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A novel pathway for morphine biotransformation

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A NOVEL PATHWAY FOR MORPHINE BIOTRANSFORMATION: IDENTIFICATION AND SIGNIFICANCE OF 10α-S-GLUTATHIONYLMORPHINE

by

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DISSERTATION

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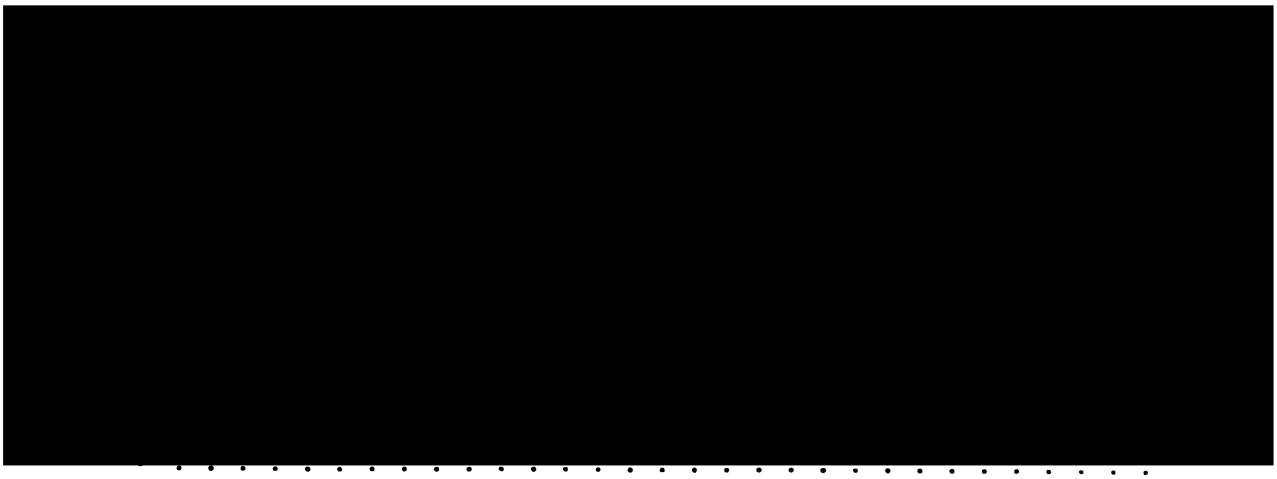

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ABSTRACT

Previous results from our laboratory have shown that incubation of morphine with rat liver microsomes in the presence of NADPH resulted in irreversible binding of the drug to microsomal protein. This process was found to be not only cytochrome P-450 dependent but also GSH inhibitable thereby indicating the possibility that morphine is metabolized to a reactive intermediate which could be trapped with GSH. To examine this possibility, rat liver microsomes were incubated with morphine, GSH and NADPH. Isolation of a morphine-GSH adduct from such aerobic incubations provided evidence that morphine was indeed metabolized to a highly reactive intermediate. The adduct was isolated by high performance liquid chromatography (HPLC) and structurally characterized by ^{1}H NMR spectroscopy to determine the site of nucleophilic attachment to the reactive morphine species. NMR spectral analysis of morphine, GSH and the morphine-GSH adduct narrowed the possible sites of GSH attachment to C_9, C_{10}, and C_{16}. Overlap of GSH proton signals with those of morphine prevented their assignment. In order to obtain more conclusive spectra of the morphine adduct, morphine-cysteine and morphine-N-acetylcysteine adducts were isolated from rat liver microsomal incubations. In the absence of the GSH proton signals, C_9 and C_{16} were ruled out as sites of GSH attachment, and the adduct was identified as 10α-N-acetylcysteinylmorphine thereby demonstrating nucleophilic attack at the benzylic C_{10} position.

The in vitro and in vivo significance of the 10α-morphine-GSH pathway was investigated in human and rat liver microsomes, in rat bile, and in freshly isolated rat

hepatocytes. In human liver microsomes, the extent of formation of the 10α-morphine adduct was at least equal to but usually greater than the extent of N-demethylation, the other microsomal cytochrome P-450 dependent morphine metabolic pathway. In liver microsomes from phenobarbital-pretreated rats, 10α-morphine-GSH formation was at least twice as great as N-demethylation. In rat bile, 10α-morphine-GSH was identified as a minor metabolite. In freshly isolated hepatocytes, the metabolite was most significant at high morphine concentrations, increasing by 4-fold over that formed at low morphine concentrations. This dramatic increase appeared to occur when glucuronidation was overwhelmed.

Hepatocyte incubations with morphine resulted in a profound dose-dependent depletion of GSH. The pattern of GSH depletion paralleled that of 10α-morphine-GSH formation.

Together these findings suggest that cytochrome P-450 activates morphine at the benzylic C_{10}-position in what appears to be a novel pathway for its metabolism. This metabolic pathway is viable both in vivo and in vitro and appears to contribute, at least in part, to the observed morphine-mediated hepatic GSH depletion and liver damage.

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List of Abbreviations

ACTH, adrenocorticotropic hormone

FABMS, fast atom bombardment mass spectrometry

GSH, reduced glutathione

GSSG, oxidized glutathione

M-3-G, morphine-3-glucuronide

M-6-G, morphine-6-glucuronide

MES, morphine-3-ethereal sulfate

Morphine-N-AcCys, morphine-N-acetylcysteine

PB, phenobarbital

SGOT, serum glutamic oxaloacetic transaminase

SGPT, serum glutamic pyruvic transaminase

TMA, tetramethylammonium hydroxide

1.0 METABOLISM OF MORPHINE

The metabolism of morphine (Figure 1.1) has been studied for well over a century. Morphine is extensively metabolized in most mammalian species with less than 10% of an administered dose being recovered unchanged in the urine (Yeh *et al.*, 1977). The known metabolites of morphine are shown in Figure 1.2.

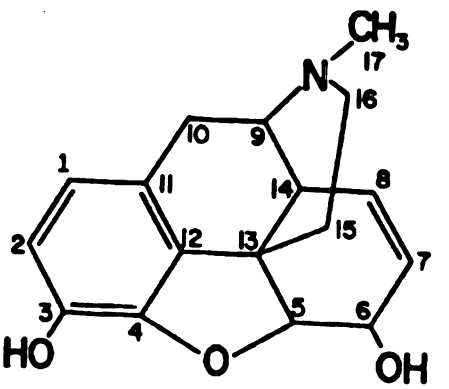


Figure 1.1 Structure of Morphine.

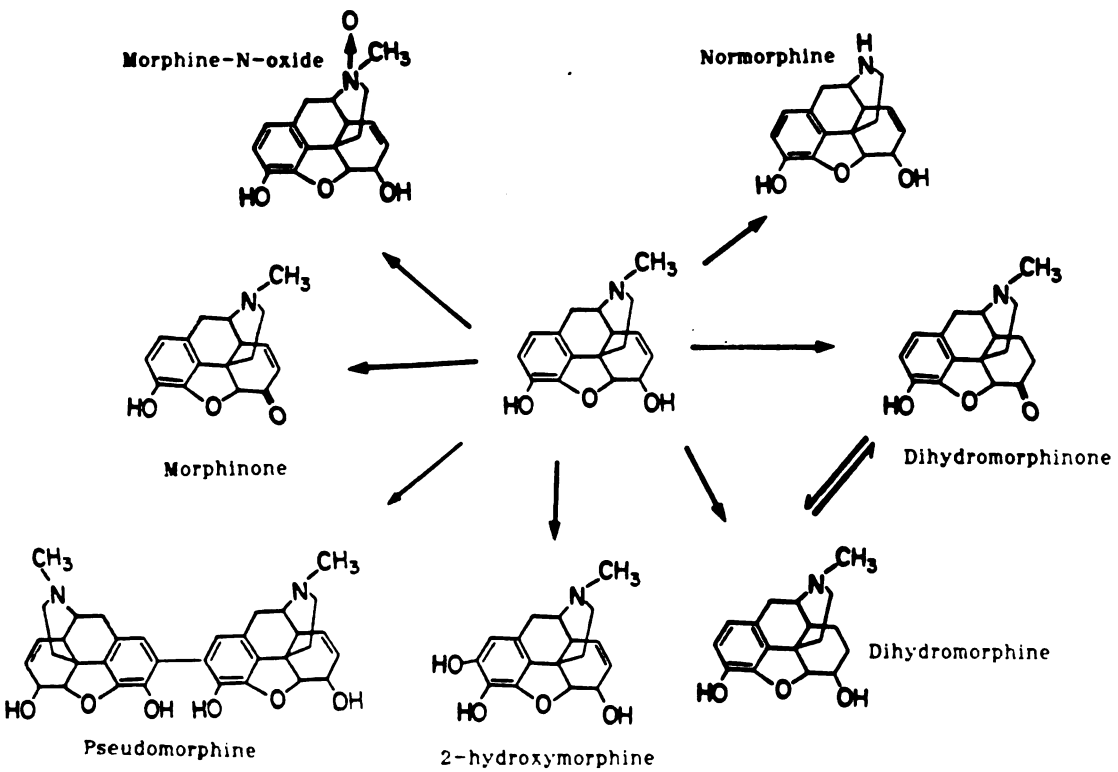
In all species studied, conjugation appears to be the major route of metabolism. Conjugation of morphine had been postulated as early as the 1880's, but it was not until 1940 that the biological fate of morphine was clarified. Oberst (1940) and Gross and Thompson (1940), studying human and dog urine respectively, offered the first evidence for the excretion of morphine in "bound" form. It was demonstrated that urine collected after morphine administration yielded increased quantities of morphine when subjected to acid hydrolysis. In 1954, Woods isolated a monoglucuronide

Figure 1.2 **Known metabolites of morphine.**

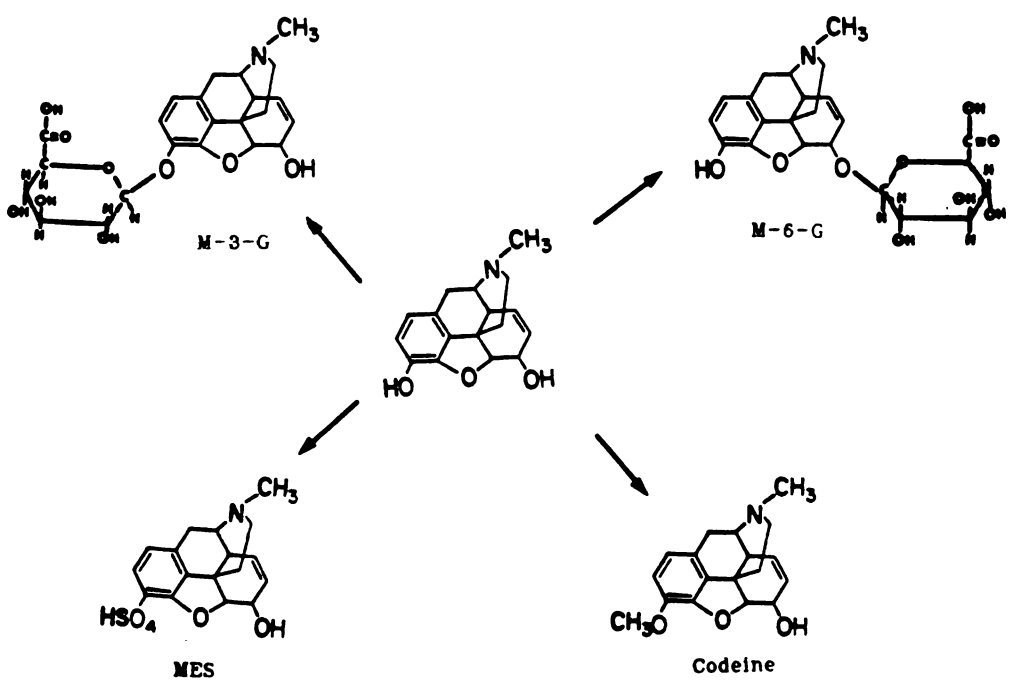
(A) Phase 1 metabolites

(B) Phase 2 metabolites

Phase 1 Reactions



Phase 2 Reactions



conjugate from dog urine and demonstrated that conjugation with UDP-glucuronic acid occurred at the phenolic position. Fujimoto and Way (1957) isolated and crystallized "bound" morphine from the urine of human addicts and established that this too was a monoglucuronide conjugate. Concurring with Woods, Fujimoto and Way showed that the glucuronide was bound at the phenolic position.

Since then, morphine-3-glucuronide (M-3-G) has been shown to constitute the major morphine metabolite in humans and in most animal species. In the rat, within 1 h of [^{14}C]morphine administration (15mg/kg), 87% of total [^{14}C]morphine in the liver, 86% of total [^{14}C]morphine in the plasma and 58% of total [^{14}C]morphine in the urine was found to be M-3-G (Liu and Wang, 1980). In human urine, 54-74% of an administered dose was recovered as M-3-G (Yeh, 1975; Sawe *et al.*, 1985).

A second glucuronide metabolite, morphine-6-glucuronide (M-6-G), was isolated and identified as a minor metabolite of morphine in rabbit urine and bile by Yoshimura *et al.*, (1969). Analysis by thin layer chromatography showed that it was also a minor metabolite in guinea pig, mouse and human urine (Oguri *et al.*, 1970). M-6-G has since been identified in human urine by GC-Mass spectrometry (Yeh *et al.*, 1977a) and tentatively in human plasma (Svensson *et al.*, 1982). Morphine 3,6-diglucuronide has also been identified in human urine by GC-mass spectrometry (Yeh *et al.*, 1977a). Although M-6-G was identified as a morphine metabolite in rat urine by Yeh *et al.* (1979), no other study has supported this finding. Rane *et al.* (1985) reported that natural (-)morphine was glucuronidated in rat liver microsomes only at position 3.

Morphine is converted to a sulfate conjugate to form morphine-3-ethereal sulfate (MES). MES was isolated from the urine of the chicken and the cat by Fujimoto and Haarstad in 1969. It is the major metabolite in both species as each is deficient in glucuronidation. Yeh *et al.* (1977a) estimated that the amount of MES in human urine was about 1% that of M-3-G. MES has not been detected as a metabolite in the rat.

Morphine is also N-demethylated by the cytochrome P-450 monooxygenase system. N-demethylation of morphine was first proposed by March and Elliot (1954) who collected $^{14}CO_2$ in expired air from rats treated with N-$^{14}CH_3$-morphine. In 1956, Axelrod measured formaldehyde liberation in rat liver homogenate and, using paper chromatography, also identified normorphine as a metabolite. Normorphine was identified in vivo by Misra *et al.* (1961) who found [^{3}H]normorphine in the urine of of [^{3}H]morphine treated rats.

