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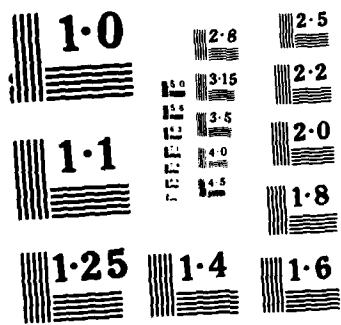
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**COMPARATIVE BIOCHEMISTRY AND METABOLISM
PART II: NAPHTHALENE LUNG TOXICITY**



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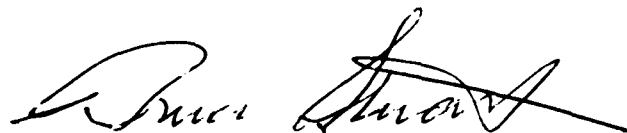
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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



BRUCE O. STUART, PhD
Director Toxic Hazards Division
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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The studies reported in this document were to quantitatively examine the diffusion of naphthalene oxide and reactive metabolites from naphthalene in suspensions of isolated mouse hepatocytes and to determine whether the efflux of these metabolites was dependent upon the depletion of intracellular glutathione. The efflux of naphthalene oxide from intact hepatocytes was monitored in incubations containing ^3H -glutathione (this tripeptide will not cross the intact hepatocellular membrane) and was based on the measurement of ^3H -labelled glutathione adducts. Between 17 and 35% of the total naphthalene oxide formed during the metabolism of naphthalene in the cell was trapped on the exterior of the cell with ^3H -glutathione. The fraction of epoxide exiting the hepatocytes as a percentage of the total amount formed did not vary with increasing naphthalene concentrations between 0.005 and 1.5 mM. These data in conjunction with the studies showing that incubation of hepatocytes with naphthalene at concentrations of 0.05 mM or greater caused a significant depletion of intracellular glutathione,					
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19. Abstract

19. Abstract
Suggested that the quantity of naphthalene oxide diffusing as a percentage of the total formed was not dependent upon the intracellular glutathione status. In contrast, the ratio of extracellular vs intracellular covalent binding was dependent on the concentration of naphthalene in the incubation. At lower substrate concentrations where glutathione levels were unaffected by the presence of naphthalene, the ratio of extracellular to intracellular covalent binding was > 1 . In higher substrate concentrations where glutathione is depressed, intracellular covalent binding was substantially greater than extracellular binding. These studies suggest that the fate of reactive naphthalene metabolites that become bound covalently, unlike naphthalene oxide, is dependent on the levels of intracellular glutathione.

PREFACE

This is the final report of a series that has focussed on the comparative biochemistry and metabolism of the volatile aromatic hydrocarbon, naphthalene. These studies have been done in an effort to understand some of the underlying mechanisms by which this compound produces a highly selective lesion in murine Clara cells. It covers work conducted by the Department of Community and Environmental Medicine, University of California, Irvine under Contract No. F33615-80-C-0512, Work Unit No. 63020115. This document describes the accomplishments of the subprogram from June 1984 through December 1984.

A. R. Buckpitt served as coordinator of the program. Technical contract monitors for the Air Force were K. C. Back, Ph.D. and M. K. Pinkerton of the Toxicology Branch, Toxic Hazards Division, Harry G. Armstrong Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio 45433. The authors wish to thank Ms. Linda Bahnson for her efforts in a portion of the HPLC thiol analysis described in this report.

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INTRODUCTION

This report summarizes the work conducted from June 1984 through December 1984 in the Subprogram on Comparative Biochemistry and Metabolism, Part II: Naphthalene Lung Toxicity. The intent of the work described in this report was to provide a better understanding of the quantitative significance of the release of reactive naphthalene metabolites by intact isolated hepatocytes and to determine potential mechanisms by which a chemically unstable metabolite which is an excellent substrate for the intracellular enzymes, epoxide hydrolase and glutathione transferases, can be formed on the endoplasmic reticulum and be released to the exterior of the cell. The studies reported here also were intended to examine the interrelationships of metabolic activation, alteration in cellular thiol status and loss of cellular viability for naphthalene in freshly isolated hepatocyte preparations.

Previous studies conducted under support from the Air Force provided evidence that reactive metabolites resulting from cytochrome P450 dependent metabolism of naphthalene in the liver were capable of leaving their site of formation and becoming covalently bound to macromolecules in extrahepatic organs such as lung and kidney (Buckpitt and Warren, 1983). The significance of the circulation of such metabolites could be two-fold. Efflux of these metabolites from the liver could serve either to cause direct cytotoxicity to cells in extrahepatic tissues or alternatively could deplete these tissues of cellular detoxication capabilities thereby rendering them considerably more vulnerable to toxic metabolites generated in situ.

RESEARCH PROGRAM

General Methods

Animals

All mice were male Swiss Webster purchased from Charles River Breeding Laboratories, Wilmington, MA. All animals were held in the vivarium of the animal resources division at the University of California. Mice were housed on hardwood bedding, were given free access to food and water, and were kept in barrier facilities.

Radiochemicals

¹⁴C-Naphthalene was purchased from California Bionuclear, Sun Valley, CA. The radiochemical purity of this material was

shown to be >99.5 % by high pressure liquid chromatography on a C18 column with water/methanol as the eluting solvent. L-Glutathione (glycine -2-[³H]), reduced form, 240 mCi/mmol was purchased in 50 uCi batches from New England Nuclear Corporation, Boston, MA. The material was stored at -80°C and was flushed with N₂ after use to prevent oxidation to the disulfide. Stock solutions of the material were prepared immediately before use.

Chemicals

Components of the NADPH generating system (NADP, glucose 6-phosphate dehydrogenase and glucose 6-phosphate), and glutathione reductase (Sigma Chem Co., Type III, yeast, Cat.# G-4751) were purchased from Sigma Chemical Company, St. Louis, MO. Collagenase (from *Clostridium Histolyticum*) was bought from ICN, Cleveland, OH. All other reagents were obtained from commercial suppliers and were reagent grade or better.

Preparation of Semi-Purified Glutathione Transferases from Mouse Liver

The 100,000 x g supernatant fraction prepared during centrifugal preparation of microsomes from mouse liver was dialyzed against 3 x 50 volumes of 25 mM sodium phosphate buffer, pH 7.0 to remove glutathione. One to three mL of the supernatant prepared in this fashion were chromatographed on 1 x 6.5 cm columns of glutathione agarose as described by Simons and Van der Jagt (1981). Glutathione containing (5 mM) Tris buffer (0.05 mM, pH 9.6) was used to elute the transferase enzymes and the activity was assayed after pressure dialysis with 1-chloro-2,4-dinitrobenzene as described previously (Habig et al., 1974).

Preparation of Isolated Hepatocytes from Mouse Liver

Hepatocytes were prepared by retrograde perfusion of the mouse liver with collagenase. Briefly, the mouse was treated intravenously 30 min prior to the start of surgery with 100 units heparin. The animal was anesthetized with ether, the abdomen and chest cavity were opened, and a cannula was inserted into the vena cava through the right atrium. The portal vein was severed and Hanks buffer containing 15 mM HEPES and 0.5 mM EGTA was perfused through the liver at the rate of 10 mL/min for 4 minutes. This was followed by a 12 minute perfusion with Hank's buffer containing 0.1 mM CaCl₂ and 10 units/mL collagenase at 37°C. The liver was removed, submerged in additional quantities of Hank's buffer and the liver cells were dispersed gently with a pair of forceps. The cells then were filtered through cheese cloth and were allowed to settle for 5 min on ice. They were then resuspended in Fischer's medium containing 10% heat inactivated fetal

calf serum, 15 mM HEPES and gentamycin (10 ug/mL) at pH 7.4. The cells were then layered on a solution of 36% Percoll in Fischer's medium (bubbled with 95% O₂/5% CO₂) in a centrifuge tube and viable hepatocytes were separated from nonviable cells by centrifugation at 20,000 x g in a Beckman SW 27 rotor. The bottom layer, containing viable hepatocytes, was centrifuged and the hepatocytes were washed and resuspended in Fischer's medium. Centrifugation through Percoll resulted in the preparation of hepatocytes with viabilities > 96% as assessed by trypan blue dye exclusion.

Measurement of Cellular Thiol Status

Oxidized and reduced glutathione, cysteine and cysteine-glutathione mixed disulfide were measured in the cells and medium by the HPLC technique of Reed et al. (1980) with the modifications published recently (Fariss et al., 1984). Briefly, at the termination of the incubations, cells were layered over 0.35 mL dibutyl phthalate/0.4 mL 10% perchloric acid containing 2 mM EDTA in a microcentrifuge tube. Cells were separated from the incubation media by centrifugation at 10,500 rpm (Sorvall SS-34 rotor) for 1 min. An aliquot of the hepatocyte media was removed, 50 uL internal standard (γ -glutamyl-glutamic acid) and 50 uL 70% perchloric acid (to bring the medium to 10%) were added and the samples were derivatized by the addition of iodoacetic acid and 2,4-dinitrofluorobenzene. Standards were prepared with oxidized and reduced glutathione, cysteine and cysteine-glutathione mixed disulfide (a gift of Dr. John Livesey, Department of Biochemistry, Oregon State University, Corvallis, Oregon). Quantitation of the thiols in the sample was based on peak heights of the derivatized thiols eluted from a 5 u aminopropyl column purchased from LC Associates, Houston, Tx. Column eluate was monitored at 365 nm. Derivatized thiols were eluted from the column using the following solvent program: 75% solvent A/25% solvent B for 5 min followed by linear programming to 1% solvent A/99% solvent B over 20 min. Solvent A was 80% methanol/20% distilled water; solvent B was prepared by mixing 272 g sodium acetate trihydrate, 122 mL distilled water and 378 mL glacial acetic acid and diluting 200 mL of this mixture with 800 mL of solvent A. The derivatized thiols eluted with the following retention times: glutathione-cysteine mixed disulfide-12 min, internal standard (γ -glutamyl-glutamic acid)-22 min, reduced glutathione-25 min and oxidized glutathione 28 min. All of the peaks were completely separated from each other and from interfering derivatized amino acids.

