat and mouse cytosols.

Rat	Released inorganic bromide[1]			
Time (min)	liver	kidney	testes	stomach
2	32.6±9.0	15.8±6.6	14.4±6.0	11.5±3.8
5	81.3±13.6	17.2±12.7	32.6±15.0	33.3±5.0
20	314.0±76.7	160.0±32.0	136.0±95.4	112.0±36.0
Mouse				
Time (min)	liver	kidney	testes	stomach
2	3.2±0.7	0.9±0.1	1.0±0.2	--
5	7.5±3.2	1.2±0.7	2.1±1.3	0.1±0.0
20	34.1±1.1	6.3±3.6	11.4±1.2	0.3±0.1

[1] nmoles bromide/mg cytosolic protein.

Table A.3. Hepatic and extra-hepatic GSH-dependent water-soluble product formation using ^{14}C-1,2-dibromoethane with rat and mouse cytosols.

Rat	Water-soluble product formed[1]			
Time (min)	liver	kidney	testes	stomach
2	5.9±0.9	5.8±0.5	3.1±0.2	0.9±0.1
5	14.9±0.3	13.5±4.8	6.0±1.0	2.1±0.1
20	60.4±17.7	57.6±3.9	26.5±8.5	8.2±0.8
Mouse				
Time (min)	liver	kidney	testes	stomach
2	7.7±0.9	2.6±0.2	1.9±0.2	1.0±0.0
5	19.6±2.5	6.2±0.6	5.3±0.8	2.9±0.3
20	21.3±0.3	23.3±6.0	17.8±4.0	11.0±0.0

[1] nmoles product/mg cytosolic protein

Table A.4. Hepatic and extra-hepatic GSH-dependent water-soluble product formation using ^{14}C-1,2-dichloroethane with rat and mouse cytosols.

Rat	Water-soluble product formed[1]			
Time (min)	liver	kidney	testes	stomach
2	0.4±0.1	0.3±0.0	0.2±0.1	0.1±0.0
5	1.1±0.3	0.7±0.1	0.5±0.2	0.2±0.0
20	4.0±1.2	2.9±0.2	2.3±0.3	0.9±0.1
Mouse				
Time (min)	liver	kidney	testes	stomach
2	0.3±0.1	0.1±0.0	0.1±0.0	--
5	0.8±0.2	0.3±0.1	0.2±0.1	0.1±0.0
20	3.1±1.5	1.0±0.2	0.8±0.2	0.3±0.1

[1] nmoles product/mg cytosolic protein

Table A.5. Hepatic GSH-dependent debromination of 1,2-dibromoethane and 1,2-dibromo-3-chloropropane as a function of cytosolic protein concentration.

1,2-Dibromoethane (DBE)

protein conc[1]	Rate of debromination[2] Rat	Mouse[3]
0.5	2.9±0.7	38.0±7.0
1.0	6.4±3.1	77.1±14.1
2.0	11.8±1.0	148.0±12.0

1,2-Dibromo-3-chloropropane (DBCP)

protein conc[1]	Rate of debromination[2] Rat	Mouse
0.5	7.6±3.1	1.6±0.2
2.0	14.0±6.0	2.7±1.0
3.0	27.3±7.7	4.5±1.1

[1] mg/ml

[2] nmoles bromide/min

[3] data from early time points, where reaction was shown to be linear with time

Table A.6. Hepatic GSH-dependent water-soluble product formation from ^{14}C-1,2-dibromoethane and ^{14}C-1,2-dichloroethane as a function of cytosolic protein concentration.

^{14}C-1,2-Dibromoethane (DBE)		
	Rate of water-soluble[2] product formation	
protein conc.[1]	Rat	Mouse[3]
0.5	0.9±0.6	1.9±0.8
1.0	2.2±0.2	4.2±0.7
2.0	3.9±1.1	--
5.0	--	20.1±9.0
^{14}C-1,2-Dichloroethane (DCE)		
protein conc.[1]	Rat	Mouse
0.5	0.08±0.06	--
1.0	0.2 ±0.01	0.2±0.1
2.0	0.4 ±0.02	0.5±0.06
3.0	--	1.1±0.2

[1] mg/ml

[2] nmoles product/min

[3] data from early time points, where reaction was shown to be linear with time

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