Normorphine has since been found to be a minor but consistent metabolite in vivo in the mouse, rat, rabbit, guinea pig, cat, dog and monkey (Misra *et al.*, 1973; Klutch, 1974; Yeh *et al.*, 1977). In the rat, between 2-10% of a low morphine dose has been found to be excreted in the urine as total normorphine (Misra *et al.*, 1973; Klutch, 1974; Yeh *et al.*, 1977). Yeh (1975) identified normorphine and normorphine conjugates in human urine and reported that they accounted for 1 and 4%, respectively, of the injected dose in morphine-dependent subjects. Normorphine-6-glucuronide was identified in human urine by GC-mass spectrometry by Yeh *et al.* (1977a).

Dihydromorphinone has also been identified as a minor morphine metabolite in the rat, mouse, rabbit, guinea pig, cat, dog and monkey (Klutch, 1974; Yeh, 1977;

Yeh *et al.*, 1979). Klutch found that this metabolite accounted for 2-6% of a low dose of morphine given chronically to the rat. No dihydromorphinone has been detected in human urine (Yeh *et al.*, 1977a; Yeh *et al.*, 1979).

Morphine also undergoes hydrogenation of the 7,8-double bond to form dihydromorphine. α- and β-dihydromorphine were identified as morphine metabolites in the guinea pig, rat and rabbit by GC-mass spectrometry (Yeh *et al.*, 1979). Dihydromorphine has also been isolated from rabbit urine after administration of dihydromorphinone (Roerig *et al.*, 1973), thereby suggesting that hydrogenation of the 7,8-double bond may take place either before or after oxidation of the 6-hydroxyl group.

Morphine N-oxide was identified as a minor metabolite in rat liver homogenates by Misra *et al.* (1973) using TLC and paper chromatography. It was also identified as a urinary metabolite in guinea pigs (Yeh *et al.*, 1979) and in cancer patients treated with morphine in combination with certain drugs (Woo *et al.*, 1968). Although the N-oxide has not been found as an in vivo metabolite in most species, it may be reduced in the body. The in vivo reduction of morphine-N-oxide to morphine and the consequent formation of M-3-G in rats (Misra and Mitchell, 1971) suggests that metabolically formed morphine N-oxide may itself be transformed.

O-Methylation of morphine to codeine has also been reported. The formation of codeine was reported as a metabolite in the rat and the dog by Elison and Elliott (1964) who further showed that Gunn rats, deficient in glucuronidation, exhibited enhanced codeine formation. The presence of codeine in human urine has been the subject of some debate (Boerner and Abbott, 1973; Yeh, 1974; Boerner and Roe,

1975). Boerner and Abbott (1973) reported that 85% of urine samples of heroin addicts studied contained codeine. Between 12 and 15% of total morphine recovered was identified as codeine by gas chromatography. Yeh (1974), however, found no evidence of codeine formation in the urine of heroin addicts.

There have also been reports of morphine metabolites which would suggest the formation of reactive intermediates. Pseudomorphine, a dimerized oxidation product of two molecules of morphine, has been reported in a number of in vitro systems (Hosoya and Brody, 1957; Roerig *et al.*, 1976). Roerig *et al.* (1976) suggested that the conversion of morphine to pseudomorphine in the presence of horseradish peroxidase (HRP) and H_2O_2 involved a free radical intermediate. This argument was supported by the findings that morphine became irreversibly bound to human serum albumin in the presence of HRP and $MnCl_2$ or H_2O_2 (Misra and Mitchell, 1971) and that morphine became strongly bound to specific amino acids in a system containing HRP and H_2O_2 (Deutsch *et al.*, 1977).

A morphine metabolite with a catechol structure has also been proposed. Catechols have been previously shown to be converted to semiquinone radicals and to bind irreversibly to tissue macromolecules (Dybing *et al.* 1976; Remmer *et al.*, 1977). Misra *et al.* (1973) reported the identification of a [^{14}C]morphine metabolite containing vicinal phenolic groups which was isolated from incubations of rat brain or liver homogenates. Liver homogenates convert approximately 6-7% of morphine to this metabolite. Interestingly, Daly *et al.* (1965) also identified 2-hydroxymorphine in rabbit liver microsomes.

Yamano *et al.* (1985) purified a morphine 6-dehydrogenase from guinea pig liver which catalyzes the oxidation of morphine to morphinone. Morphinone has been reported to bind covalently to protein thiols (Nagamatsu *et al.*, 1982, 1983).

On retrospective analysis, proposals of reactive morphine metabolites provide a plausible explanation for hepatic damage induced by morphine. However, the effects of morphine on the liver were first noticed in the 1950's, nearly twenty years before research established a link between hepatotoxicity and metabolism of certain xenobiotics to reactive electrophilic intermediates. Consequently, the effects of morphine on the liver were historically examined from a different perspective.

2.0 HISTORICAL ASPECTS OF THE EFFECTS OF MORPHINE ON THE LIVER

The study of morphine metabolism took on a new direction after Axelrod reported that morphine tolerant rats N-demethylated morphine at a significantly slower rate than nontolerant rats (1956*a*). Axelrod proposed the depression of N-demethylation as a model for morphine tolerance since N-demethylating enzymes were stereospecific towards levo-isomers, and since nalorphine, a pharmacological antagonist of morphine analgesia, inhibited N-demethylation and blocked both the development of tolerance and the reduction of enzyme activity (Axelrod, 1956*a*; 1956*b*; Axelrod and Cochin, 1956). It was suggested that continuous interaction of narcotic drugs with their receptors and/or enzymes for N-demethylation led to their inactivation.

Studies by Cochin and Axelrod (1959) supported Axelrod's initial hypothesis. In a comparison of in vitro reduction of enzyme activity and in vivo development of tolerance, the degree of depression of N-dealkylation was shown to parallel the degree of tolerance developed. However, the theory was challenged in a number of articles published in the following years (Takemori and Mannering, 1958; Mannering and Takemori, 1959; Way and Adler, 1960; Ellison *et al.*, 1963; Clouet and Ratner, 1964).

Takemori and Mannering (1958) disputed the assertion that the N-demethylase was stereospecific toward levo-isomers since the stereoisomers levorphanol and dextrorphan were N-demethylated with equal ease. This result questioned Axelrod's proposed mechanism for the depression of N-demethylation since even though dextrorphan was N-demethylated to the same extent as levorphanol, this dextro-isomer failed

to depress N-demethylation of narcotic drugs to the same extent as either of the levo-isomers levorphanol or morphine (Mannering and Takemori, 1959)

Elison *et al.* (1963), studying the kinetics of N-demethylation in rat liver microsomes, demonstrated that there was no difference in substrate binding between normal and morphine-tolerant rats and thus no alteration in enzyme structure. On the other hand, it was shown that the decreased N-demethylation in tolerant rats was due to reduced levels of enzymes (Elison *et al.*, 1963) since the V_{max} was 50% lower in morphine-tolerant rats.

References had been made since 1959 (Cochin and Axelrod) to a decrease in liver enzymes as the causal factor for depressed N-demethylation in morphine-tolerant rats. Clouet (1965) reported that chronic morphine treatment decreased the level of the microsomal carbon monoxide binding pigment (later named cytochrome P-450) and that this decrease in the amount of the hemoprotein paralleled the decrease in enzyme activity.

Sladek *et al.* (1974) subsequently reported that morphine decreased hepatic cytochrome P-450 levels and the hepatic metabolism of non-narcotic as well as narcotic drugs in adult male rats. Depression of aminopyrine N-demethylation, hexobarbital hydroxylation, and steroid hormone hydroxylation was also reported in liver microsomes of morphine-treated rats (Kato and Gillette, 1965; Kato *et al.*, 1970).

In the 1960's and early 1970's, the effect of morphine on hepatic drug metabolism in rats was found to be associated with the long recognized sex differences in drug metabolism in this species. For example, N-demethylase activity in female rat liver was known to be a fraction of that found in males. These differences were

presumed to result from androgen-induced effects on both cytochrome P-450 synthesis and substrate binding (Schenkman *et al.*, 1967; Kato and Onoda, 1970).

Much of the research on the morphine effect on hepatic drug metabolism focused on the relationship of morphine treatment to known androgen-induced effects on liver drug metabolizing enzymes. Studies by Kato and Gillette (1965), Kato and Onoda (1970), Kato *et al.* (1970, 1971) and Sladek *et al.* (1974) reported that depression of mixed-function oxidations after morphine treatment occurred in mature male but not immature male or female rats. Administration of testosterone to developing female rats increased the rates of ethylmorphine and hexobarbital metabolism to levels seen in male rats. Morphine administration to these female rats depressed the testosterone-stimulated metabolism. In male rats, the effects of morphine administration appeared to parallel the effects of castration. Morphine treatment was found to decrease sharply the binding capacity of cytochrome P-450 for specific substrates. These results led to the conclusion that morphine treatment depressed sex dependent drug metabolism in mature male rats by impairing the androgen effect on the hepatic mono-oxygenase system.

Morphine was also found to activate the pituitary-adrenal axis via the hypothalamus (George and Way, 1957, 1959) and was postulated to have an effect on drug metabolism similar to that of adrenalectomy (Kato *et al.*, 1971). Kato *et al.* (1971) had shown that adrenalectomy decreased drug metabolism in the rat. In addition, pituitary hormones were shown to be intimately involved in the regulation of hepatic drug metabolism (Wilson, 1969, 1970). Morphine treatment was shown to block pituitary ACTH, and morphine-induced increases in hexobarbital sleeping time were

largely reversed by exogenous administration of ACTH (Munson and Briggs, 1955; Bousquet *et al.*, 1964). Thus, it was suggested that not only androgenic hormones but neurohumoral factors were involved in the morphine-mediated depression of drug metabolism in the rat.

In the late 1970's, new evidence suggested that morphine-induced depression of drug metabolism could be related to hepatic damage. Morphine was found to depress the metabolism of ethylmorphine in both male and female mice (Ho *et al.*, 1976). Mice implanted with a morphine pellet for one day had significantly decreased liver weight, and hepatic drug metabolizing enzymes were significantly lowered as compared to those in animals implanted with a placebo (Ho and Takanaka, 1977). Since there are no sex differences in drug metabolism in the mouse, other factors were clearly responsible for the hepatic effects of morphine in mice.

The possibility that such effects might be due to morphine-induced hepatic damage was indicated by two-fold increases in SGOT and SGPT levels in mice implanted with a morphine-pellet for 1 day (Chang and Ho, 1979). Increases in the activities of these marker enzymes were significantly attenuated by concurrent administration of naloxone, completely prevented by hypophysectomy and partially blocked by adrenalectomy leading to the conclusion that the morphine-induced hepatic damage in mice might be of CNS origin.

Gurantz and Correia (1981) also reported 2-3 fold increases in SGOT and SGPT levels in mature male rats after morphine treatment. As in the mouse, these increases were significantly lessened by administration of naloxone. Increases in SGOT and SGPT were accompanied by an acute reduction of hepatic cytochrome P-450 (Gurantz

and Correia, 1981). It was demonstrated that the cytochrome P-450 loss resulting from a single dose of morphine occurred through increased degradation of the heme moiety of cytochrome P-450. This morphine effect was also blocked by administration of naloxone. While naloxone inhibition of morphine action in the CNS was well known, it was also possible that naloxone prevented the loss of cytochrome P-450 heme and the increases in serum transaminase levels by competing with morphine either as a substrate for cytochrome P-450 or at an as yet unidentified hepatic receptor site.

Concurrent morphine-induced hepatic glutathione (GSH) depletion, long associated with hepatic drug metabolism, also suggested a direct effect of the drug on the liver. James *et al.* (1982) reported that morphine treatment decreased hepatic GSH in mice to approximately 50% of basal values within 3 to 6 hours after morphine treatment. This depletion was dose-dependent and abolished by the narcotic antagonist, naltrexone. Correia *et al.* (1984*a*) reported that acute treatment of male rats with morphine for only an hour depleted their hepatic GSH content by 50-60%.

Although inhibition of these hepatotoxic effects by naloxone or naltrexone is consistent with CNS involvement morphine-mediated hepatotoxicity, it was also possible that these antagonists, by acting at morphine metabolizing sites, could impede the formation of a toxic intermediate in the liver. The hepatic effects observed in adult male rats might be explained by the extensive metabolism in mature male rats as compared to that in female or immature male rats. For example, in female rat liver, morphine N-demethylase was reported to be about one-tenth that found in male liver (Axelrod, 1956b). Thus, if metabolism to a reactive intermediate were the cause of

these effects and if it were to be similarly low in female rats, then the hepatic damage would of course be significantly greater in male than female rats.

James and Harbison (1982) reported the depletion of hepatic GSH and increases in SGPT levels after administration of the narcotic agonists SKF 525A, L-α-acetylmethadol (LAAM) or norLAAM. These effects were found to be strain and species dependent. The hamster, mouse and Fisher 344 rat were affected, but the guinea pig and Sprague-Dawley rat were not. The agonists were analgesic in all species and strains but species and strain selective in hepatic effects. Drug metabolism, on the other hand, is known to be species and strain dependent. This paradox further supported the argument that the hepatic effects induced by morphine were at least partly the result of morphine metabolism in the liver.

The possible formation of reactive morphine metabolites by rat liver microsomes was investigated by Correia *et al.* (1984*a*) using [^{3}H]dihydromorphine as a radiolabelled tracer. Preliminary investigations showed that [^{3}H]dihydromorphine caused GSH depletion and increased serum transaminases comparably to that precipitated by morphine. The studies revealed that [^{3}H]dihydromorphine bound covalently to microsomal protein when incubated in the presence of NADPH and O_2. Such irreversible binding was inhibited in the presence of CO and was augmented in hepatic microsomes of rats pretreated with the cytochrome P-450 inducer, phenobarbital. These findings strongly suggested that such irreversible binding to hepatic microsomal protein was a cytochrome P-450 dependent process.