Measurement of Naphthalene Metabolites by HPLC

The methods for measuring the rate of metabolism of naphthalene to naphthalene 1,2-dihydrodiol and naphthalene glutathione adducts have been described previously (Buckpitt et al., 1984). Briefly, incubations were quenched by the addition of 2 volumes ice cold methanol, denatured protein was removed by centrifugation, and an aliquot of the supernatant was evaporated to dryness under vacuum. The residue was reconstituted and an aliquot was injected onto a C18 Novapak column (Waters Associates). Metabolite elution was monitored by UV absorbance at 254 nm, and the conjugates and dihydrodiol were quantitated by collecting the compounds and measuring the radioactivity eluting from the column. All three conjugates have been identified as monogluthathione derivatives of hydroxy-1,2-dihydronaphthalene by fast atom bombardment mass spectrometry (Buckpitt and Richieri, 1984; Castagnoli et al., unpublished results). The absolute stereochemistry of these metabolites remains to be determined.

RESULTS

Metabolism of Naphthalene in Isolated Hepatocyte Incubations

To determine the time course, the effect of varying substrate concentrations, the relative amounts of dihydrodiol vs glutathione conjugates formed, and the relative distribution of these metabolites intra- and extracellularly during the metabolism of naphthalene in freshly isolated hepatocyte incubations, mouse hepatocytes were incubated in the presence of 0.05 mM naphthalene for times up to 4 hours and with concentrations of naphthalene from 0.05 to 1.5 mM. As indicated by the data in Figure 1, naphthalene is rapidly metabolized to all three glutathione conjugates. Formation of all three glutathione adducts appears to level off after 15 min of incubation. Approximately 10% of the substrate is metabolized to glutathione adducts in 15 min and thus, naphthalene or glutathione are probably not rate limiting under these conditions. The intracellular concentrations of conjugates 1 and 2 remain low throughout the incubation; once formed they appear to rapidly leave the hepatocyte. Adduct 3 remains in the hepatocyte longer than adduct 1 or 2. The ratio of adduct 2 to 1 formation in isolated hepatocyte incubations is approximately 2:1 which is similar to that found in incubations of hepatic microsomes from the mouse.

The rate of naphthalene metabolism to the dihydrodiol and to glutathione adducts increases with increasing substrate concentrations up to a concentration of 0.5 mM naphthalene (Figure 2).

There was virtually no difference in the amounts of any of the metabolites present in incubations performed at 0.5 vs 1.5 mM naphthalene. The ratio of dihydrodiol to glutathione adduct formation changed substantially with increasing substrate concentrations and this is most likely a reflection of the K_m values for epoxide hydrolase vs glutathione transferase-mediated naphthalene oxide metabolism.

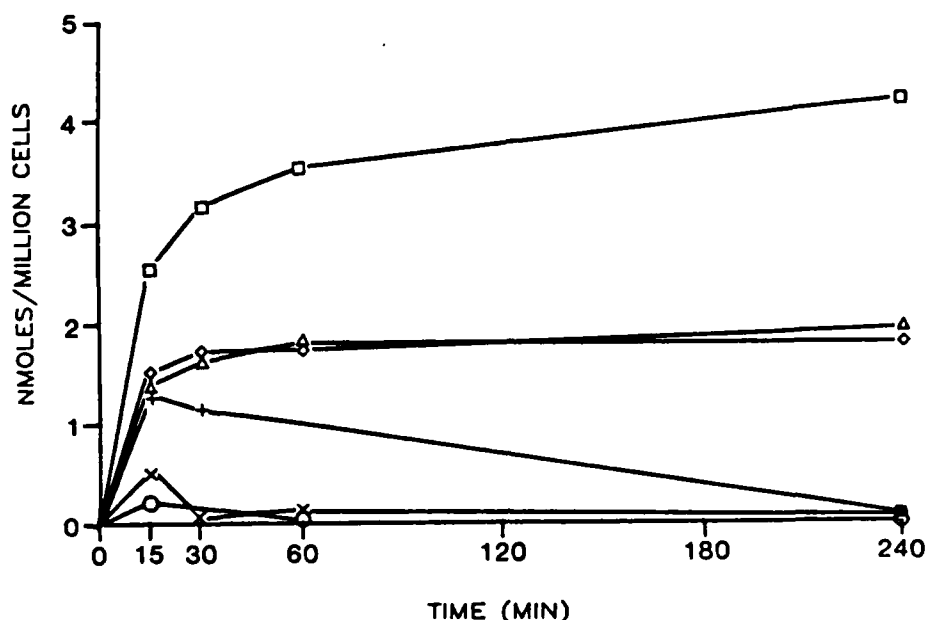


Figure 1. Time course metabolism of naphthalene (0.05 mM) to glutathione conjugates in isolated hepatocyte incubations. Glutathione adducts 1, 2 and 3 were isolated from the medium and from washed hepatocytes after the incubation. The symbols denote the following: intra-cellular conjugates 1 (o), 2 (X) and 3 (+); extra-cellular conjugates 1 (Δ), 2 (□), and 3 (◇). Values are the mean of 2 incubations.

Effect of Varying Concentrations of Naphthalene on the Viability of Mouse Hepatocytes.

Prior to the start of studies to examine the efflux of naphthalene oxide from isolated hepatocytes, the effect of naphthalene on the integrity of the hepatocellular membrane was examined. The major focus of the studies described in this report was to examine the diffusion of naphthalene oxide from isolated intact cells and thus, incubation conditions had to be

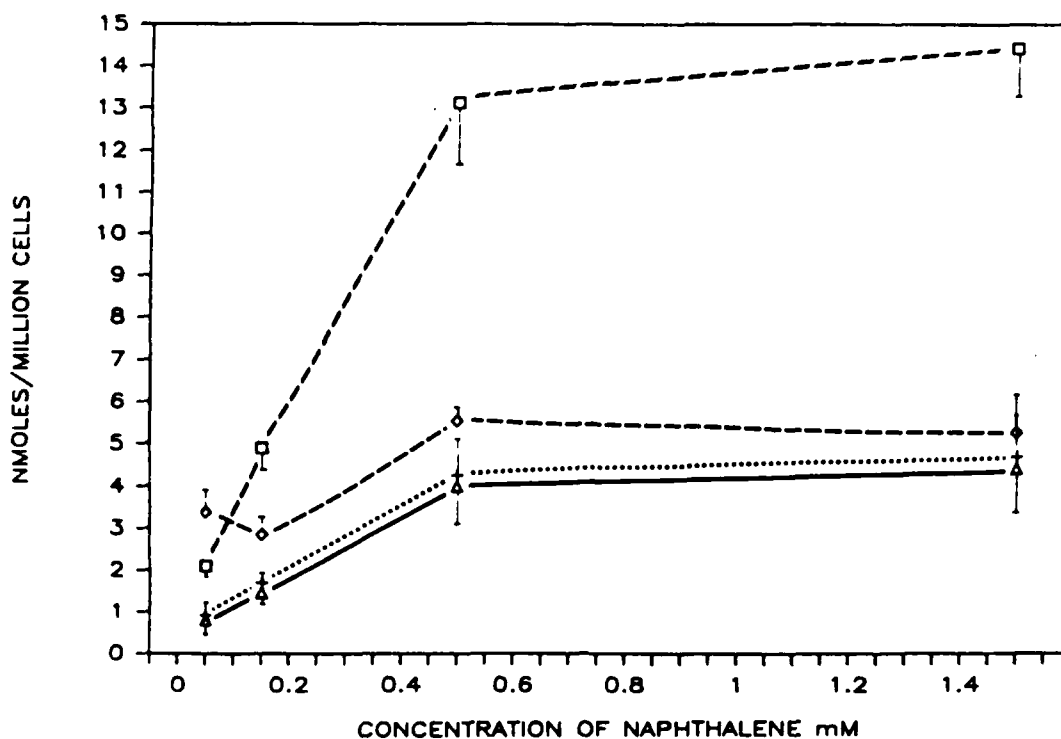


Figure 2. Rates of formation of naphthalene dihydrodiol and naphthalene glutathione conjugates in isolated hepatocytes incubated in the presence of varying concentrations of naphthalene. Incubations were conducted for 15 min. with 1×10^6 hepatocytes/mL. Values are the mean from 3 separate incubations. Symbols denote: (□) dihydrodiol, (+) naphthalene glutathione adduct 1, (◇) naphthalene glutathione adduct 2 and (Δ) naphthalene glutathione adduct 3.

established which would afford the use of cells with intact membranes. As indicated by the data in Figure 3, mouse hepatocytes incubated in the absence of substrate retain their ability to exclude trypan blue dye throughout the four hour incubation. Incubations conducted with increasing concentrations of naphthalene showed no effect on the viability at 1 hour but at 4 hours the viability of hepatocytes decreased with increasing substrate concentration. These data indicate that the studies to determine the rate of efflux of naphthalene oxide from isolated hepatocytes would have to be conducted with relatively short incubation times (15 and 30 min). The data from this study contrast with the observed lack of hepatotoxicity with naphthalene in vivo. These data combined with recent studies by Cohen and his coworkers (Doherty et al., 1984; Miller et al., 1985) who have demonstrated substantial losses in hepatocyte viability

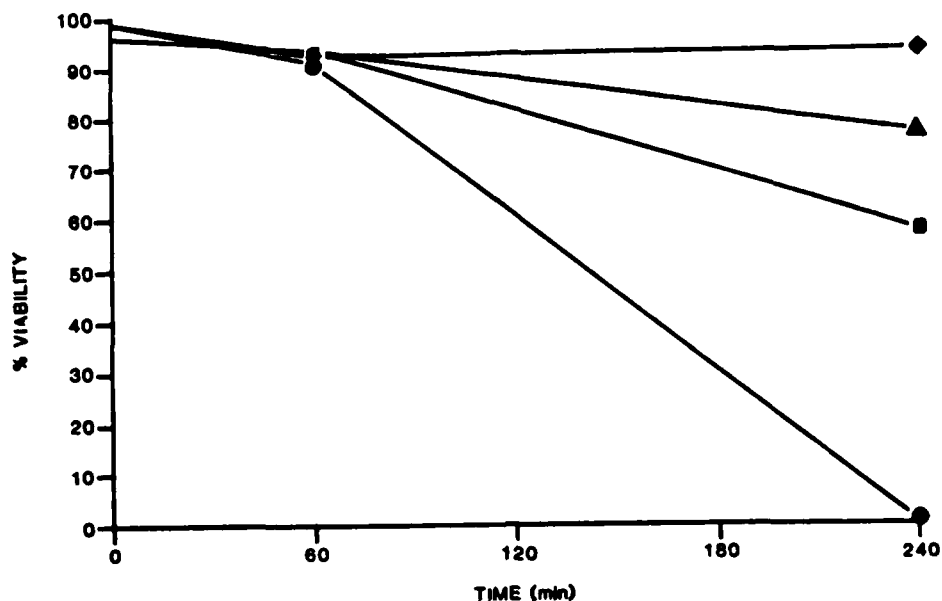


Figure 3. Viability of isolated mouse hepatocytes incubated in the presence of naphthalene (0.05 mM-(▲), 0.1 mM-(■) and 0.5 mM-(●) or vehicle only (♦)). Viabilities are expressed as the % hepatocytes excluding trypan blue dye.

during incubations with 1-naphthol (which is not hepatotoxic in vivo, (Buckpitt et al., 1985) suggest that extrapolation of toxicity data obtained in isolated hepatocyte studies to the in vivo situation must be done with considerable caution, if at all.

Quantitation of Naphthalene Oxide Efflux from Isolated Hepatocytes

The overall goal of these studies was to quantitatively determine the rate of transfer of naphthalene 1,2-oxide across intact hepatocyte membranes and to determine whether this process was dependent upon the substantial depletion of cellular glutathione. To do this a system had to be developed which was capable of trapping naphthalene oxide on the exterior of the cell. The approach used in the current studies is outlined in Figure 1 and is based upon the fact that reduced glutathione is unable to penetrate the intact hepatocellular membrane (Hahn et al., 1978; Liebler et al., 1985). As indicated in Figure 4, ^{14}C -naphthalene added to the hepatocyte incubations would diffuse across the hepatocyte membrane and undergo epoxidation by the cytochrome P450 monooxygenases. This would be a substrate for

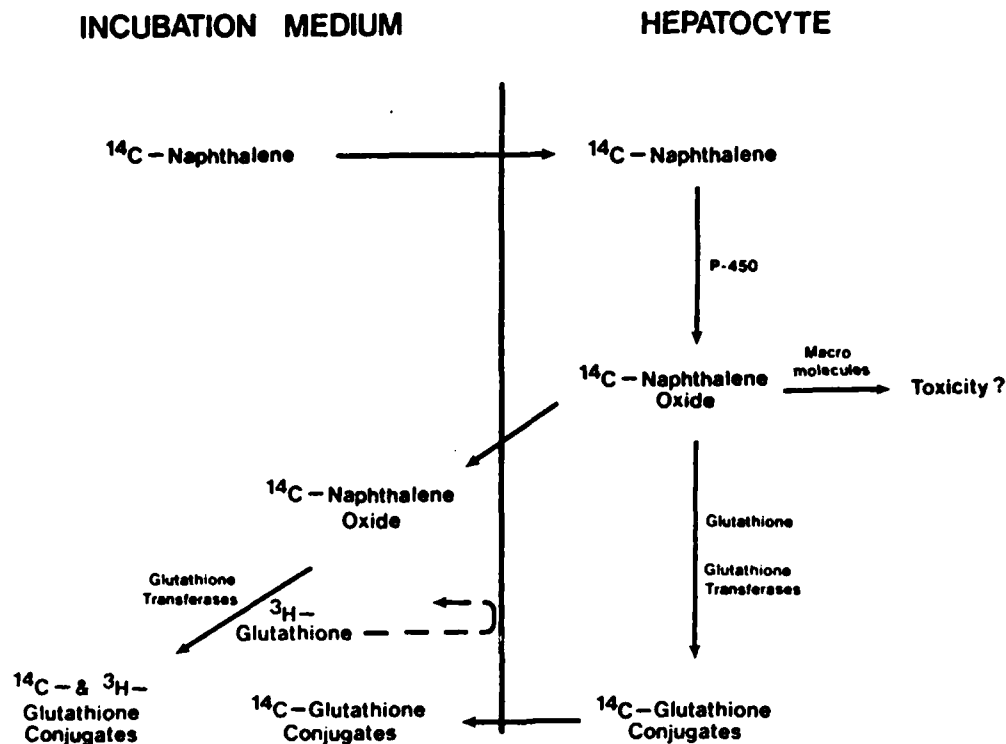


Figure 4. Schematic diagram indicating the experimental approach used for trapping naphthalene oxide diffusing from intact hepatocytes.

the intracellular epoxide hydrolase and for cytosolic glutathione transferases and would result in the formation of ^{14}C -labelled glutathione adducts and dihydrodiol. Any naphthalene oxide capable of escaping the intact hepatocyte could be trapped by including ^3H -glutathione and glutathione transferases in the incubation medium. Measurement of the relative amounts of naphthalene oxide leaving the intact cell vs that metabolized intracellularly could be based upon the ratios of ^3H DPM associated with the glutathione conjugates to ^{14}C DPM in the combined dihydrodiol and conjugate fractions.

Since reduced glutathione is rapidly oxidized to glutathione disulfide in solution and because the reduced form of glutathione is a substrate for the glutathione transferases, steps had to be taken to maintain this sulfhydryl compound in the reduced state. To determine whether the loss of glutathione was a substantial problem in 15 or 30 minute incubations, 1 mM reduced

glutathione was added to Fischer's medium containing 10% fetal calf serum and the colorimetric procedure of Ellman (1959) was used to determine the loss of reduced sulfhydryl levels. As indicated from the data in Figure 5 reduced glutathione is rapidly lost from hepatocyte medium incubated in an atmosphere of 95% O₂/5% CO₂. Fifty percent of the dithiobis-nitrobenzoic acid reactants were lost within the first 15 min of the incubation, and by 60 min less than 5% of the original free sulfhydryl content remained. In contrast, maintaining the medium on ice in

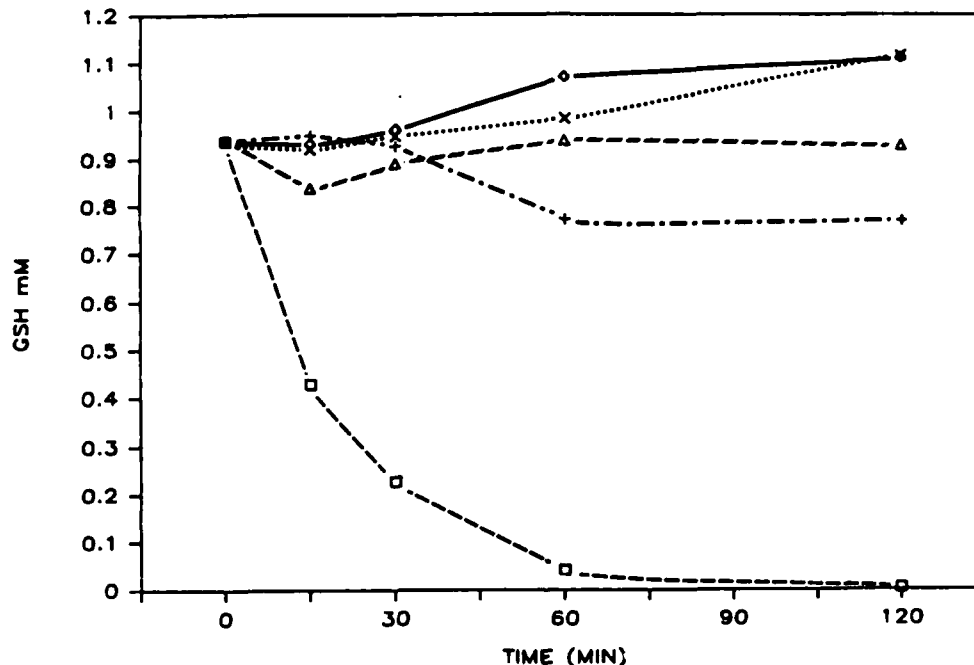


Figure 5. Reduced sulfhydryl levels (measured by the Ellman procedure, 1959) in Fischer's medium to which 1 mM reduced glutathione was added at T=0. Incubations were done either in an atmosphere of 95% O₂/5% CO₂ at 37°C or in air at ice temperatures. At the indicated time, an aliquot of the incubation mixture was removed, proteins were precipitated by the addition of 4% sulfosalicylic acid and reduced sulfhydryl levels were assayed in an aliquot of the supernatant. Incubations were performed either at 37°C (□, ◇, Δ, X) under a O₂/CO₂ atmosphere or at 0°C in air. Incubations contained: (□) no GSSG reductase, no NADPH; (◇) 1.5 units reductase, NADPH regenerating system, (Δ) 3.0 units reductase +NADPH, or (X) 6.0 units of reductase plus NADPH. The NADPH regenerating system consisted of 0.9 mg NADP, 9 mg glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase. Values are the mean from two incubations.

an air atmosphere resulted in little loss of free sulfhydryl content. Greater than 90% remained after 2 hours. In an attempt to determine whether glutathione could be maintained in the reduced form by inclusion of glutathione reductase, hepatocyte incubation medium was incubated with 1 mM glutathione in the presence of varying amounts of glutathione reductase with a NADPH regenerating system. Inclusion of as little as 1.5 units glutathione reductase in the presence of an NADPH regenerating system was capable of maintaining glutathione levels at the initial concentrations. Thus, ^3H -GSH concentrations can be maintained at high levels throughout a 120 min incubation period by the inclusion of glutathione reductase and an NADPH regenerating system.