Such irreversible binding of morphine to microsomal protein was also markedly inhibited by the inclusion of GSH or naloxone (Correia *et al.*, 1984*a*). Inhibition of

covalent binding by GSH suggested the formation of a reactive metabolite. The effect of naloxone suggested competitive substrate inhibition, since naloxone is also known to inhibit morphine N-demethylation competitively. Moreover, it was also shown that covalent binding was enhanced in rats whose glucuronidation was compromised by pretreatment with galactosamine, perhaps suggesting that inhibition of glucuronidation shunted metabolism to the pathway leading to a reactive intermediate.

Irreversibly bound adducts of radiolabelled morphine (or its metabolites) have been reported previously. Misra *et al.* (1971) reported covalent binding [^{14}C]morphine in rat brain, whereas Mullis *et al.* (1979) reported irreversible binding of [^{3}H] and [N-$^{14}CH_3$]morphine not only in the rat brain, but liver and lung tissue as well. The nature of the reactive intermediate(s), however, was then unknown.

Nagamatsu *et al.* (1982, 1983) argued that morphine is metabolized to morphinone in the mouse by the cytosolic enzyme 6-β-dehydrogenase and that morphinone was responsible for the hepatic depletion of GSH. These authors suggested (Nagamatsu *et al.*, 1986) that morphine-induced GSH depletion is due to the formation of an adduct formed by the Michael addition of GSH to the C_8 position of morphinone.

The findings of Correia *et al.* (1984*a*), on the other hand, suggest that the hepatotoxic effects of morphine are at least in part mediated by the cytochrome P-450 monooxygenase system. By a comparison of structural features of morphine analogs with hepatotoxic indices, it was shown that the (-)3-hydroxy-N-methylmorphinan moiety of morphine was responsible for the liver damage. HPLC analysis of incubations of rat liver microsomes (Correia *et al.*, 1984*b*) with [^{3}H]morphine and GSH or morphine and [^{3}H]GSH revealed the formation of a radioactive polar metabolite

which contained both morphine and GSH.

Recently, Roberts *et al.* (1987) reported that intracerebro-ventricular injection of morphine produced a 25% depletion of hepatic GSH in mice over a period of 3 hours. Although morphine-mediated effects in the central nervous system may well account for some hepatic GSH depletion, the rapid morphine-induced depletion of hepatic GSH in vivo, as well as in the isolated perfused rat liver, the evidence for cytochrome P-450 involvement in morphine-induced hepatic effects and the formation of a morphine-GSH adduct strongly argue that morphine is transformed to a reactive electrophilic metabolite which binds to GSH in a process which may contribute to GSH depletion and consequent binding of reactive metabolites to tissue nucleophiles in the rat liver.

3.0 HEPATIC DEPLETION OF GLUTATHIONE

Glutathione is involved in numerous aspects of cell function. In all of these aspects, cell function depends on the proper ratios of GSH and glutathione disulfide (GSSG). The maintenance of cell and organelle membrane integrity, the polymerization of tubulin and disassembly of microtubules, and the conformation of proteins and modulation of protein function all depend on the GSH status of the cell, and specifically require that GSSG be maintained at very low levels (Kosower and Kosower, 1978).

In the liver, GSH performs a crucial role in the detoxification of reactive oxygen species as well as xenobiotics. GSH protects cells against lipid peroxidation by reducing both H_2O_2 and organic hydroperoxides in conjunction with GSH peroxidase. In the process, GSH is oxidized to GSSG.

GSH is also involved in the conjugation of metabolic intermediates. Many xenobiotics are transformed by the cytochrome P-450 monooxygenase system to reactive electrophilic metabolites. Conjugation with the nucleophilic GSH detoxifies these reactive intermediates before alkylation of tissue macromolecules occurs.

GSH levels in rat liver have been estimated to range from 4.5 mM (Kosower and Kosower, 1978) to 10 mM (Sies *et al.*, 1983). Despite the high basal level of GSH, both conjugation and oxidation to GSSG may result in profound decreases in GSH levels. The depletion of GSH has been associated with the hepatotoxicity induced by many xenobiotics. This review will discuss the mechanisms of hepatic GSH depletion as well as the early consequences of such depletion.

Mechanisms of GSH depletion

Hepatic GSH may be depleted by three separate mechanisms: decrease in synthesis, conjugation with electrophilic compounds, and oxidation to GSSG.

GSH synthesis and breakdown

The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine and L-glycine involves two enzymatic steps, both requiring ATP. In the first step, catalyzed by the enzyme τ-glutamylcysteine synthetase, the linkage between L-glutamate and L-cysteine is formed. The second step involves the formation of a peptide bond between τ-glutamylcysteine and L-glycine which is catalyzed by GSH synthetase. The rate limiting step is the first and is governed both by feedback inhibition (Richman and Meister, 1975) and by the availability of L-cysteine (Thor *et al.*, 1978a; Reed and Beatty, 1980.)

Under conditions of stimulated GSH consumption, the resynthesis of GSH is largely governed by the amount of L-cysteine available. In freshly isolated hepatocytes, normal GSH synthesis can be maintained by including either L-cysteine or L-methionine in the incubation medium. Thor *et al.* (1978) reported that in incubations with bromobenzene, hepatic GSH levels did not decrease below 30% of basal levels when incubated in a medium containing these amino acids. In these incubations, no cell toxicity was observed in contrast to that in incubations in which L-cysteine and L-methionine were not included. Similarly, in hepatocytes isolated from rats pretreated with the GSH depleting agent diethylmaleate, GSH was resynthesized to normal levels

when either L-cysteine or L-methionine was included in the incubation medium (Thor *et al.*, 1979).

Under normal conditions, GSH efflux is the major factor in intracellular turnover, and the rate of GSH efflux into plasma was found to approximate the turnover rate of hepatic GSH (15-20% h^{-1}) (Lauterberg *et al.*, 1984; Kaplowitz, 1985). GSH breakdown is initiated by the enzyme γ-glutamyltransferase which is located mainly in the kidney and intestinal mucosa. The active site of the enzyme faces the extracellular space (Horiushi *et al.*, 1978) rendering only extracellular GSH accessible to the enzyme.

Conjugation with GSH

Many xenobiotics are metabolized to highly reactive electrophilic intermediates. These electrophiles will bind irreversibly to cellular nucleophiles including protein, RNA and DNA. GSH acts to intercept these reactive species by nucleophilic attack before covalently binding to tissue nucleophiles can occur.

GSH conjugation is catalyzed by GSH transferases, a group of multiple (at least seven) inducible cytosolic enzymes which display overlapping substrate specificity (Jakoby, 1978; Ketterer, 1986). Many electrophiles also bind non-enzymatically to GSH. The distinction between those electrophiles that rapidly bind to GSH in the absence of GSH transferase and those that require the enzyme for conjugation has been suggested by Coles (1985) to rest with the hardness or softness of the electrophile. GSH has been termed a soft nucleophile because of the large atomic volume of sulfur and its consequent diffuse electron density. According to this theory, GSH reacts most

rapidly with electrophiles of similar softness. N-acetyl-p-benzoquinone imine (NAPQI), the acetaminophen reactive intermediate, is one such electrophile; NAPQI is also a substrate for GSH transferase. This combination has the effect, that with low doses of the substrate most if not all NAPQI will be detoxified as a GSH conjugate (Ketterer, 1986).

In the case of hard electrophiles, enzymatic conjugation is essential if detoxification is to occur. Hard electrophiles, such as benzo(a)pyrene-7,8-diol-9,10-oxide, preferentially bind to hard nucleophilic positions of nucleic acids (Cooper, 1980; Ketterer, 1986). This metabolite of benzo(a)pyrene reacts poorly with GSH non-catalytically but as Jernstrom *et al.* (1985) and Meyer *et al.* (1985) have shown, it is conjugated by all GSH transferases. Ketterer (1986) suggested that the abundance of hepatic GSH transferases renders the rate of enzymatic detoxication sufficient to prevent benzo(a)pyrene from being a hepatocarcinogen in the rat. On the other hand, aflatoxin B_1-8,9-oxide, another hard electrophile, has been shown to be a substrate for only specific GSH transferases. The inability of aflatoxin B_1 oxide to react with GSH transferases has been suggested to allow DNA to effectively compete with GSH transferases for binding with this electrophile (Coles, 1985; Ketterer, 1986).

GSH conjugates are excreted in the bile or are subsequently transformed in the kidney and excreted in the urine as mercapturic acids. Bourne and Young (1934) were the first to isolate a mercapturic acid, l-α-napthylmercapturic acid, from rabbit urine. At the time, it was believed that mercapturic acids were formed via direct conjugation with cysteine. It is now known that transformation of GSH conjugates to the mercapturic acid requires the activity of three enzymes, all predominantly

located in the proximal straight tubules of the kidney: γ-glutamyltransferase, a dipeptidase and N-acetyltransferase. The initial step is catalyzed by γ-glutamyltransferase. In the kidney, the active site of this enzyme was shown to face the luminal side of the proximal straight tubule (Hughey *et al.*, 1978; Horiuchi *et al.*, 1978). The dipeptidase which catalyzes the removal of glycine is also believed to be oriented toward the lumen of the tubule (Hughey *et al.*, 1978), while the N-acetylation of the resulting cysteine conjugate is believed to be associated with the renal tubular endoplasmic reticulum (Hughey *et al.*, 1978). The resulting N-acetylcysteine (mercapturic acid) conjugate is then excreted in the urine.

Much of the early knowledge of GSH conjugation and consequent GSH depletion was obtained from early studies of acetaminophen-induced hepatic necrosis in mice. Mitchell *et al.* (1973) demonstrated an inverse relationship between hepatic GSH levels and irreversible binding of [^{3}H]acetaminophen to tissue proteins. Pretreatment with cysteine, leading to increased GSH synthesis, decreased both the covalent binding of the acetaminophen metabolite and the severity of liver necrosis, suggesting that binding of the reactive acetaminophen metabolite to GSH instead of tissue protein, prevented hepatic necrosis. Later studies with freshly isolated hepatocytes confirmed a direct correlation between the loss of GSH and formation of the acetaminophen-GSH conjugate (Moldeus, 1978).

In vivo studies (Jollow *et al.*, 1974) of bromobenzene-induced liver necrosis revealed parallel changes in hepatic GSH and the excretion of the mercapturic acid conjugate. Immediately after bromobenzene injection, when the liver was replete with GSH, the mercapturic acid conjugate constituted nearly 75% of urinary metabolites

and very little covalent binding was detected. At later time points, both GSH and the mercapturic acid declined as covalent binding increased. In vitro studies demonstrated that the formation of bromophenyl-GSH conjugate was dependent on the hepatic content of GSH. At low concentrations of GSH, very little GSH conjugate was formed; protein bound bromobenzene was at its highest level. As the GSH concentration increased, the formation of the bromophenyl GSH conjugate increased while that of the protein bound bromobenzene decreased.

Jernstrom *et al.* (1982) identified GSH conjugates in isolated rat hepatocytes incubated with benzo(a)pyrene. When hepatocytes were pretreated with diethylmaleate, the intracellular GSH level was lowered to about 35% of the normal level. This decrease in GSH resulted in a 2-fold increase in benzo(a)pyrene induced DNA binding. Concurrently, there was an approximate 2-fold decrease in the amounts of GSH conjugates formed thereby demonstrating the relationship between DNA binding and GSH conjugation.

Many other xenobiotics are transformed to reactive metabolites which are then conjugated with GSH and in high doses result in GSH depletion. The studies of acetaminophen, bromobenzene and benzo(a)pyrene hepatotoxicity described above are three notable examples of the inverse relationship between covalent binding of reactive intermediates and hepatic GSH levels.

Oxidation to GSSG

GSH is also involved in the reduction of H_2O_2. The reaction, catalyzed by the enzyme GSH peroxidase, involves the oxidation of GSH to GSSG. The GSSG so formed is reduced back to GSH by the enzyme GSH reductase. In the process, NADPH is oxidized.

Under conditions of oxidative stress, large amounts of H_2O_2 are produced, and the rate of GSH oxidation may exceed the rate or capacity for reduction of GSSG and/or GSH synthesis de novo. This inability of GSH reductase to reduce GSSG may result from inhibition of the enzyme by oxidation (or alkylation) of critical sulfhydryl groups (Bellomo *et al.*, 1987) or inadequate amounts of NADPH (Eklow *et al.*, 1984), a cofactor in the reaction. Under either of these conditions, GSSG has been found to accumulate.

Accumulation of hepatic GSSG appears to trigger its efflux which may contribute to GSH depletion by removal of intracellular GSSG. Release of GSSG into the bile has been well documented (Sies *et al.*, 1978; Akerboom *et al.*, 1982). More recently, a GSSG-stimulated ATPase activity was found in the plasma membrane fraction of rat liver (Nicotera *et al.*, 1985). Nicotera *et al.* (1985*b*) showed that this activity is stimulated by several GSH- conjugates as well. The SG-ATPase is activated rather than inhibited by thiol-depleting agents, and it has been suggested that the ATPase may function to protect the cell by active extrusion of both GSSG and GSH-conjugates and thereby maintain a normal cellular GSH/GSSG redox level even under conditions of GSH depletion (Nicotera and Orrenius, 1986).

Early Effects of GSH Depletion

Covalent binding to cellular nucleophiles

Early studies of acetaminophen hepatotoxicity in mice documented that at low doses of the drug, trapping of reactive intermediates by GSH protected the liver from damage. At higher doses when GSH was depleted, covalent binding to cellular macromolecules occurred (Mitchell *et al.*, 1973). Mitchell *et al.* (1973) reported that significant covalent binding did not occur in vivo until initial GSH levels were depleted by at least 70%. Pretreatment of mice with diethylmaleate caused a marked increase in covalent binding and dramatically potentiated hepatic necrosis. Jollow *et al.* (1973) reported a correlation between the extent of covalent binding and the degree of liver necrosis, and others have suggested covalent binding is the cause of hepatotoxicity. However, the critical question that remains is whether alkylation per se is the ultimate cause of hepatotoxicity or is hepatic damage a result of the loss of GSH protection to other cell nucleophiles and/or critical proteins.

Studies by Streeter *et al.* (1984) indicated that protein thiol groups are major sites of covalent binding of NAPQI in vitro. And Tsokos-Kuhn *et al.* (1985) suggested that alkylation caused decreased Ca^{2+} uptake in plasma membrane vesicles in rats pretreated with CCl_4, bromobenzene or acetaminophen.

Albano *et al.* (1985) reported enhanced covalent binding of NAPQI in isolated hepatocytes preincubated with diethylmaleate. However, cytotoxicity, measured by LDH release, correlated better with decreases in total protein thiol groups than with increases in covalent binding. It was suggested that the loss of protein sulfhydryl groups was due in large part to oxidation of essential thiol groups since the addition of dithiothreitol after NAPQI incubation resulted in a dramatic decrease in cell death.

Lipid Peroxidation

GSH is thought to play a major role in preventing lipid peroxidation by reducing H_2O_2 in conjunction with Se-dependent GSH peroxidase and by reducing organic hydroperoxides in conjunction with GSH transferase/GSH peroxidases.

Studies by Anundi *et al.* (1979) demonstrated that GSH depletion per se led to lipid peroxidation which was followed by cell lysis in isolated rat hepatocytes. The addition of the GSH precursor methionine increased GSH levels and inhibited lipid peroxidation and the consequent cell lysis. Younes and Siegers (1980) demonstrated that GSH depletion in vivo led to increased spontaneous lipid peroxidation in liver homogenates of rats and mice. An inverse relationship between hepatic GSH concentration and lipid peroxidation was subsequently reported and it was shown that lipid peroxidation did not occur until GSH levels were depleted to about 20% of the initial GSH concentration (Younes and Siegers, 1981). Further, in vitro addition of GSH following GSH depletion in vivo was shown to abolish spontaneous lipid peroxidation in a concentration-dependent manner (Younes and Siegers, 1983). Similarly, Tan *et al.* (1984) showed that lipid peroxidation initiated in vitro in rat liver microsomes was inhibited by the rat liver soluble fraction. Upon further analysis, the inhibitory activity was found to be due to Se-dependent GSH-peroxidase and GSH transferases with GSH peroxidase activity.

Formation of mixed disulfides

Concomitant with GSSG formation, protein-GSH mixed disulfides are formed. The reaction which may occur spontaneously or via hepatic thioltransferase (Axelsson *et al.*, 1978) takes place as follows:

$$\text{protein-SH} + \text{GSSG} \rightleftharpoons \text{protein-SSG} + \text{GSH}$$

By removing GSSG and producing GSH, the formation of mixed disulfides could function as another way of altering the GSSG/GSH balance.

Brigelius *et al.* (1983) reported that the amount of protein-mixed disulfides formed is linearly related to the amount of intracellular GSSG. The linearity of this relationship was corroborated by Bellomo *et al.* (1987) who suggested that a critical GSH/GSSG ratio must be reached in order for protein-GSH mixed disulfides to be formed.

Loss of protein thiol protection

Many enzymes depend on free sulfhydryl groups for their structure and activity. One effect of GSH depletion may be the loss of free thiol groups of proteins. Phosphofructokinase, glycogen synthetase, glycogen phosphorylase phosphatase, hexokinase, and pyruvate kinase have all been found to be inactivated by small disulfides including GSSG (Ziegler, 1985). Activity of these enzymes is restored upon incubation with thiols.

Studies of menadione metabolism in isolated rat hepatocytes (Di Monte *et al.*, 1984a) demonstrated that the rapid GSH depletion triggered by menadione metabolism was nearly complete before protein sulfhydryls were decreased. Cell toxicity, measured

as trypan blue uptake, correlated better with a loss of protein thiols than with the early depletion of GSH, suggesting that sulfhydryl groups in proteins critical for cell survival were normally protected by the presence of GSH. The marked increase in protein thiol loss in hepatocytes of rats pretreated with diethylmaleate further supported this proposal (Di Monte *et al.*, 1984*b*).

Loss of protein sulfhydryl groups can take place either by alkylation or by formation of protein mixed disulfides. Streeter *et al.* (1984), as mentioned earlier, demonstrated that protein thiol groups were major sites of covalent binding of NAPQI in vitro. In isolated hepatocytes, Moore *et al.* (1985) reported a decrease in protein thiols in incubations with NAPQI. The loss of protein sulfhydryl groups was considered consistent with either covalent binding of NAPQI to protein sulfhydryls or with the formation of protein mixed disulfides.

GSH depletion, thus, due to either alkylation or mixed disulfide formation, appears to result in a loss of protein thiol groups. Protein thiol depletion as well as GSH depletion by itself have been linked to the disruption of Ca^{2+} homeostasis in the hepatocyte.

Loss of Protein Thiols and the Disruption of Ca^{2+} Homeostasis

Free Ca^{2+} in cytosol of most cells is maintained between 0.05 and 0.2 μM (Murphy *et al.*, 1980). In hepatocytes, 60% and 80% of the total cell Ca^{2+} is located in the mitochondria (Murphy *et al.*, 1980; Jewell *et al.*, 1982; Joseph *et al.*, 1983) with the remainder primarily located in the endoplasmic reticulum (Murphy *et al.*, 1980; Joseph *et al.*, 1983). A Ca^{2+}-ATPase in the plasma membrane functions to reverse

the slight membrane permeability to extracellular Ca^{2+} and to maintain the low intracellular cytosolic Ca^{2+} concentrations (Joseph *et al.*, 1983).

The breakdown of these mechanisms is thought to precipitate the cell damage associated with a variety of hepatotoxins. In mitochondria, release of sequestered Ca^{2+} is believed to be linked to an accumulation of $NADP^+$, a result of enhanced GSSG reduction (Lehninger *et al.*, 1978; Bellomo *et al.*, 1982*a*). However, Beatrice *et al.* (1984) proposed that the inability to retain Ca^{2+} within mitochondria was directly related to GSH depletion.

Inactivation of the microsomal $Ca^{2}+$ pump has also been shown to follow GSH depletion. In studies with t-butylhydroperoxide (Jones *et al.*, 1983), H_2O_2 and menadione (Thor *et al.*, 1985) in isolated hepatocytes, addition of either GSH or DTT to the incubation medium prevented the impairment of Ca^{2+} sequestration and suggested that the inhibition resulted from modification of critical protein thiols. Inhibition of the microsomal Ca^{2+} pump has also been reported in studies of CCl_4, $CHCl_3$, 1,1-dichloroethylene, and carbon disulfide hepatotoxicity (Moore, 1980, 1982*a*,1982*b*), thereby suggesting that such inhibition can occur either through alkylation or the formation of mixed disulfides.

Impairment of the plasma membrane Ca^{2+}-ATPase was also shown to follow GSH depletion (Di Monte *et al.*, 1984*b*; Moore *et al.*, 1985) and be a result of the loss of critical protein thiols (Nicotera *et al.*, 1985*a*). Inhibition of such activity by formation of protein mixed disulfides was demonstrated by Nicotera *et al.* (1986*a*) who showed that cystamine, a disulfide which does not affect GSH levels, inhibited plasma membrane Ca^{2+}-ATPase activity in plasma membrane fragments by formation

of protein-cysteamine mixed disulfides. Tsokos-Kuhn *et al.* (1985) demonstrated that alkylating agents also caused inhibition of the Ca^{2+}-ATPase activity.

Recent evidence suggests that the rise in cytosolic Ca^{2+} concentration which appears to precipitate cytotoxicity may also result in enhanced rates of proteolysis. Such proteolytic stimulation may induce lysis of cell structures and irreversible cell damage (Nicotera *et al.*, 1986*b*).

4.0 RESEARCH OBJECTIVES

The depletion of GSH may be a requisite step in a cascade of events leading to cell toxicity. Reports of morphine-induced GSH depletion and the identification of a GSH conjugate clearly suggest the potential for cell damage. Earlier studies by Correia *et al.*,(1984*a*) showed that morphine is transformed by cytochrome P-450 mediated metabolism to a highly reactive electrophilic intermediate which was quenched by GSH. Such quenching was subsequently found to be due to formation of an isolable morphine-GSH adduct. The main objectives of this research were to structurally characterize such a morphine-GSH conjugate, to examine the formation of this metabolite in vitro by human and rat liver microsomes and in vivo in rat bile, and to explore the importance of this metabolite in overall morphine metabolism and in morphine-induced GSH depletion in isolated rat hepatocytes.

5.0 ISOLATION AND IDENTIFICATION OF A MORPHINE-GSH ADDUCT

Introduction

The morphine-GSH adduct, formed via cytochrome P-450 mediated metabolism, was isolated from aerobic incubations of rat liver microsomes with morphine, NADPH and GSH. The structure of morphine suggested many sites where metabolism to an electrophilic center might occur. Reactive intermediates thus formed would be capable of attacking cellular nucleophiles (i.e. GSH or protein).

Initially, morphine 2,3-catechol (Figure 5.1) was regarded as one possible reactive intermediate. This possibility was supported by the fact that catechols are

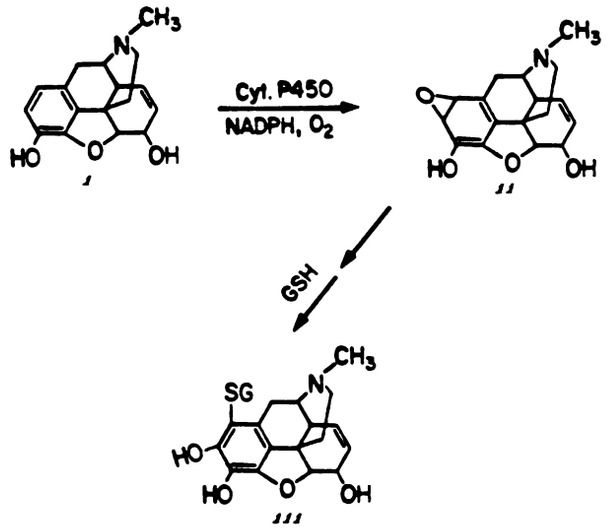


Figure 5.1 Pathway for metabolism of morphine to a morphine-catechol GSH conjugate

known to undergo oxidation to semiquinone free radicals or to quinones both of which have been demonstrated to bind to tissue macromolecules (Dybing *et al.*, 1976). Further, catechol estrogen-GSH conjugates had been identified (Numazawa and

Nambara, 1977), and it had been suggested that conjugation with GSH occurred after oxidation of the catechol to a semiquinone radical. A morphine-catechol GSH adduct (Figure 5.1, *[iii]*) would most likely arise via metabolism of morphine *[i]* to a 1,2-arene oxide (Figure 5.1, *[ii]*). Reports in the literature of a morphine metabolite with a catechol structure (Daly *et al.*, 1965; Misra *et al.*, 1973) also strengthened the possibility of such a GSH adduct, as did studies of hepatotoxicity of morphine congeners which showed that the absence of a free 3-OH group in ethylmorphine and codeine greatly attenuated GSH depletion (Correia *et al.*, 1984a).

A morphine iminium species (Figure 5.2) was also considered as another likely intermediate. Metabolism to electrophilic iminium species has been demonstrated for a number of tertiary amines (Nguyen *et al.*, 1976; Ziegler *et al.*, 1981; Ward *et al.*, 1982). These reactive species are formed via initial hydroxylation to the carbinolamine.

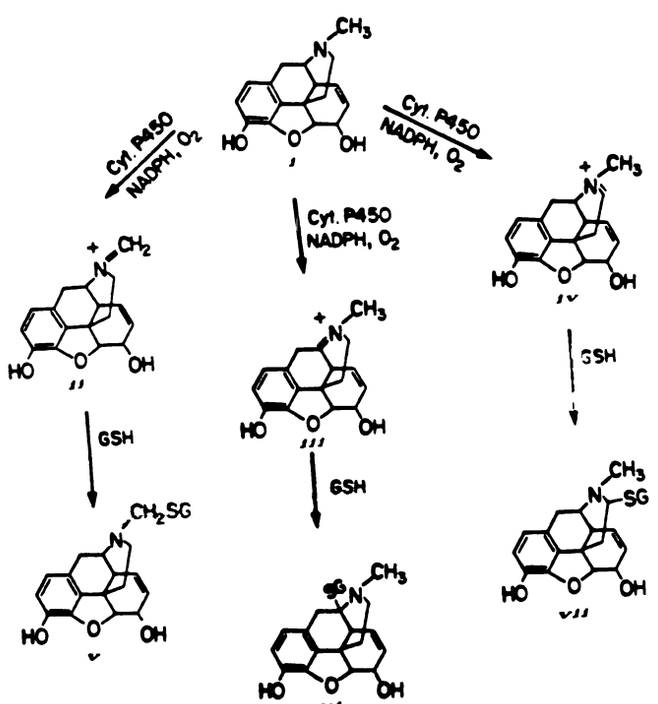


Figure 5.2 Pathway for GSH conjugate formation resulting from the metabolism of morphine to a reactive iminium species

In morphine, hydroxylation at C_9, C_{16} or the N-methyl carbon would lead to an iminium species (Figure 5.2, *[ii]*, *[iii]* or *[iv]*) which then might bind to nucleophilic GSH (Figure 5.2, *[v]*, *[vi]* or *[vii]*) and/or protein residues. A N-methylene GSH conjugate of N,N-dimethyl-4-aminoazobenzene has indeed been identified by Ketterer *et al.* (1982) who hypothesized that this metabolite was derived from a methylene iminium intermediate.

A third possibility that was also considered was metabolism at the 7,8-unsaturated bond (Figure 5.3). As such, Nagamatsu *et al.* (1983) suggested that morphine was converted to a morphinone-GSH adduct (Figure 5.3, *[iii]* via the Michael addition of GSH to the C_8 position of metabolically generated morphinone (Figure 5.3, *[ii]*). Metabolism to morphine 7,8-epoxide was also hypothesized (Figure 5.3, *[iv]*). The 7,8-epoxide of codeine has been detected in rat urine after codeine

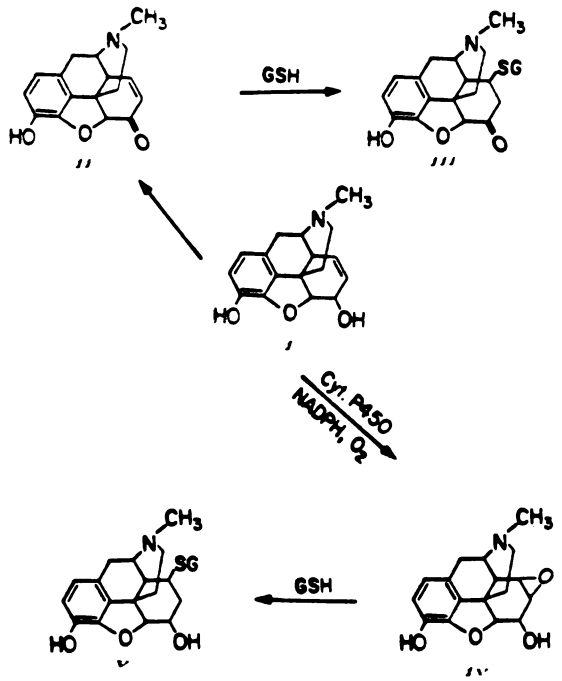


Figure 5.3 Possible pathway for metabolism of morphine to a C_8 morphine-GSH adduct

administration and also in vitro, after incubations of codeine with rat liver microsomes (Miyata *et al.*, 1981). It was thus conceivable that a morphine 7,8-epoxide could be formed as well. Earlier work by Correia *et al.* (1984*a*), however, demonstrated that morphine congeners levorphanol and hydromorphine, lacking the 7,8 unsaturated bond, were not only as hepatotoxic as morphine but that [^{3}H]dihydromorphine was equivalent to morphine in binding irreversibly to microsomal protein (in the absence of GSH). These results indicated that the 7,8-unsaturated bond was not necessary for morphine-induced liver damage and questioned the notion that a 7,8-epoxide intermediate or even morphinone could be responsible for morphine-induced hepatotoxicity.