To quantitatively determine naphthalene oxide effluxing from isolated intact hepatocytes, a total of 6 separate hepatocyte preparations were made. Three of these preparations contained ^{14}C -naphthalene in concentrations ranging from 0.005 to 1.5 mM (concentrations are specified in the legend to Figure 6). In the remaining three preparations, unlabelled naphthalene (at the same concentrations as above) was incubated in the presence of ^3H -glutathione (1 mM). ^3H -Glutathione preparations were made

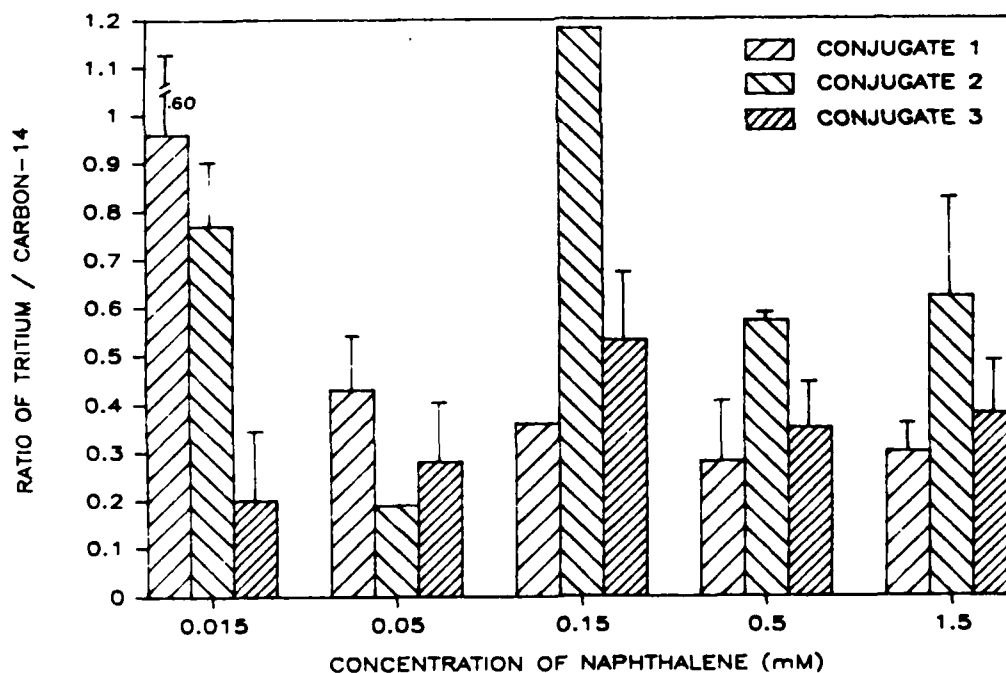


Figure 6. Ratio of ^3H to ^{14}C -labelled naphthalene glutathione adducts in hepatocyte incubation medium after 15 min incubation with naphthalene at concentrations ranging from 0.015 mM to 1.5 mM. Values are the mean of 2 or mean + S.E. for 3 separate incubations.

immediately before use and the specific activity of the material used in incubations containing 0.005 and 0.015 mM naphthalene ranged from 7387 to 7582 DPM/nmole; in incubations containing 0.05 and 0.15 mM the specific activity was from 2317 to 3914 DPM/nmole, and in incubations containing 0.5 and 1.5 mM naphthalene, the specific activity of the added 1 mM glutathione was 1275-2435 DPM/nmole. Naphthalene was added last to the incubations, the cell suspension was gassed, capped tightly, and the incubation vessels were transferred to a shaking water bath. At 15 min 0.5 mL cells were removed and the cells were pelleted by centrifugation. The cell medium was removed and methanol was added to precipitate protein. A further 2 mL buffer was added to the cells and they were resuspended and pelleted by centrifugation. The supernatant was discarded and methanol was added to the cell pellet to lyse the cells and precipitate protein. ^3H and ^{14}C -labelled naphthalene glutathione conjugates were quantitated by HPLC on Waters Novapak columns (0.8 x 10 cm).

The data in Figures 6 and 7 show the ratio of ^3H -labelled to ^{14}C -labelled glutathione conjugates in the cell medium of isolated hepatocyte incubations done in the presence of ^3H -GSH or ^{14}C -naphthalene for 15 and 30 min, respectively. The ratio of ^3H to ^{14}C provides an indication of the quantity of conjugates formed extracellularly vs intracellularly. Although there was some variability in the data obtained for $^3\text{H}/^{14}\text{C}$ -labelled conjugates at the 15 min incubation time particularly at low naphthalene concentrations (Figure 6), it appeared that between 30 and 60% of total conjugates formed were produced extracellularly. At the two lowest substrate concentrations, only small quantities of naphthalene oxide were being produced, and measuring small amounts of ^3H -conjugate in the presence of large amounts of ^3H -glutathione was subject to some error. At the higher concentrations of naphthalene at both the 15 and 30 min time points the ratio of ^3H to ^{14}C for glutathione adduct 2 appeared to be greater than adducts 1 or 3. This would indicate that the epoxide, which is the precursor to glutathione adduct 2, diffuses more easily than the epoxide which is responsible for the formation of glutathione adduct 1. It should be noted that hepatocyte incubations vary moderately from one another. Orrenius and his coworkers often present the data from a "typical" experiment (Thor et al., 1982; Orrenius et al., 1983). Thus, the standard errors reported in these studies are consistent with data from other laboratories studying xenobiotic metabolism in isolated hepatocytes.

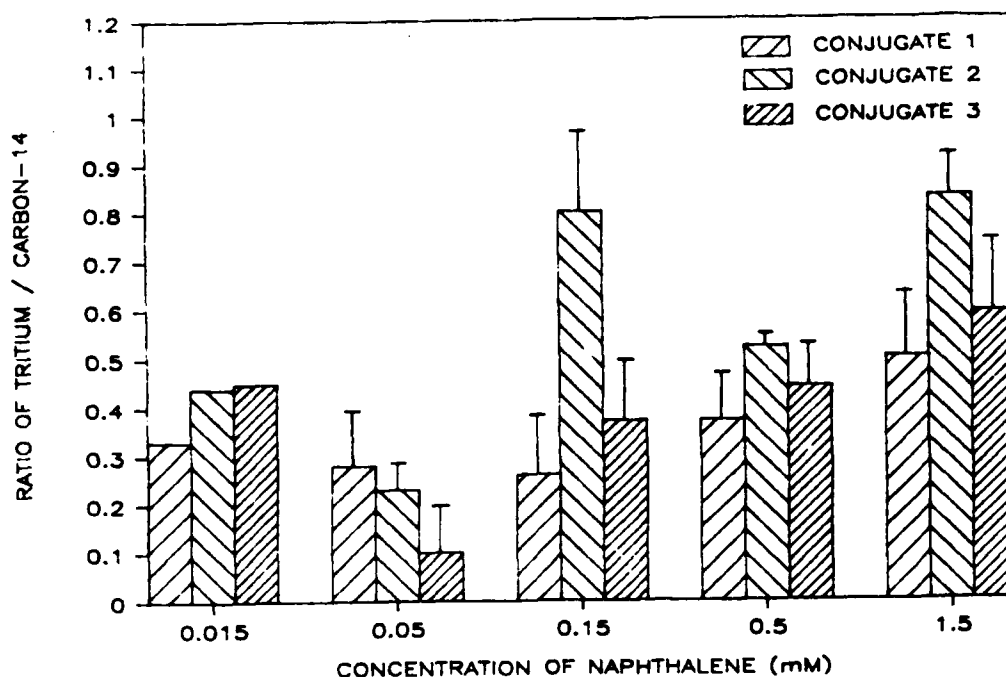


Figure 7. Ratio of ^3H to ^{14}C -labelled naphthalene glutathione adducts in hepatocyte incubation medium after 30 min incubation with naphthalene at concentrations ranging from 0.015 mM to 1.5 mM. Values are the mean of 2 or mean + S.E. for 3 separate incubations.

To calculate the total amount of naphthalene oxide leaving intact hepatocytes over 15 and 30 min incubation periods as a percentage of the total amount of naphthalene oxide formed intracellularly, the quantity of tritium labelled glutathione conjugates was summed and divided by the sum of total ^{14}C -labelled dihydrodiol and glutathione conjugates. As indicated by the data in Figure 8, between 17 and 35% of the total naphthalene oxide formed intracellularly was capable of leaving the intact hepatocyte. The percentage of the epoxide leaving the cell did not depend upon the concentration of naphthalene in the incubation and therefore, as discussed later in this report, did not appear to depend upon the substantial depletion of intracellular glutathione levels. Thus, the efflux of naphthalene oxide from isolated hepatocytes does not show a glutathione dose threshold. In addition, there was little change in the percentage of naphthalene oxide leaving the hepatocyte from 15 to 30 min indicating that ^3H -glutathione was not slowly penetrating the cells and resulting in the formation of tritium glutathione conjugates.