Finally, hydroxylation at the benzylic carbon, C_{10}, (Figure 5.4, *[ii]*) was considered as a fourth possibility. Subsequent loss of this hydroxyl group could very well lead to a reactive species which could be detoxified by GSH at C_{10} (Figure 5.4, *[iii]*).

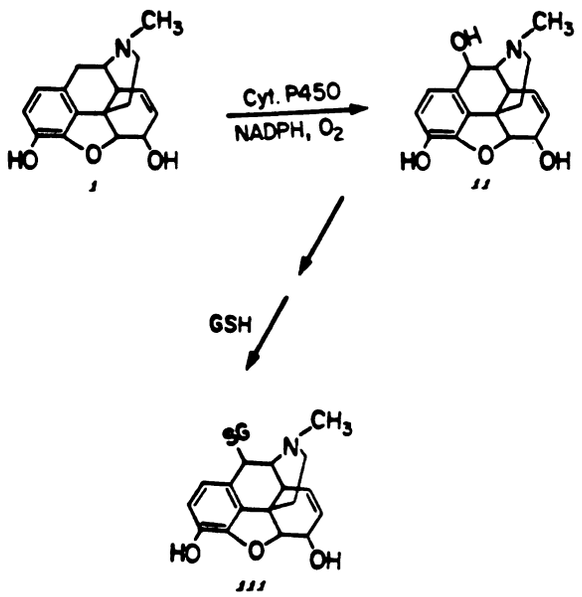


Figure 5.4 Possible pathway for formation of a morphine-GSH adduct generated by hydroxylation the C_{10} position

The procedures for the generation of the morphine-GSH adduct in rat liver microsomes as well as for isolation of the metabolite are described below. Characterization of mass spectral and NMR analyses of the isolated morphine-GSH adduct (Correia *et al.*, 1984*b*) confirmed that this metabolite contained both morphine and GSH in a 1:1 ratio. The studies reported in this chapter were undertaken to elucidate the structure of this morphine-GSH adduct.

Materials and Methods

Generation of morphine adducts in rat liver microsomal preparations

Because phenobarbital (PB) pretreatment of rats was found to stimulate formation of the reactive metabolite (Correia *et al.*, 1984*a*), male Sprague-Dawley rats were treated with PB (80 mg/kg, i.p.) for 3 days. After sacrifice, livers were removed, weighed and perfused with ice-cold 1.15% KCl. The liver was cut into small pieces and a 50% w/v homogenate was prepared in phosphate buffer (0.1 M, pH 7.4) using a large glass homogenizer fitted with a loose plunger. The homogenate was then centrifuged at 9000 x g at 4° C for 20 min in order to sediment nuclei, mitochondria, and unbroken or fragmented cells. The 9000 x g supernatant was recentrifuged at 100,000 x g for 1 h at 4° C in order to pellet the microsomes. After the 100,000 x g supernatant was decanted, the microsomes were resuspended in 1.15% KCl and recentrifuged at 100,000 x g at 4° C for 30 min. The pellet was resuspended in phosphate buffer (0.1 M, pH 7.4), and the protein content was determined by the method of Lowry (1951) using bovine serum albumin as a standard.

Microsomes (2 mg protein/ml) were incubated in a final volume of 10 ml of phosphate buffer (0.1 M, pH 7.4) with [C_1 - ^{3}H]morphine (0.5μCi, 1.0 mM), NADPH (1 mM), l-isocitrate (2.5 mM), l-isocitrate dehydrogenase (50 units) and GSH (2.5 mM), cysteine (2.5 mM) or N-acetylcysteine (7.5 mM) in a shaking water bath at 37° C for 15 min. All systems included an incubation mixture which lacked NADPH. Reaction mixtures containing morphine-GSH and morphine-cysteine adducts, reactions were terminated by the addition of ice-cold methanol. The precipitated protein was then

removed by centrifugation and the methanol by rotoevaporation. Since the metabolites were not to be analyzed quantitatively, the procedure for isolating the morphine N-acetylcysteine (morphine-N-AcCys) adduct was modified. In systems containing morphine-N-AcCys adduct, incubations were terminated by chilling the reaction vials in ice. Morphine and its metabolites were separated from microsomes by ultracentrifugation at 100,000 x g at 4° C for 1 h. In all preparations, the supernatant (or remaining aqueous phase) was passed through a Waters Sep-Pak C_{18} cartridge, washed with water (2 ml) and eluted with methanol (4 ml). The methanol eluate was evaporated to dryness on a rotoevaporator.

Isolation and purification of metabolically generated morphine adducts

Residues were chromatographed on an Altex reverse phase C_{18} Ultrasphere ODS (5μ, 10 mm i.d. x 250 mm) column. The procedure for isolation of the morphine cysteine-adduct was identical to that of the morphine-GSH adduct reported earlier (Correia *et al.*, 1984b). The adducts were initially chromatographed with a mobile phase of methanol/water (20:80, v/v), tetramethylammonium hydroxide (TMA, 25 mM) with the pH adjusted to 6.1 with aqueous phosphoric acid. At a flow rate of 2 ml/min, morphine and normorphine eluted at 16 and 11 min, respectively. Both GSH and cysteine eluted at 4-5 min. HPLC analysis of incubation mixtures lacking NADPH revealed peaks corresponding only to morphine and the nucleophile. In the presence of NADPH, peaks corresponding to morphine, normorphine and the morphine adduct were observed. The adduct of GSH eluted at 7-9 min whereas that of cysteine eluted at 9-10 min.

The morphine-N-AcCys adduct was initially chromatographed in acetonitrile/water (10:90, v/v) containing 0.5% ammonium acetate (pH 5.5). HPLC chromatograms of preparations containing N-acetylcysteine are shown in Figure 5.5A and 5.5B. At a flow rate of 2 ml/min, N-acetylcysteine eluted with the solvent front at 4-5 min. Authentic normorphine eluted at 12 min and morphine at 14 min. In microsomal preparations where [^{3}H]morphine was incubated in the absence of NADPH, chromatograms showed only UV absorbing peaks corresponding to morphine and N-acetylcysteine (Figure 5.5A). The radioactivity coincided only with the morphine peak. In preparations incubated in the presence of NADPH, three peaks were found to be radioactive: morphine, normorphine and the morphine-N-AcCys adduct. This adduct eluted at 9-10 min (Figure 5.5B).

After collection of the peak containing the morphine adduct, the methanol was removed by rotoevaporation and the remaining water by lyophilization. Residues containing either the morphine-GSH or morphine-cysteine adduct were redissolved in methanol/water (20:80, v/v) containing ammonium formate (10 mM, pH 6.1) and rechromatographed on the same HPLC column with the above mobile system. Collection of the radiolabeled adduct separated it from residual normorphine. The collected material was rotoevaporated and lyophilized as before and redissolved in the same solvent system for further purification. This process was continued through three separate collections.

Figure 5.5 HPLC profiles of morphine incubated with liver microsomes from PB-pretreated rats.

(A) Incubations contained [^{3}H]morphine (1.0 mM), N-acetylcysteine (7.5 mM) and NADPH (1.0 mM).

(B) Incubations contained [^{3}H]morphine and N-acetyl-cysteine in the absence of NADPH. Shaded areas indicate radioactivity of the HPLC peaks.

Radioactivity (cpm)
2000
1500
1000
500
36,058
40,595
2,714
A
B
0
4
8
12
16
20
Time (min)
Absorbance at 282 nm

The morphine-N-AcCys adduct was rechromatographed first with a mobile phase composed of methanol/water (20:80, v/v) containing TMA (25mM, pH 6.1) at a flow rate of 2 ml/min. Under these conditions, the adduct eluted at 13 min. The collected material was rotoevaporated, lyophilized, redissolved and then chromatographed in methanol/water (20:80, v/v) containing ammonium formate (20 mM, pH 6.0).

Mass spectral analysis of morphine-N-AcCys adduct

The adduct was analyzed by fast atom bombardment mass spectrometry (FABMS) in a glycerol or thioglycerol matrix and recorded on a Finnigan-MAT triple stage quadrupole instrument. In a FABMS/MS experiment, the protonated molecular ion (m/z 447) was passed into an argon filled collision cell as previously described (Krowech *et al.*,1986)[1].

^{1}H NMR analysis

Sample extracts were prepared in D_2O (99.99%). Repeated exchanges with D_2O followed by lyophilization of the liquid assured an extremely small H_2O content in the sample as well as deuteration of all exchangeable protons. After the final lyophilization, the residues were dissolved in D_2O in a N_2 atmosphere, and the material was transferred to NMR tubes.

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[1] Spectra were recorded and analyzed by Dr. Kenneth Straub, Department of Drug Metabolism, Smith, Kline and French Laboratories

Reference samples including morphine, normorphine, GSH, cysteine and N-acetylcysteine were prepared using the same HPLC solvent systems as the morphine-adduct being analyzed and were prepared in D_2O as described above.

NMR spectra were recorded using either a Nicolet NT-360 spectrometer or a Nicolet NM500 spectrometer[2]. Spectra were recorded in D_2O and chemical shifts were measured relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DDS).

[2]The samples were analyzed by Dr. J. Dallas and Dr. G. Matson, University of California, Davis NMR facility.

Characterization of Adducts Formed during Incubations with Morphine and GSH, Cysteine and N-Acetylcysteine

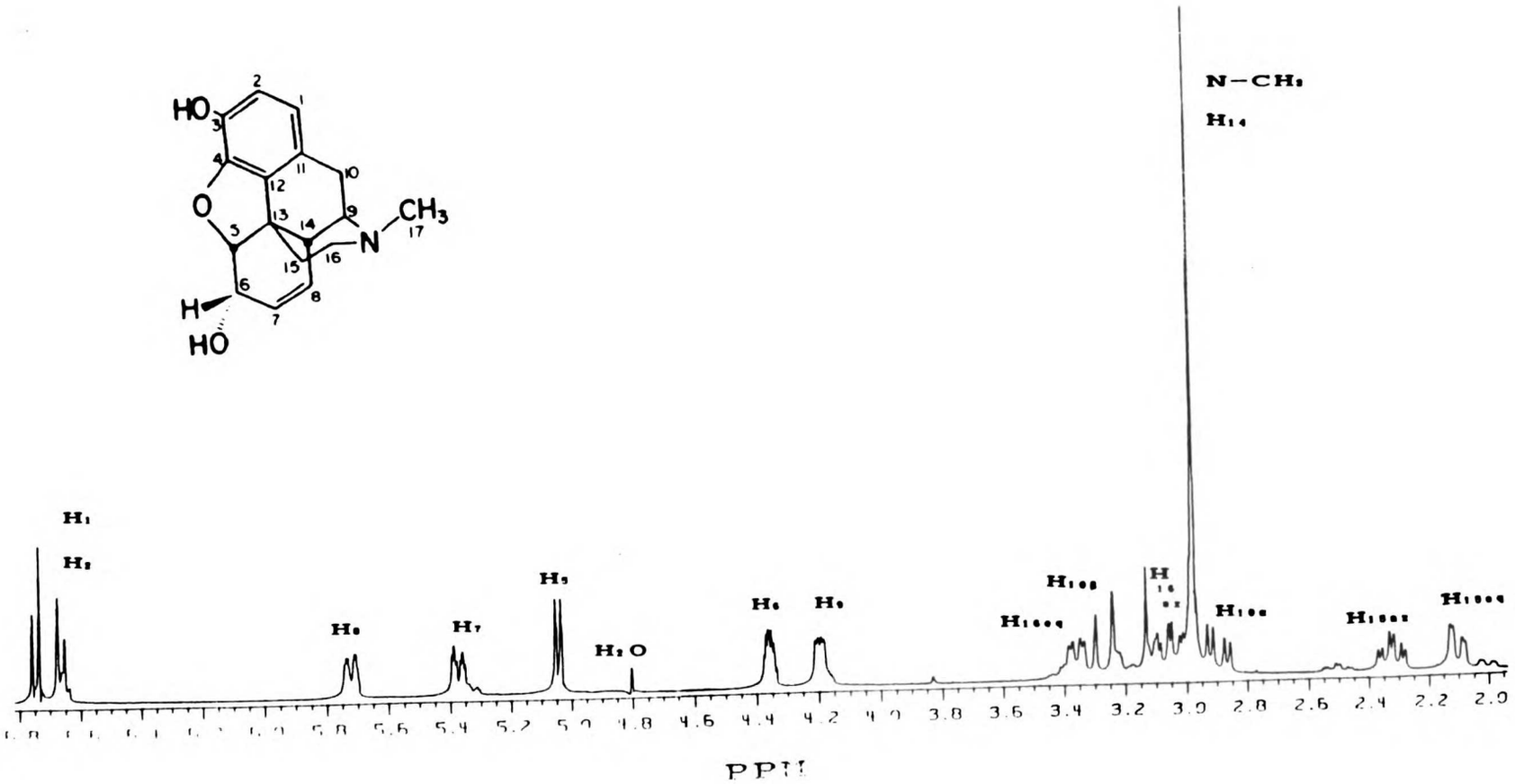
In order to determine the site of nucleophilic attack on the reactive morphine intermediate, the NMR spectra of morphine, GSH, morphine-GSH adduct as well as adducts prepared using the nucleophiles cysteine and N-acetylcysteine were analyzed. The mass spectrum of morphine-GSH has been reported earlier (Correia *et al.*, 1984*b*). The mass spectrum morphine-N-AcCys is described in the discussion below.