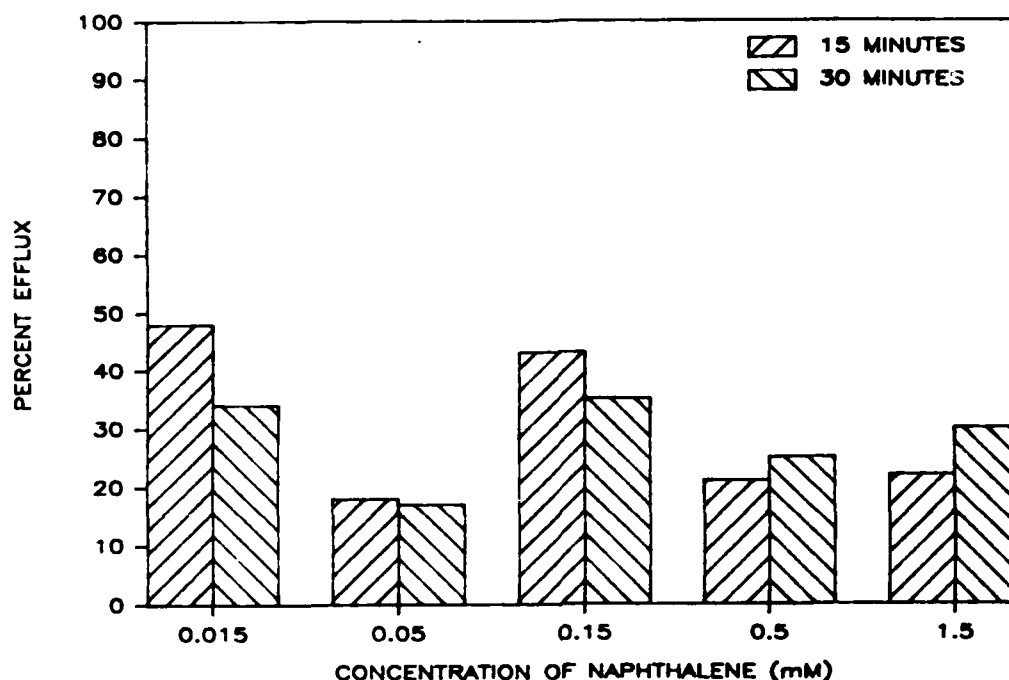


Figure 8. Fraction of naphthalene oxide formed intracellularly which diffuses from isolated hepatocytes in a 15 or 30 min incubation period. Data are calculated as the total metabolite present in ^3H -labelled glutathione conjugates divided by the sum of ^{14}C -labelled dihydrodiol and naphthalene conjugates. Values are the mean of 3 incubations.

These data compare favorably with those reported by Monks et al. (1984) who monitored the diffusion of reactive metabolites of bromobenzene (consisting of bromobenzene oxides and reactive metabolites formed from further metabolism of bromophenol) and showed that up to 35% of reactive bromobenzene metabolites formed intracellularly were capable of leaving hepatocytes. Two of the problems associated with these studies which were avoided in the experimental protocol used in the studies described here are that the efflux of reactive metabolites was monitored by measuring the amount of radioactivity from ^{14}C -bromobenzene covalently bound to glutathione transferase B, and reactive metabolites may not have been completely trapped in the extracellular matrix. Moreover, several different reactive metabolites are responsible for the covalent binding of radioactivity from bromobenzene, and the

studies reported by Monks et al. (1984) were unable to determine what contribution each of the metabolites made to the overall diffusion of reactive metabolites.

Covalent Binding of Reactive Naphthalene Metabolites to Intra- and Extracellular Proteins.

Like bromobenzene, naphthalene is metabolized to a number of different reactive metabolites that are capable of becoming bound covalently to protein (Hesse and Mezger, 1979; Schwarz et al., 1980; Buckpitt et al., 1985). The role of each of these metabolites in naphthalene-induced lung injury is uncertain. Earlier studies, however, have demonstrated a good correlation between the overall covalent binding levels in the target tissue with the extent and severity of the pulmonary lesion by naphthalene (Warren et al., 1982; Buckpitt and Warren, 1983). Moreover, these early studies provided evidence that reactive naphthalene metabolites were capable of effluxing from the liver and becoming bound covalently in extrahepatic tissues in vivo (Buckpitt and Warren, 1983). Thus, additional studies were done to determine whether reactive naphthalene metabolites were capable of diffusing from intact hepatocytes and becoming bound covalently intra- and extracellularly. Additionally, it was of interest to determine whether there is a glutathione dose threshold in the covalent binding of metabolites to nucleophilic sites intra- and extracellularly.

Hepatocytes were incubated for various times ranging from 30 to 240 min with concentrations of naphthalene from 0.005 to 0.5 mM. Cells were separated from the medium by centrifugation and the cells were washed once with fresh incubation medium. Sufficient SDS (sodium dodecyl sulfate) was added to the cells and medium to bring the solution to 2% and the samples were placed in a boiling water bath for 10 min to denature the proteins. The entire sample was placed in a dialysis bag (Spectrapor 3-MW cut-off 3,000) and the samples were dialyzed against 1000 volumes of 0.01 M sodium phosphate, pH 7.4 containing 0.1% SDS. The samples were dialyzed until no further radioactivity could be detected in the dialysate. An aliquot was removed for protein determination (Lowry et al., 1951) and a further aliquot was counted in a liquid scintillation counter. Data were expressed as nmoles covalently bound/ mg protein. This technique for measuring covalent binding was developed by Sun and Dent (1980, 1982). Comparative studies using the dialysis and traditional exhaustive solvent extraction techniques have shown that the dialysis technique yields higher levels of covalent binding probably because many small molecular weight proteins are lost during the solvent extractions (Dent and Sun, 1982).

The data in Figures 9-12 show that the formation of covalently bound naphthalene metabolites both intra- and extracellularly occurs rapidly, and at the lower substrate concentrations (Figure 9-0.005 mM, Figure 10-0.05 mM) the formation of these metabolites appears to level off after the first 30 min incubation. At the two higher substrate concentrations, additional quantities of reactive metabolite are being formed throughout the time period of the incubation (Figure 11-0.25 mM; Figure 12-0.5 mM). While the covalent binding of reactive naphthalene metabolites to proteins inside and outside of the cell increased with increasing concentrations of naphthalene, the relative amounts of metabolite bound extracellularly vs intra-cellularly changed markedly with substrate. At the low substrate concentrations, extracellular binding was substantially higher than at high naphthalene concentrations (Figure 13). These data indicate that there may be a glutathione threshold for intracellular covalent binding of reactive naphthalene metabolites. At the low substrate concentrations, intracellular glutathione levels are high and this nucleophile affords protection from reactive metabolites in the cell. However, at the higher substrate concentrations, where glutathione is depleted, intracellular covalent binding is substantially greater than extracellular binding (Figures 11-13).

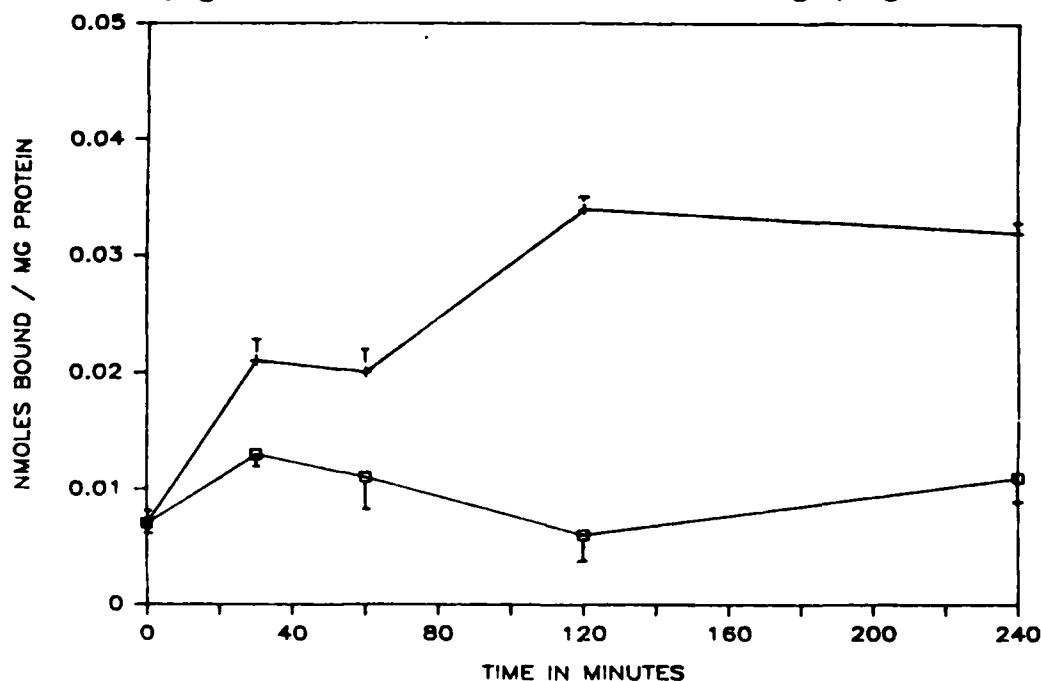


Figure 9. Time course formation and covalent binding of reactive naphthalene metabolites to intracellular (□) and extracellular (+) proteins in isolated hepatocytes incubated with 0.005 mM naphthalene. Values are the mean + S.E. for 3 incubations.

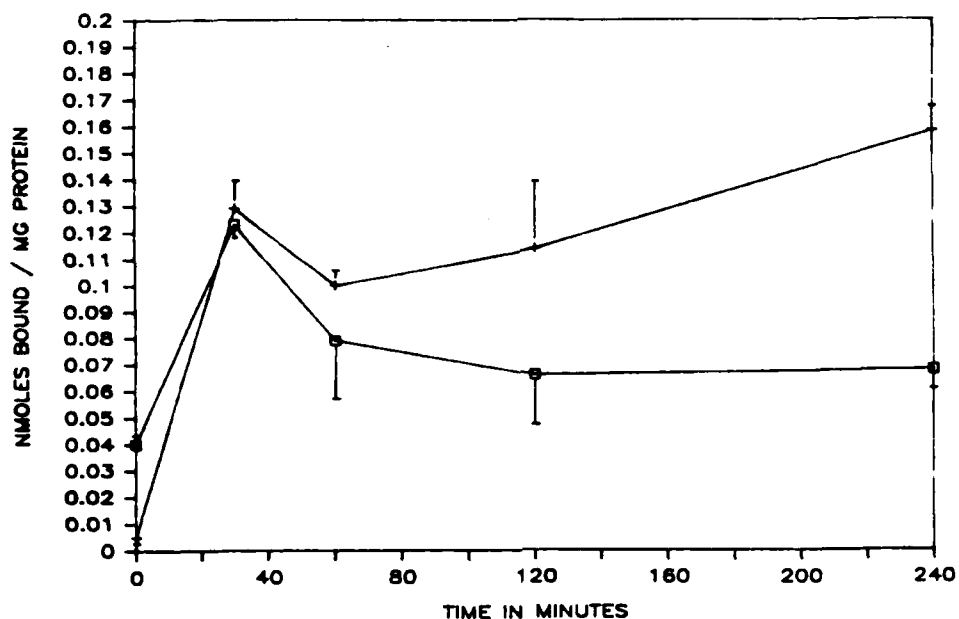


Figure 10. Time course formation and covalent binding of reactive naphthalene metabolites to intracellular (□) and extracellular (+) proteins in isolated hepatocytes incubated with 0.05 mM naphthalene. Values are the mean + S.E. for 3 incubations.