^{1}H NMR spectrum of Morphine

The ^{1}H NMR spectra of morphine at pH 1.5 and pH 7.4 are shown in Figures 5.6 and 5.7, respectively. While the spectrum at pH 7.4 provided information about chemical shifts and signal shapes under the conditions in which the adducts were prepared, the superior resolution in acid pH permitted a detailed analysis of signal coupling and multiplicity.

The downfield signals at 6.74 ppm and 6.66 ppm (Figure 5.7) were assigned to protons H_1 and H_2. Such assignments are consistent with the downfield chemical shifts expected for aromatic protons. In spin-spin decoupling experiments, the doublet at 6.74 ppm collapsed to a singlet upon irradiation of the signal at 6.66 ppm. Conversely, irradiation of the latter doublet resulted in a singlet at 6.74 ppm, thus indicating the coupling of these two protons to each other. These assignments concurred with those of Okuda *et al.* (1964).

Figure 5.6 360 MHz ^{1}H NMR spectrum of morphine (pH 1.5).



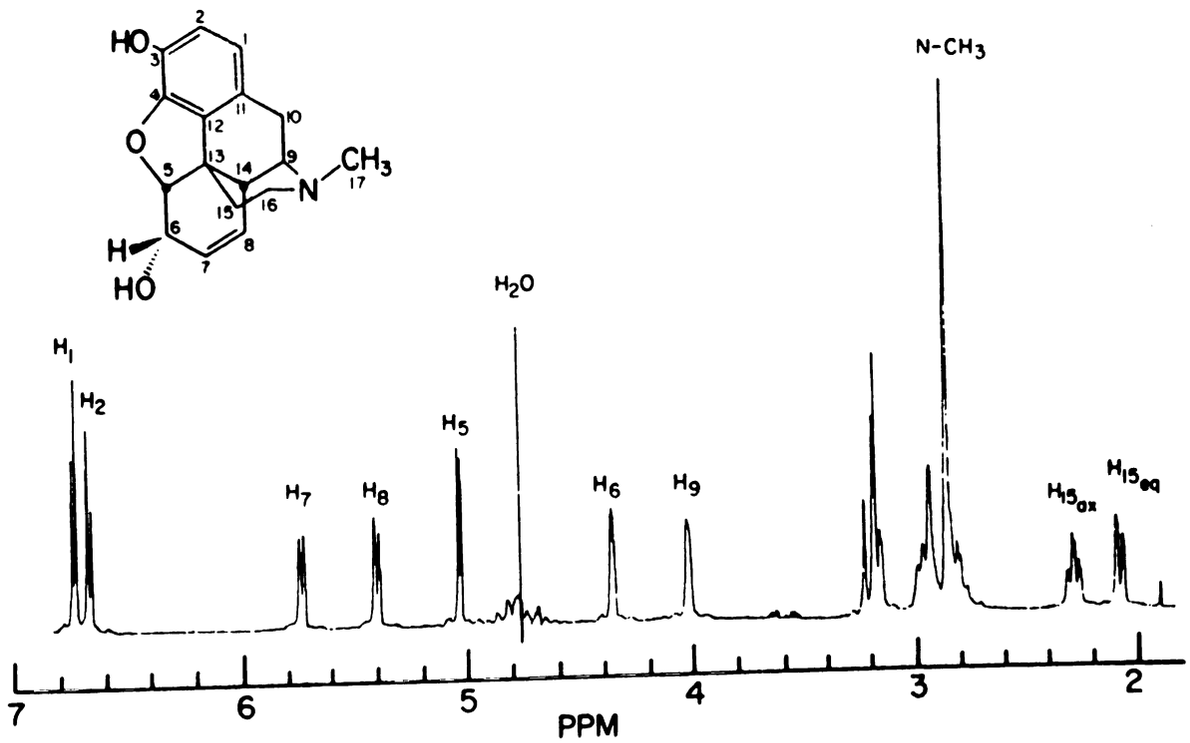
42a

The doublets centered near 5.7 ppm and 5.4 ppm were assigned to H_7 and H_8, respectively. The downfield chemical shifts are consistent with predictions for these olefinic protons. Decoupling experiments demonstrated the coupling of these two protons to each other. In addition, irradiation of the signal at 5.7 ppm resulted in the sharpening of the multiplet near 4.35 ppm. Based on this coupling, the doublet at 5.7 ppm was assigned to H_7. The signal at 4.35 ppm was identified as H_6. This signal was coupled both to H_7 and to the doublet near 5 ppm. This doublet collapsed to a singlet when the H_6 multiplet was irradiated. The doublet near 5 ppm was thus assigned to H_5. This assignment was also supported by predictions of a downfield resonance for H_5 due to deshielding by the adjacent ether oxygen. The above assignments confirmed those of Okuda *et al.* (1964).

The signals centered near 2.1 and 2.3 ppm were assigned to H_{15} protons. From chemical shift predictions, these protons were expected to be the most upfield signals in the spectrum. Examination of signal multiplicity demonstrated the coupling of these two protons. The doublet of doublets resonating near 2.1 ppm revealed a proton coupled to two other protons; the triplet of doublets resonating near 2.3 ppm indicated a proton coupled to three other protons. In decoupling experiments, irradiation of the 2.1 ppm signal collapsed the signal at 2.3 ppm to a doublet of doublets.

Analysis of the ^1H NMR spectrum recorded at pH 1.5 (Figure 5.6) permitted further characterization of the H_{15} protons. Irradiation of the signal near 2.1 ppm also sharpened the triplet of doublets centered at 3.1 ppm. Irradiation at 2.3 ppm collapsed the 2.1 ppm doublet, transformed the signal at 3.1 ppm into a multiplet, and

Figure 5.7 360 MHz ^{1}H NMR spectrum of morphine (pH 7.4).


HO
O
N
CH3
H
HO
H1
H2
H7
H8
H5
H2O
H6
H9
N-CH3
H15ax
H15eq
7
6
5
4
3
2
PPM

converted the doublet of doublets near 3.4 ppm to a simple doublet. Irradiation of the signals at 3.1 and 3.4 ppm reflected the same couplings. Based on these experiments and in agreement with Glasel (1981), signals at 2.1 and 2.3 ppm were assigned to H_{15eq} and H_{15ax}, respectively. Signals at 3.1 and 3.4 ppm were assigned to H_{16ax} and H_{16eq}. These experiments demonstrated then, that H_{15ax} was coupled to both H_{16} protons while the H_{15eq} proton was coupled to only one.

The multiplet at 4.2 ppm was assigned to H_9. Upon irradiation of this signal, the doublet of doublets at 2.9 ppm (Figure 5.6) collapsed to a simple doublet. This doublet of doublets in turn was coupled to the simple doublet centered at 3.3 ppm. These latter signals were assigned to the H_{10} protons. Although H_9 should have theoretically been coupled to both H_{10} protons, the projections of the dihedral angle between $H_{10\beta}$ and H_9 indicated a small coupling constant for these two protons which was apparently too small to be detected at the field strength used. Hence, the simple doublet (at 3.3 ppm), uncoupled to H_9, was assigned to $H_{10\beta}$. The doublet of doublets at 2.9 ppm was assigned to $H_{10\alpha}$.

The intense singlet near 2.8 ppm was assigned to the N-CH_3 protons. The chemical shift was consistent with the combined effect of methyl shielding and deshielding by the nitrogen atom. This assignment was confirmed in the literature (Okuda *et al.*, 1964; Glasel, 1981).

The integrated intensity of the N-CH_3 signal indicated four underlying protons. The signal was thus also assigned to H_{14}, the only unidentified morphine proton, an assignment also previously suggested by Glasel (1981).

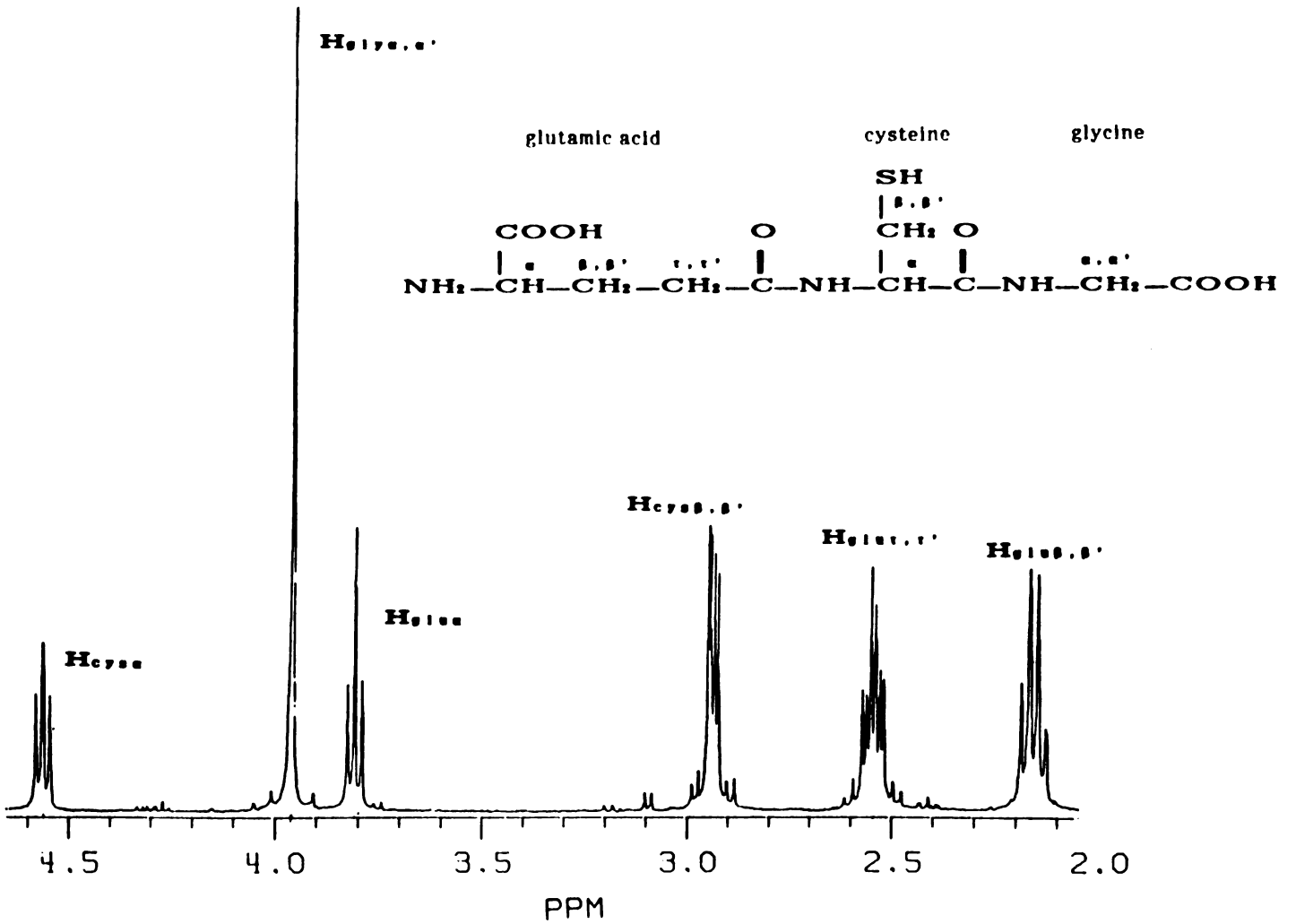
^{1}H NMR spectrum of glutathione

The ^{1}H NMR spectrum of GSH is shown in Figure 5.8. Proton assignments based on signal multiplicity and spin-spin decoupling experiments. Decoupling experiments indicated that the triplet at 4.56 ppm and the doublet of doublets at 2.94 ppm were coupled to each other but to no other protons. The integrated intensity of the signals indicated two protons underlying the doublet of doublets at 2.94 ppm and one proton underlying the triplet at 4.56 ppm. The triplet was thus assigned to $H_{cys\alpha}$ and the doublet of doublets to $H_{cys\beta,\beta'}$. The coupling of the quartet at 2.15 ppm with both the triplet at 3.8 ppm and the triplet of doublets at 2.54 ppm indicated that these were H_{glu} protons. The triplet at 3.81 ppm, which was coupled only to the signal at 2.15 ppm and whose integrated intensity revealed one underyling proton, was assigned to $H_{glu\alpha}$. Irradiation of the signal (triplet of doublets) at 2.54 ppm revealed coupling only to the quartet at 2.15 ppm. The signal at 2.54 ppm was assigned to $H_{glu\gamma,\gamma'}$ and the quartet to $H_{glu\beta,\beta'}$. The integrated intensity of the singlet at 3.96 ppm indicated two underyling protons; this signal was assigned to $H_{gly\alpha,\alpha'}$. These assignments confirmed those of Pacheka *et al.* (1979).

^{1}H NMR spectrum of morphine-GSH adduct

The spectrum of the morphine-GSH adduct is shown in Figure 5.9. The downfield signals were all identified by chemical shifts, spin-spin decoupling experiments and comparison with the ^{1}H NMR spectrum of morphine. Thus, H_1, H_2, H_5, H_6, H_7, and H_8 were all unambiguously identified.