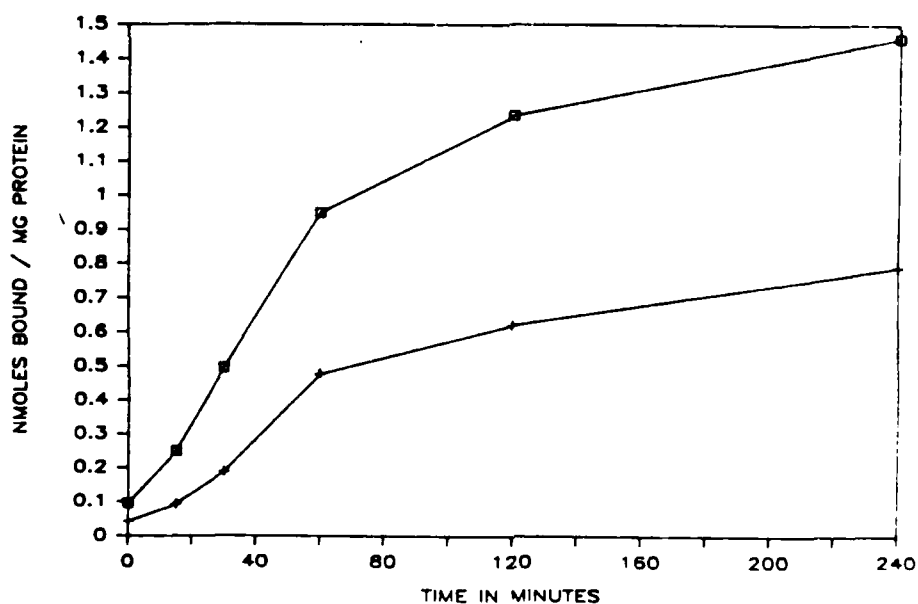


Figure 11. Time course formation and covalent binding of reactive naphthalene metabolites to intracellular (□) and extracellular (+) proteins in isolated hepatocytes incubated with 0.25 mM naphthalene. Values are the mean for 2 incubations.

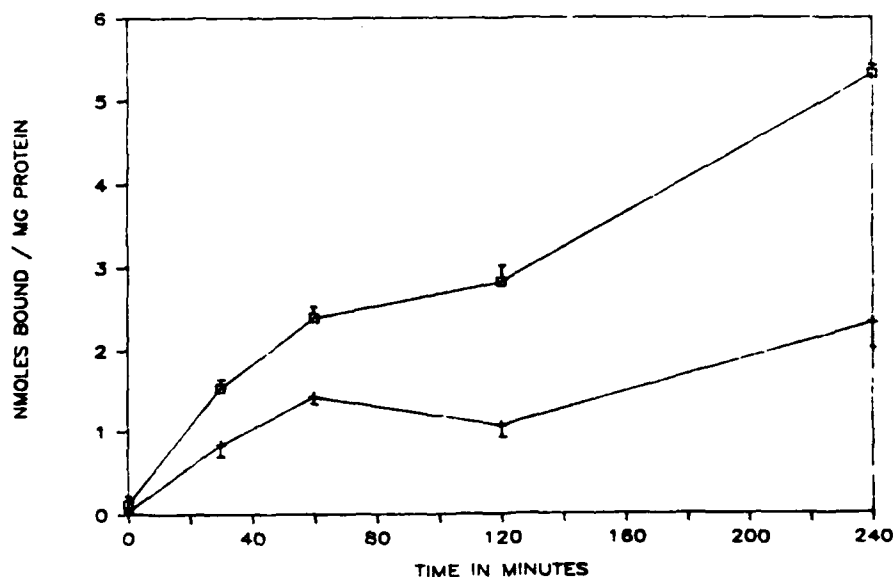


Figure 12. Time course formation and covalent binding of reactive naphthalene metabolites to intracellular (□) and extracellular (+) proteins in isolated hepatocytes incubated with 0.50 mM naphthalene. Values are the mean for 2 incubations.

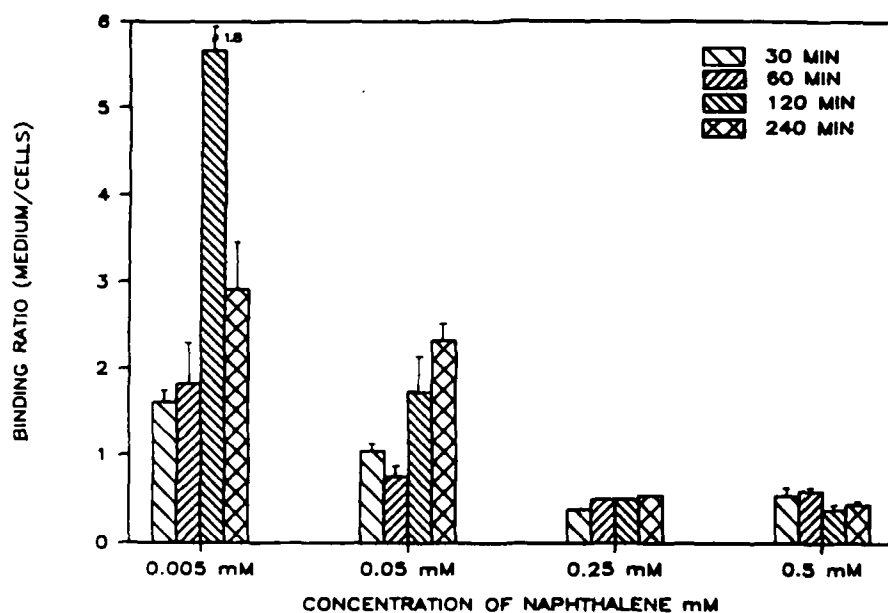


Figure 13. Ratio of covalently bound metabolites extracellularly vs intracellularly in isolated hepatocyte incubations. Values are from either single determinations (0.25 mM) or are the mean + range for 2 incubations (0.5 mM) or mean + S.E. for 3 incubations.

The relative levels of intracellular vs extracellular covalent binding must be interpreted with considerable caution because it is likely that the number of nucleophilic sites available for covalent interactions with reactive naphthalene metabolites differs on the interior vs the exterior of the cell. The number of nucleophilic sites could drastically alter the relative abilities to trap reactive naphthalene metabolites. However, the relative numbers of these sites would not differ between different substrate concentrations and thus the comparisons at different concentrations are likely to be informative. The other possibility which must be considered in interpreting these data is that the reactive naphthalene metabolite covalent protein complex may be formed intracellularly and may be excreted intact from the liver cell. The liver is responsible for the synthesis of many proteins including serum albumin and it is possible that the movement of the protein with covalently adducted naphthalene residues to the exterior of the cell accounts for the observed extracellular covalent binding. This possibility will be examined in future studies.

Thiol Status in Isolated Hepatocytes Incubated with Naphthalene or Menadione.

To determine whether there are interrelationships between the diffusion of reactive naphthalene metabolites and naphthalene oxide across intact hepatocellular membranes and the levels of intracellular reduced glutathione, hepatocytes were incubated for times ranging from 30 min to 4 hours with varying concentrations of naphthalene. Menadione (2-methyl-1,4-naphthoquinone) was used as a positive control since Thor et al. (1982) have demonstrated substantial glutathione depletion by this compound in isolated hepatocyte incubations.

To be certain that substantial losses in cell viability were not responsible for the effects being noted in cellular glutathione levels, hepatocyte viability was determined at 0, 120 and 240 min. The data in Figure 14 indicate that the initial viability of isolated hepatocytes was > 95% and did not fall below 80% for hepatocytes incubated in the presence of solvent, 0.005 or 0.05 mM naphthalene. Viabilities of isolated hepatocytes incubated with 0.5 mM naphthalene were 75% at 120 min and fell to less than 10% at 4 hours. Menadione (0.5 mM) resulted in a rapid loss of cellular viability (10% at 120 min). Thus, data collected at the high concentration of naphthalene at later time points (120 and 240 min) as well as the data from incubations with menadione at 120 and 240 min probably reflect, in part, the loss in numbers of viable hepatocytes.

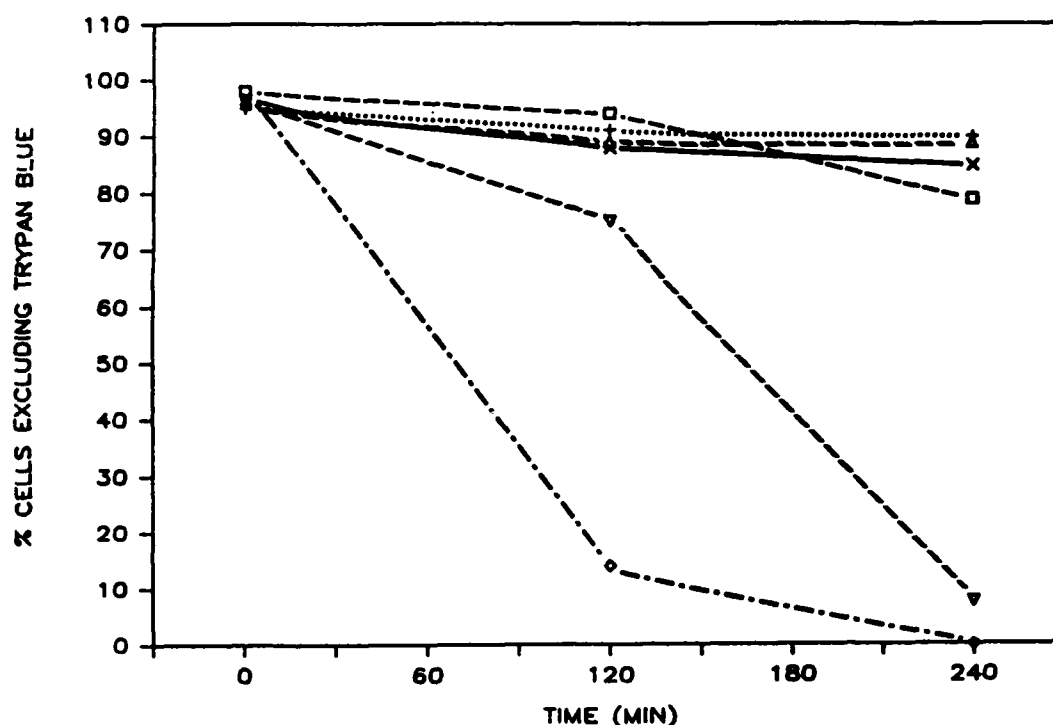


Figure 14. Viabilities of isolated hepatocytes (as assessed by trypan blue dye exclusion) incubated under the following conditions: (□) no additions, (+) methanol (vehicle) control, (Δ) 0.005 mM naphthalene, (X) 0.05 mM naphthalene, (▽) 0.5 mM naphthalene, and (◇) 0.5 mM menadione. Values are the mean from 3 incubations.

As indicated by the data in Figure 15 the reduced glutathione levels in different preparations of mouse hepatocytes at time 0 varied from 28 to 40 nmoles/million cells. This variation is probably due in part to interanimal differences in hepatic glutathione content and to small sampling errors in removing an aliquot of hepatocytes for determination of the thiol levels. Intracellular reduced glutathione levels remained constant over the first hour in cells incubated in the presence of solvent (methanol) or 0.005 mM naphthalene. Thereafter, the glutathione content increased to approximately 50 nmoles/million cells. These data are consistent with those reported by Farris and Reed (1983) who have demonstrated the synthesis of intracellular glutathione in hepatocytes incubated with media containing methionine. Addition of 0.05 mM naphthalene to the incubations resulted in a rapid depletion of glutathione to approximately 40% of the control levels 15-30 min after the start of the incubations. Intracellular glutathione levels recovered rapidly and

reached control at 120 min. Glutathione levels in hepatocytes incubated in the presence of either 0.5 mM naphthalene or 0.5 mM menadione decreased to less than 20% of control at the earliest time point studied and remained at this point throughout the incubation. These data support the view that the efflux of naphthalene oxide (Figure 8) is not dependent upon the substantial depletion of intracellular reduced glutathione levels. Moreover, the contention that the ratio of extracellular to intracellular covalent binding decreases substantially under conditions where intracellular glutathione is depleted is supported by the data in Figure 15.

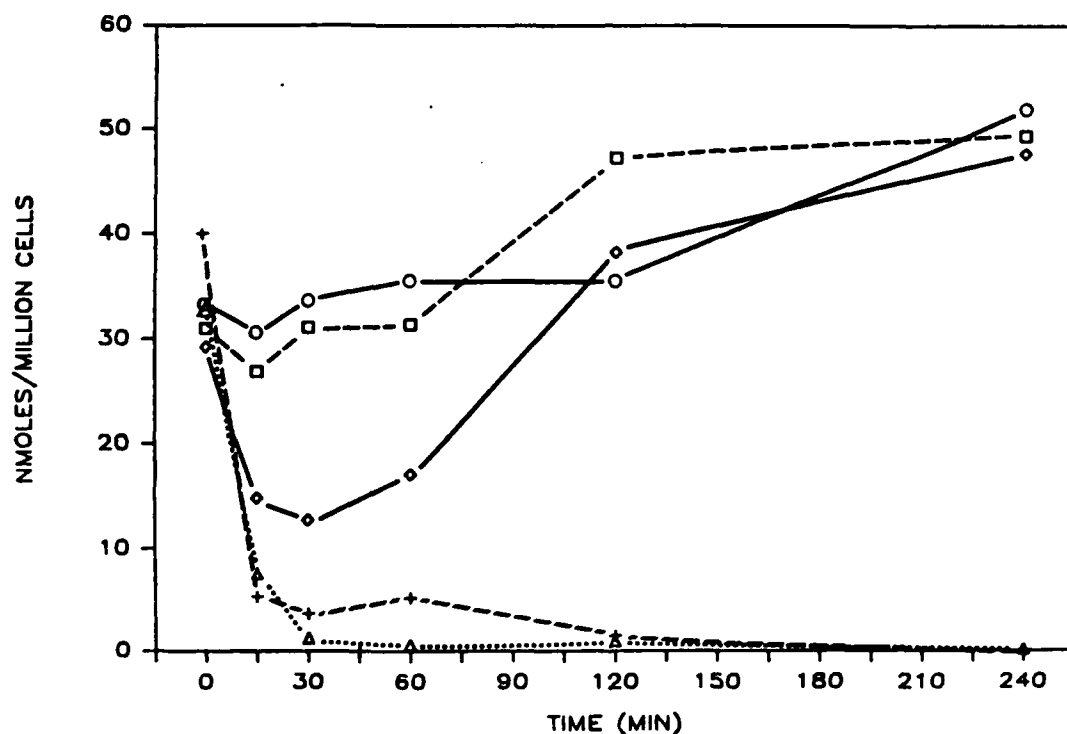


Figure 15. Intracellular reduced glutathione levels in hepatocytes incubated with menadione or varying concentrations of naphthalene. Data are for methanol control (□), 0.005 mM naphthalene (○), 0.05 mM naphthalene (◇), 0.5 mM naphthalene (X), or 0.5 mM menadione (+). Values are the mean of 3 separate hepatocyte incubations. Standard errors of the mean were less than 15% of the mean with the exception of the data for the 15 min. control, 120 min. 0.005 mM naphthalene; 15 and 30 min 0.05 mM naphthalene and 15 min 0.5 mM naphthalene which were less than 30% of the mean.

Studies with both the isolated perfused liver and with isolated hepatocytes have shown that the liver excretes large amounts of reduced glutathione. To determine whether incubation of isolated hepatocytes with naphthalene or menadione altered the rate of efflux of preformed glutathione, hepatocytes were incubated with 0.2 mM cystine in the incubation medium and glutathione was measured as the glutathione-cysteine mixed disulfide. The data in Figure 16 indicate that efflux of reduced glutathione in mouse hepatocytes is approximately 7.5 nmoles/hr/106 hepatocytes and that addition of increasing concentrations of naphthalene decreased the rate of glutathione efflux from the cells. The rate of glutathione efflux from mouse hepatocytes is approximately double that reported for rat hepatocytes (Farris and Reed, 1983). Naphthalene, at a concentration of 0.005 mM had no effect on the rate of efflux of glutathione from hepatocytes while 0.05 mM naphthalene initially decreased the rate of efflux to 2.5 nmoles glutathione per million cells in the first hour. Thereafter, the rate of efflux returned to control levels. In comparison, incubation of hepatocytes with naphthalene or menadione at 0.5 mM markedly decreased glutathione efflux. There was essentially no increase in extracellular glutathione levels in cells incubated with 0.5 mM naphthalene. There appears to be a good correlation between the time and extent of depletion of intracellular glutathione with the decrease in the rate of glutathione efflux observed in hepatocyte medium. These data are in considerable disagreement with those reported by Farris and Reed (1983) who were not able to demonstrate any change in the rate of glutathione efflux from isolated rat hepatocytes under conditions where intracellular glutathione was depleted. Other studies using isolated perfused liver have shown that the rate of release of reduced glutathione into the perfusate is highly dependent upon on the intracellular glutathione concentrations (Kaplowitz et al., 1985). Additional studies will obviously be required to resolve these issues.

It seemed possible that a portion of the intracellular glutathione depletion observed in the hepatocyte incubations could be due to naphthalene oxide being formed and released in one cell and depleting glutathione from another cell. To examine this question and provide further evidence that glutathione added to the cell incubation medium was unable to cross the intact hepatocyte membrane, hepatocytes were incubated in the presence and absence of 0.05 mM naphthalene with and without reduced glutathione and intracellular reduced glutathione levels were measured. All incubations contained glutathione reductase and 5 CDNB units of glutathione transferases to trap any naphthalene oxide leaving the cells. As noted from the data in Figure 17,

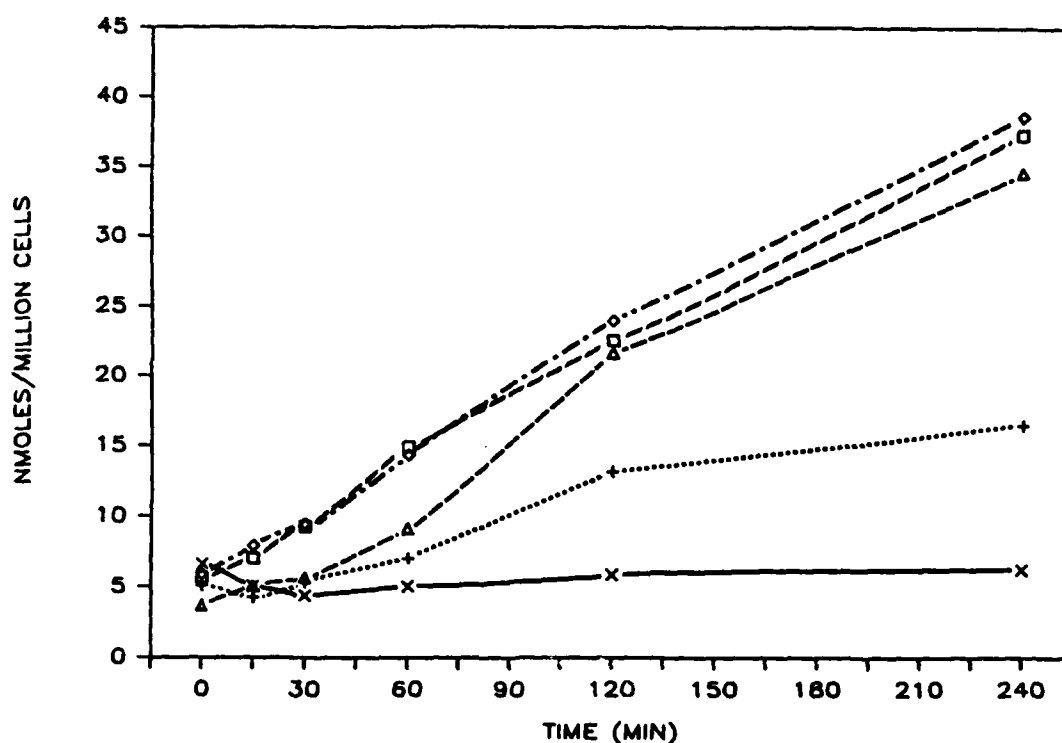


Figure 16. Extracellular reduced glutathione levels in isolated mouse hepatocytes incubated in the presence of solvent (control), menadione, or varying concentrations of naphthalene. The symbols denote the following: (□) vehicle control, (◇) 0.005 mM naphthalene, (△) 0.05 mM naphthalene, (X) 0.5 mM naphthalene and (+) 0.5 mM menadione. Values are the mean of three incubations. Standard errors did not exceed 15% of their respective means.