Figure 5.8 500 MHz ^{1}H NMR spectrum of GSH.


glutamic acid
cysteine
glycine
COOH
SH
O
O
NH2—CH—CH2—CH2—C—NH—CH—C—NH—CH2—COOH
4.5
4.0
3.5
3.0
2.5
2.0
PPM

47a

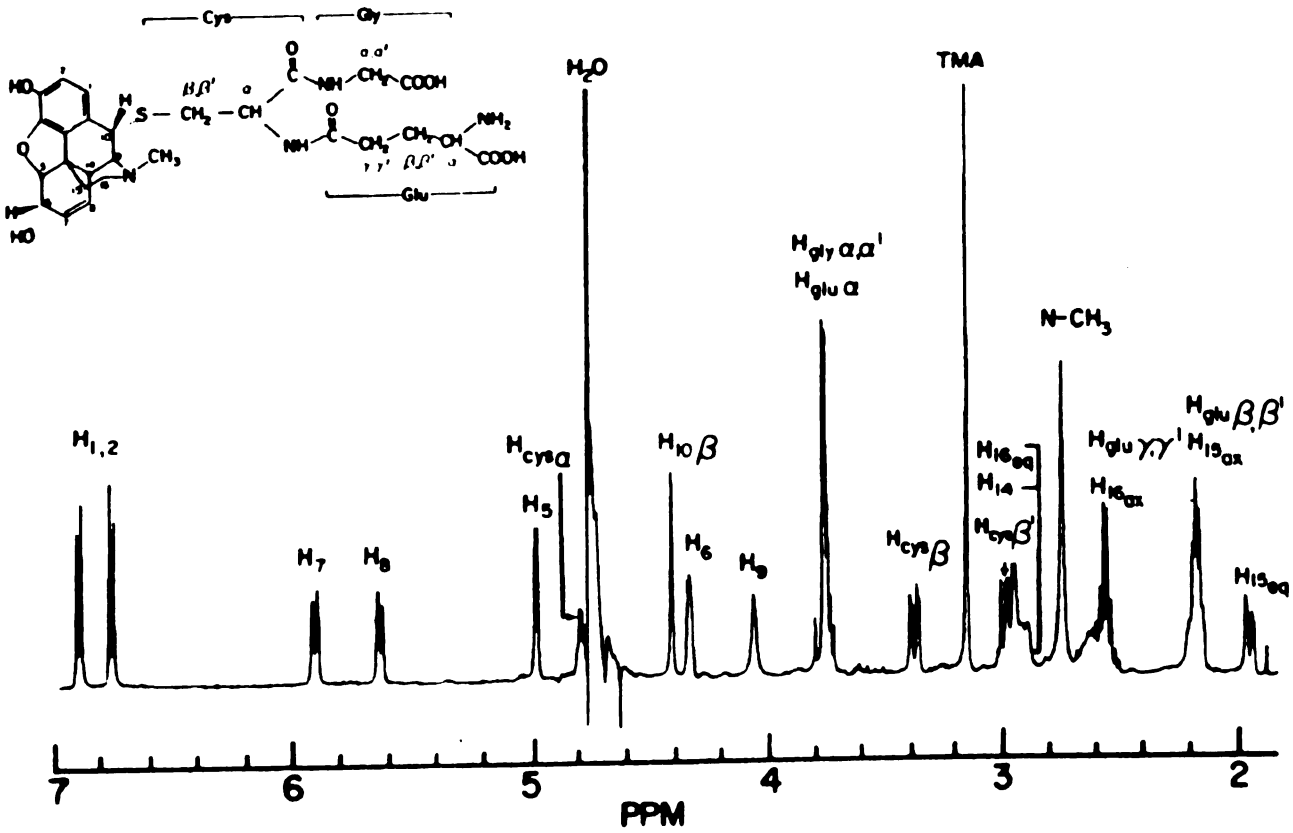
The presence of the aromatic ring protons, H_1 and H_2, in this spectrum eliminated the possibility of GSH attack at the aromatic ring, and therefore ruled out an arene oxide or catechol-quinone reactive intermediate. Identification of the olefinic protons, H_7 and H_8, eliminated the possibility of a 7,8 epoxide intermediate or a Michael addition at C_8 to metabolically generated morphinone.

As in the morphine spectrum, the singlet resonating near 2.8 ppm was assigned to the N-CH_3 protons thus ruling out the formation of a N-methylene iminium intermediate and GSH attachment to a N-CH_2 moiety.

Analysis of signals from the GSH residue was initially complicated by the inability to identify $H_{cys\,\alpha}$ and thus to positively assign $H_{cys\,\beta,\beta'}$. In the ^1H NMR spectrum of GSH, $H_{cys\,\alpha}$ appeared near 4.6 ppm. This signal was notably absent in the spectrum of the adduct. The presence of the $H_{cys\alpha}$ signal was demonstrated when the temperature of the NMR sample was increased from 22° C to 35° C (Figure 5.10). The increase to 35° C slightly shifted the position of the H_2O signal thereby exposing the previously unnoticed multiplet at 4.8 ppm. In decoupling experiments, irradiation of this signal collapsed the doublet of doublets at 3.4 ppm to a simple doublet and sharpened the signal at 3.0 ppm. Decoupling experiments had previously shown coupling of these two signals to each other. Based on these experiments, the multiplet at 4.8 was assigned to $H_{cys\,\alpha}$, the signals at 3.4 and 3.0 ppm to $H_{cys\,\beta}$ and $H_{cys\,\beta'}$.

The chemical shifts of the glycine and glutamic acid proton signals were consistent with their corresponding resonances in the ^1H NMR spectrum of GSH. $H_{gly\,\alpha,\alpha'}$ came to resonance near 3.8 ppm. Integration of this signal revealed three underlying protons, and irradiation demonstrated coupling to the signal centered at

Figure 5.9 500 MHz ^{1}H NMR spectrum of morphine-GSH adduct.


Cys
Gly
Glu
H_2O
TMA
$H_{gly\,\alpha,\alpha'}$
$H_{glu\,\alpha}$
N-CH_3
$H_{glu\,\beta,\beta'}$
$H_{glu\,\gamma,\gamma'}$
H_{19ax}
$H_{1,2}$
$H_{cys\alpha}$
H_5
$H_{10}\beta$
H_{16eq}
H_{14}
H_{16ax}
$H_{cys\beta'}$
H_7
H_8
H_6
H_9
$H_{cys\beta}$
H_{19eq}
7
6
5
4
3
2
PPM

2.16 ppm. In the ^1H NMR spectrum of GSH, the signal at 2.16 ppm was assigned to $H_{glu\beta,\beta'}$ thereby suggesting that in the spectrum of the adduct, the third proton in the multiplet at 3.8 ppm was $H_{glu\alpha}$.

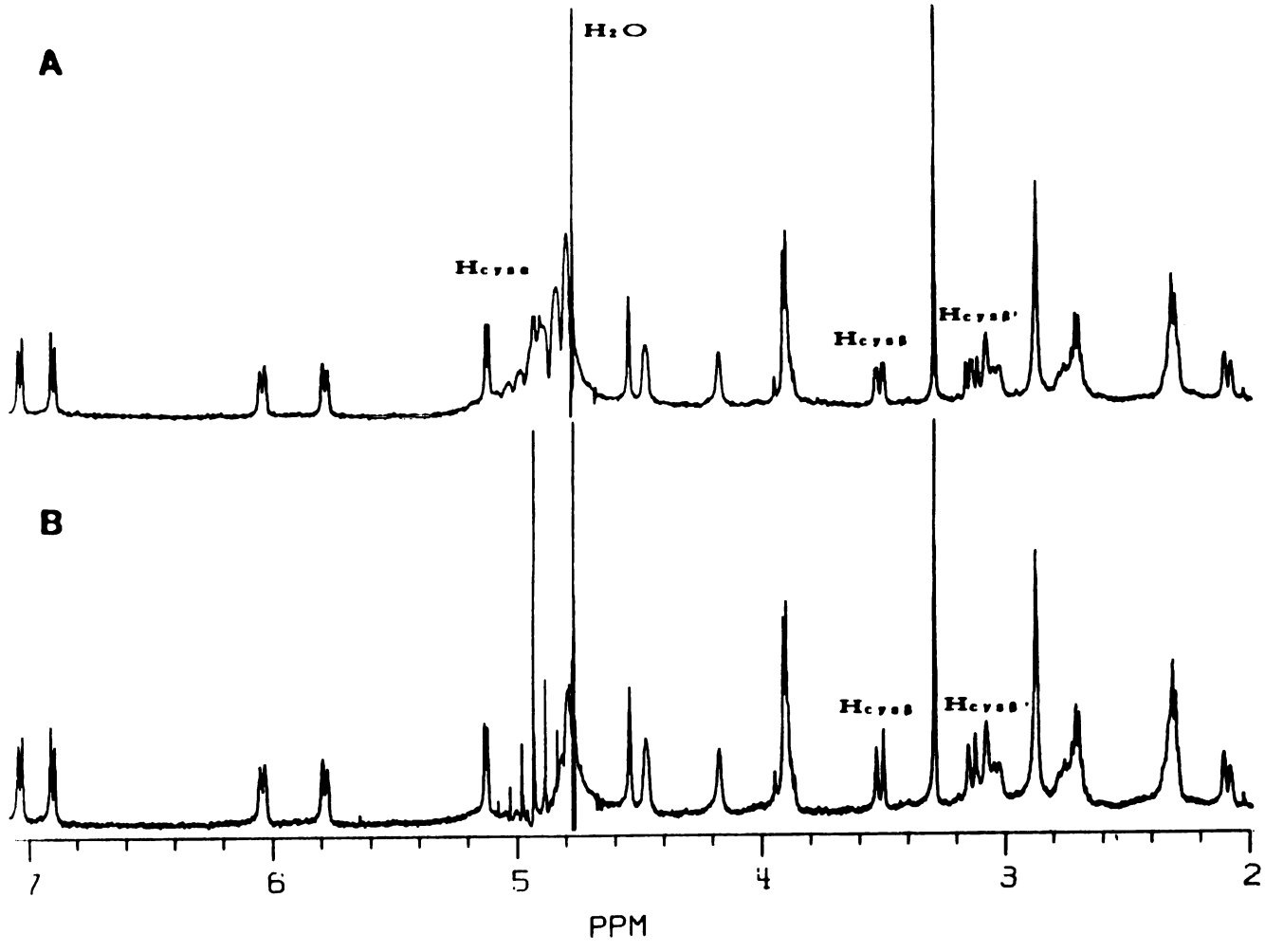
The signal centered at 2.16 ppm also integrated to three protons. The third proton underlying the signal at 2.16 ppm was clearly H_{15ax} demonstrated by its coupling to H_{15eq}. Irradiation of the signal at 2.16 ppm collapsed the H_{15eq} doublet (1.97 ppm) to a singlet. It also markedly sharpened the multiplet at 2.6 ppm and seemed to slightly sharpen the signal at 3.0 ppm. Integration of both of these later signals revealed three underlying protons. Coupling between signals at 2.16 ppm and 2.6 ppm was certain to be due to coupling between $H_{glu\beta,\beta'}$ and $H_{glu\gamma,\gamma'}$ (resonating at 2.54 in the GSH spectrum), but also suggested coupling between H_{15ax} and a H_{16} proton which possibly comprised the third proton underlying the signal near 2.6 ppm. Based on coupling to H_{15ax}, the third proton underlying the signals in the region 2.9-3.0 ppm could have been the other H_{16} proton. Unfortunately, the overlap of $H_{glu\beta,\beta'}$ with the H_{15ax} signal and the presence of $H_{glu\gamma,\gamma'}$ and $H_{cys\beta'}$ signals at 2.57 and 3.0 ppm, respectively, prevented assessment of H_{15ax} coupling.

The possible sites for GSH attachment not eliminated by this analysis were C_9, C_{10}, C_{14}, and C_{16}. Nucleophilic attack at C_{14} was ruled out due to its position in the morphine structure, a site inaccessible for metabolic activation. If nucleophilic attack were at C_{16}, then both H_{10} protons and H_9 would be observed. H_{10} protons were not identified in the spectrum although the singlet near 4.1 resonated in the same region as did the H_9 signal in the morphine spectrum. However, the inability to identify H_{10} protons precluded a definitive assignment. If attack were at C_9, both

Figure 5.10 ^{1}H NMR spectrum of morphine-GSH adduct at 35° C.

(A) Spectrum at 35° C revealed signal centered near 4.8 ppm.

(B) Irradiation at 4.8 ppm demonstrated coupling to signals at 3.4 and 3.0 ppm.



51a

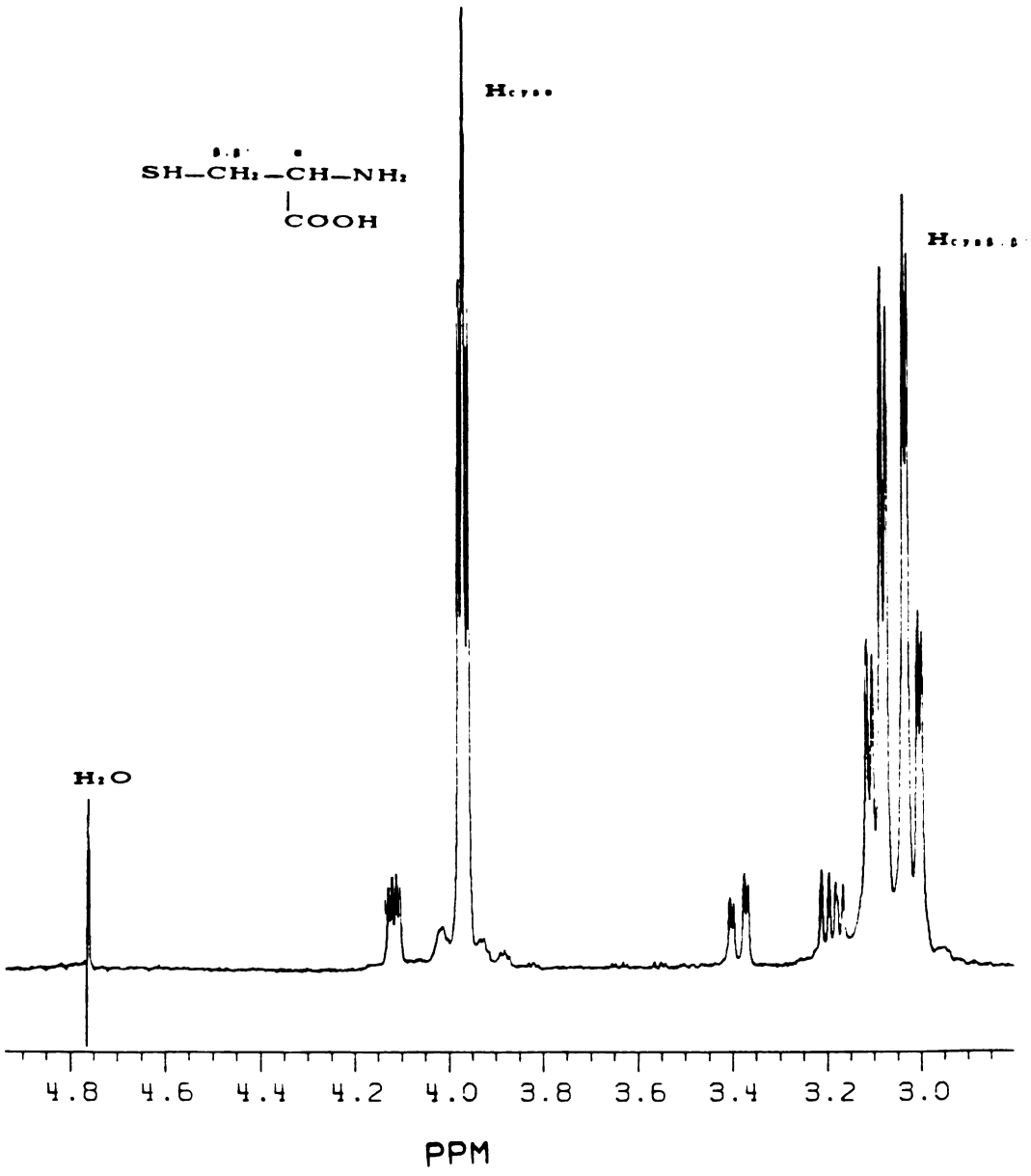
H_{10} and H_{16} protons would be present. As discussed above, the presence of the H_{16} protons could not be validated due to overlapping GSH proton signals. If GSH were attached at C_{10} , both H_{16} protons, H_9 and one H_{10} proton would be observed. Coupling between H_9 and H_{10} protons would depend on whether the $H_{10\alpha}$ or $H_{10\beta}$ proton remained. The sharp singlet near 4.4 ppm was possibly the signal of a proton geminal to the sulfur atom. If this proton were $H_{10\beta}$, then no coupling with H_9 would be expected and the signal near 4.1 ppm might reasonably be assigned to H_9.