intracellular glutathione levels in hepatocytes incubated in the presence of glutathione did not differ significantly from hepatocytes incubated in control media. Consistent with previous observations, addition of naphthalene to hepatocyte incubations resulted in a 30% depletion of intracellular glutathione. The extent of glutathione depletion, however, was not altered by inclusion of glutathione in the hepatocyte medium. These data suggest that naphthalene oxide diffusing from one cell is not a major factor in the depletion of cellular glutathione in another cell. It is possible that naphthalene oxide does not cross the hepatocyte membrane easily and thus cannot deplete intracellular glutathione. These data would be consistent with the data showing that very large doses of naphthalene oxide administered in vivo were required before significant depletion of pulmonary

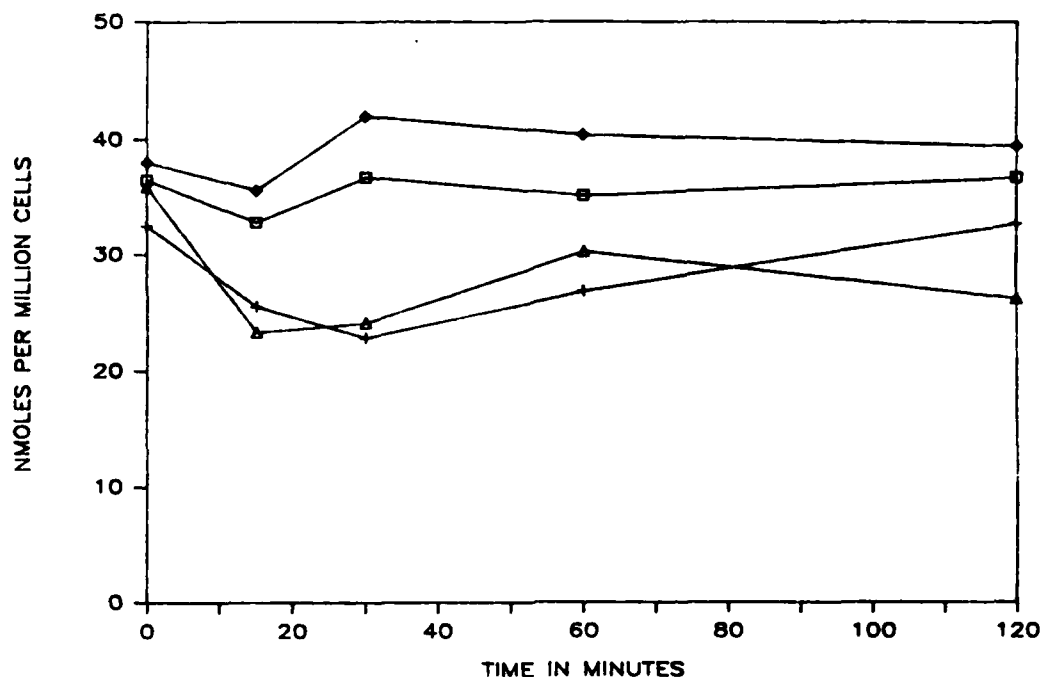


Figure 17. Intracellular reduced glutathione levels in hepatocytes incubated in the presence of naphthalene with or without glutathione. The symbol denote the following groups: (□) methanol (vehicle) control, (+) 0.05 mM naphthalene, (◇) 1 mM glutathione, or 0.05 mM naphthalene with 1 mM glutathione. Values are the mean of three separate incubations. Standard errors did not exceed 15% of the mean with the exception of data for 15, 60 and 120 min time points of the naphthalene + glutathione group and the 120 min time point for the 1 mM glutathione group which did not exceed 25% of the mean.

glutathione was observed (Richieri and Buckpitt, 1984). Preliminary studies in which naphthalene oxide was added directly to hepatocyte incubations showed very rapid and nearly complete loss of intracellular glutathione with the corresponding formation of oxidized glutathione. These data will require further confirmation before an adequate explanation can be provided.

SUMMARY AND CONCLUSIONS

Intraperitoneal administration of the volatile hydrocarbon, naphthalene, results in a highly tissue and species selective lesion of murine Clara cells (Mahvi et al., 1977, Tong et al., 1982, Warren et al., 1982). The fact that the Clara cell is a target cell for naphthalene and that this cell type has been identified as a major locus of cytochrome P450 monooxygenases in the lung (Boyd, 1977; Serabjit-Singh et al., 1980) supports the view that metabolic activation of naphthalene in murine Clara cells may play a necessary role in the lung lesion by naphthalene. Subsequent studies on the relationship between the fate and formation of highly reactive naphthalene metabolites and their covalent binding to macromolecules in the lung demonstrated a good correlation between these two events (Warren et al., 1982; Buckpitt and Warren, 1983). However, the finding of high levels of covalent binding in nontarget tissues and data indicating that reactive naphthalene metabolites appeared to circulate suggested that in vivo covalent binding was not necessarily a good measure of the formation of reactive metabolites in that tissue in situ and that possibly chemically different reactive metabolites were being formed in target vs nontarget tissues. Subsequent studies using glutathione to trap reactive naphthalene metabolites formed in microsomal incubations have demonstrated a good correlation between the formation of a specific glutathione adduct of naphthalene and the tissue selectivity for naphthalene-induced tissue necrosis (Buckpitt et al., 1984; Buckpitt and Bahnson, 1984).

Reactive and potentially toxic metabolites released from the liver could potentially interact with extrahepatic tissues/cells in a number of detrimental ways. Such metabolites could potentially be responsible for the pathologic effects observed after exposure to the parent compound. As suggested by Ottenwalder et al. (1983), the formation of reactive vinyl chloride metabolites by hepatocytes followed by the transport of these to hepatic sinusoidal cells may be involved in the hemangioendotheliomas observed after vinyl chloride exposure. Alternatively, it is possible that reactive metabolites released from one tissue could exacerbate the toxicity of reactive metabolites formed in a target tissue by altering intracellular detoxication capabilities. The studies covered in this report were done in an effort to develop and validate a method for measuring the rate of release of reactive and potentially toxic metabolites of naphthalene and to study the mechanisms for this process. These studies indicated that 17-35% of the total amount of naphthalene oxide formed intracellularly is capable of leaving intact hepatocytes and that the rate of release of the epoxide is not dependent upon the depletion of intracellular glutathione levels. In addition, the

studies reported here demonstrated the release of reactive naphthalene metabolites which are capable of becoming covalently bound to extracellular protein from hepatocytes. The relative proportions of reactive metabolite covalently bound intracellularly vs extracellularly were dependent upon substrate concentration and appeared to be related to intracellular glutathione levels. At the higher substrate concentrations where glutathione was depleted substantially, there was relatively more intracellular compared to extracellular covalent binding in comparison to low naphthalene concentrations where the covalent binding to proteins extracellularly predominated. Trapping naphthalene oxide effluxing from hepatocytes by inclusion of glutathione and glutathione transferases failed to block the depletion of intracellular glutathione, thus suggesting that naphthalene oxide released from cells cannot enter other cells easily. While this is consistent with in vivo data showing depletion of pulmonary glutathione only after the administration of very large quantities of naphthalene oxide, this observation will require further study.

In conclusion, these studies have demonstrated the release of naphthalene oxide during the incubation of naphthalene with isolated hepatocytes. How this compound, which has a half life in buffer at 37°C of less than 3 min and which is a good substrate for both the glutathione transferases (Hayakawa et al., 1975) and epoxide hydrolases (Dansette et al., 1974), is capable of diffusing from the cells deserves additional attention.

The following is a list of abstracts, published manuscripts and manuscripts in preparation which have been supported in whole or part by funds from contract #AF33615-80-C-0512:

Abstracts:

Buckpitt, A.R., Smart, G. and Baker, B.: Pulmonary bronchiolar damage of naphthalene administered by inhalation. Fed. Proc. 41: 7997 Abs, 1982.

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metabolism of naphthalene and 2-methylnaphthalene and the effect of indomethacin on the bronchiolar necrosis. *Biochem. Pharmacol.* in press.

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Buckpitt, A.R. and Bahnson, L.S.: Species differences in naphthalene-induced pulmonary bronchiolar necrosis: Correlation with the kinetics of microsomal formation of glutathione adducts., in preparation.

Buckpitt, A.R., Bahnson, L.S., and Franklin, R.B.: Effects of cobalt protoporphyrin on naphthalene and 2-methylnaphthalene-induced bronchiolar injury and on naphthalene metabolism in vitro and in vivo., in preparation.

Richieri, P.R. and Buckpitt, A.R.: Diffusion of naphthalene oxide and reactive naphthalene metabolites from hepatocytes., in preparation.

Richieri, P.R., Buonarati, M. and Buckpitt, A.R.: Intracellular glutathione levels and glutathione release in hepatocytes incubated with naphthalene, in preparation.

Buckpitt, A.R. and Bahnson, L.S.: Naphthalene metabolism by human lung microsomal enzymes. *Drug Metab. Dispo.*, submitted.

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