However, since overlap of GSH signals with those of morphine precluded analysis of H_{15ax} coupling and therefore the number of H_{16}protons, it was necessary to obtain the spectrum of a model compound that could be interpreted fully. For these reasons, the morphine-cysteine adduct was prepared and analyzed.

^{1}H NMR spectrum of cysteine

The ^{1}H NMR spectrum of cysteine is shown in Figure 5.11. The triplet near 4.0 ppm was assigned to the $H_{cys\,\alpha}$ proton. The multiplicity of the signal indicated coupling to two other protons. The quartet of doublets centered at 3.1 ppm was assigned to the $H_{cys\beta,\beta'}$ protons. Integration of these signals confirmed the presence of one proton underlying the signal near 4.0 ppm and two protons beneath the one centered at 3.1 ppm.

Figure 5.11 500 MHz ^{1}H NMR spectrum of cysteine.



53a

^{1}H NMR spectrum of morphine-cysteine adduct

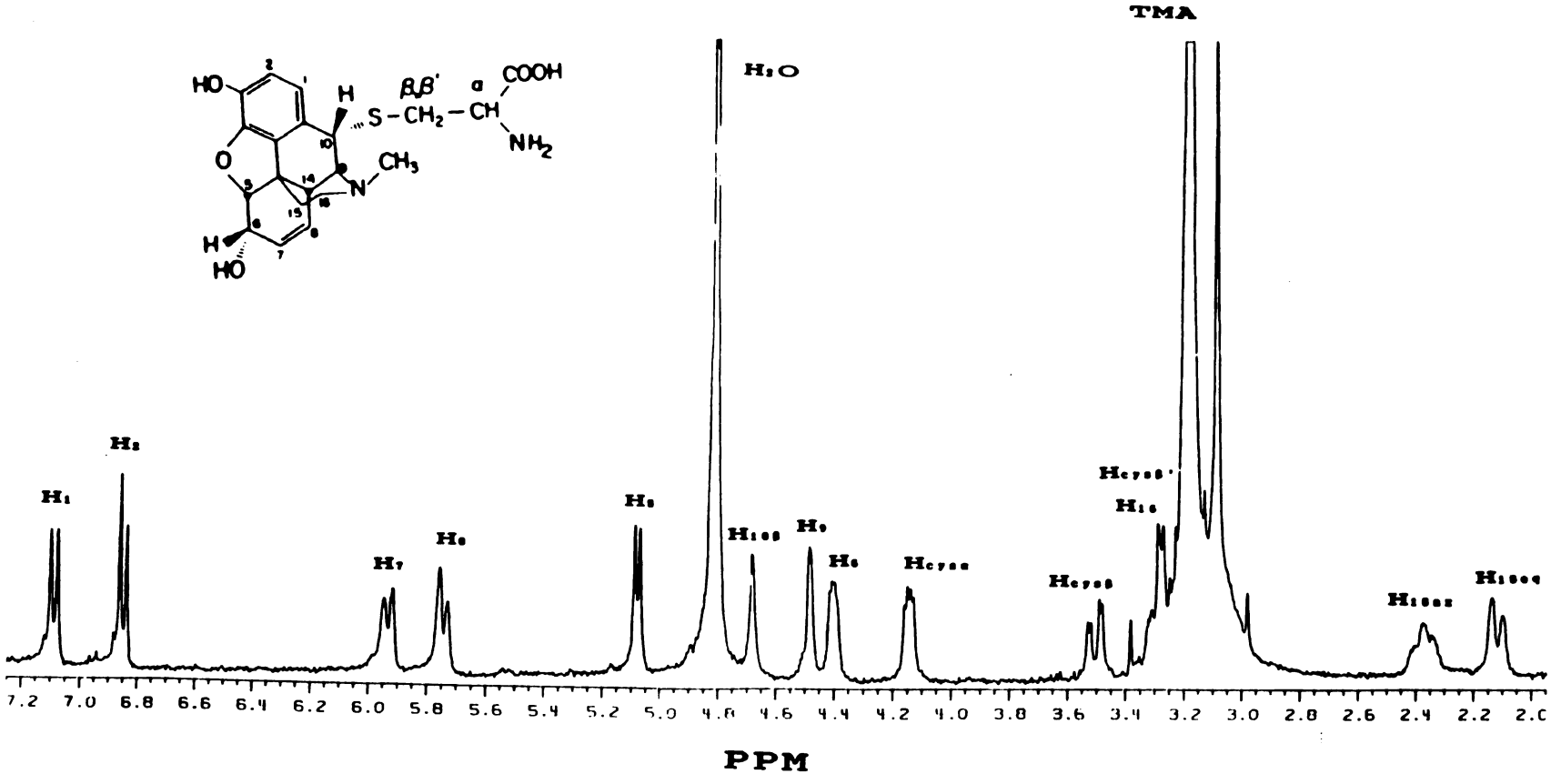
The spectrum of the morphine-cysteine adduct, Figure 5.12, showed many similarities with the spectra of morphine and morphine-GSH. As in the spectrum of morphine-GSH, the downfield signals were all consistent with those in the morphine spectrum. Thus, identification of H_1 , H_2 , H_7 , H_8 , and H_5 was unambiguous. H_6 was assigned to the signal resonating at 4.4 ppm based on its resonance in both morphine and morphine-GSH spectra (4.4 ppm) and by coupling to H_5.

In this spectrum, assignment of the $H_{cys\alpha}$ proton was clear. Based on coupling to the doublet centered at 3.5 ppm and to the signal at 3.3 ppm, the multiplet centered at 4.15 ppm was assigned to the $H_{cys\alpha}$ proton. The signals at 3.5 and 3.3 ppm were assigned to $H_{cys\beta}$ and $H_{cys\beta'}$. The identification of the doublet at 3.5 ppm as $H_{cys\beta}$ thus confirmed the assignment made in the morphine-GSH spectrum.

Irradiation of the H_{15ax} signal (2.35 ppm) demonstrated that at least one H_{16} proton resonated near 3.3 ppm. However, because of the overlap in the region between 3.0 and 3.4 ppm, the presence of the other H_{16} proton could not be determined. Without the overlap of $H_{glu\beta,\beta'}$ on the H_{15ax} signal, the number of H_{16} protons should have been elucidated by the signal splitting of H_{15ax} . If H_{15ax} were coupled to both H_{16} protons as well as H_{15eq} , a triplet of doublets would be expected, as in the morphine spectrum. Although the shape of this signal was not inconsistent with a triplet of doublets, it was far from unequivocal.

As in the spectrum of the morphine-GSH adduct, two prominent singlets (in this spectrum at 4.5 to 4.7 ppm) were unidentified. If GSH were bound at C_{16} , the remaining H_{16} proton would most likely be shifted upfield. The signal at 4.7 ppm

Figure 5.12 500 MHz ^{1}H NMR spectrum of morphine-cysteine adduct.


TMA
H₂O
H₁
H₂
H₇
H₆
H₅
H₁₀β
H₉
H₈
Hcysα
Hcysβ
Hcysβ'
H₁₀
H₁₀α
H₁₀q
PPM
7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0

could clearly not be assigned to H_{16} since the signal at 3.3 ppm included a H_{16} resonance. If GSH were bound at C_9, the H_9 signal would disappear but the signals for the H_{10} protons, coupled to each other, would be evident. The signals at 4.5 and 4.7 ppm are clearly not coupled to each other. If, however, the signal at 4.7 ppm were the $H_{10\beta}$ proton, which was not coupled to H_9, then the signal at 4.5 ppm could be tentatively assigned to H_9.

To assign with confidence the signal at 4.7 to $H_{10\beta}$, the presence of both H_{16} protons had to be irrefutably demonstrated. Only a small amount of the morphine-cysteine adduct had been isolated; and contrary to expectations, the spectral resolution of the morphine-GSH sample had been better. It was therefore decided to prepare the morphine-N-AcCys conjugate. Not only was N-acetylcysteine likely to be as good a nucleophile as GSH but also, the adduct being a mercapturic acid, its greater stability was expected to permit a larger amount of material to be isolated and thereby to provide better resolution.

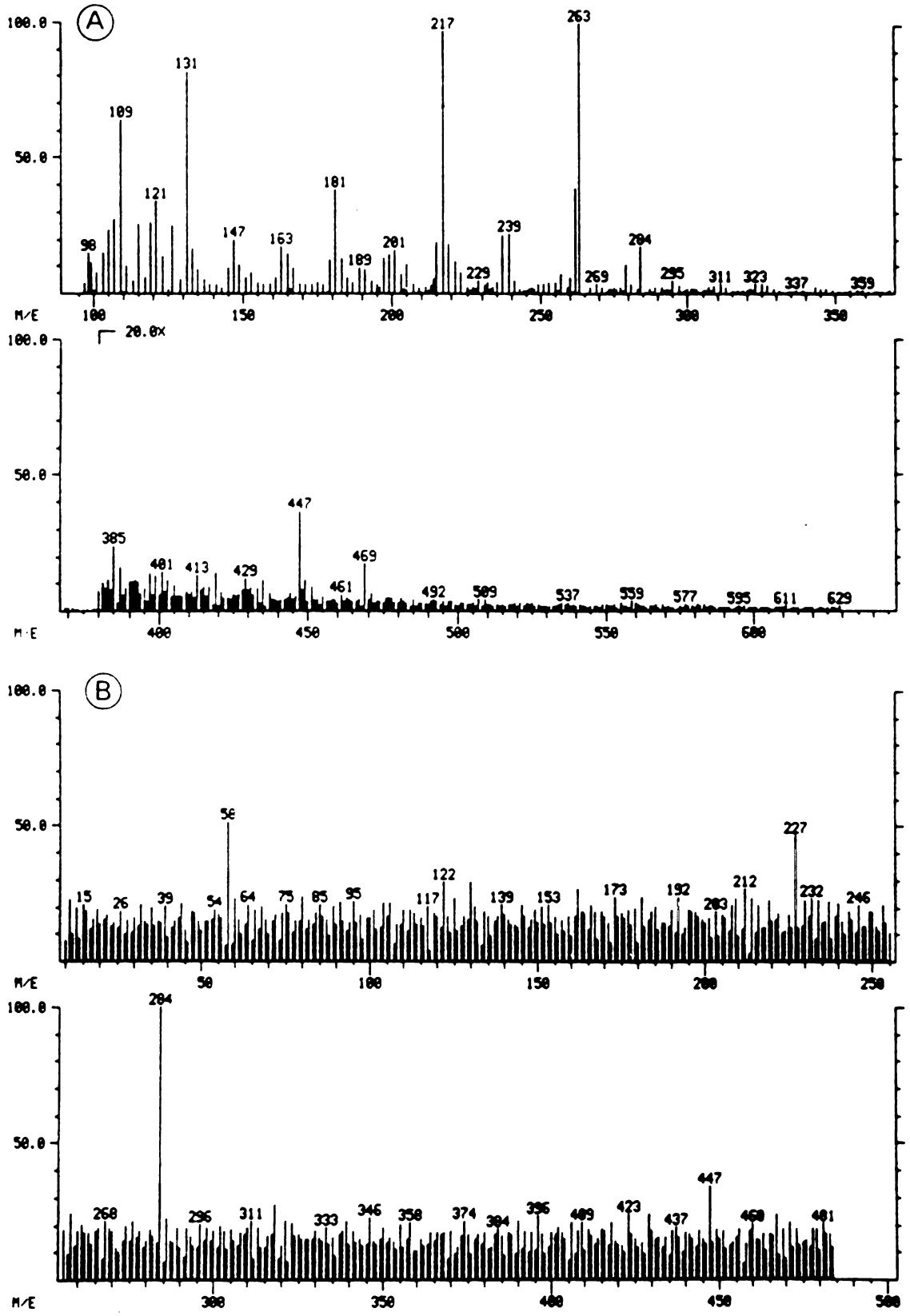
Mass spectral analysis of morphine-N-AcCys adduct

The purified metabolite was analyzed by FABMS in a thioglycerol matrix (Krowech *et al.*, 1986). The mass spectrum is shown in Figure 5.13A. The protonated molecular ion (MH+) was observed at m/z 447 and a molecular ion plus sodium $(MNa)^+$ at m/z 469. While the ions at m/z 217 and 263 could not be identified, the less intense fragment at m/z 284 was consistent with the loss of the N-acetylcysteine group. The sample was further analyzed in a FABMS/MS experiment. The FABMS/MS spectrum (Figure 5.13B) revealed fragments corresponding to the loss

Figure 5.13 Mass Spectra of morphine-N-AcCys adduct.

(A) FAB mass spectrum in thioglycerol matrix of the morphine-N-AcCys adduct.

(B) MS/MS spectrum of the morphine-N-AcCys adduct.



57a

of both N-acetylcysteine at m/z 284 and the N-methylethylamine moiety at m/z 227 and to the cyclization of the N-methylethylamine chain - $(C_3H_7N)H^+$ - at m/z 58 thereby confirming that this sample was indeed a morphine-N-AcCys adduct.

^{1}H NMR spectrum of N-acetylcysteine

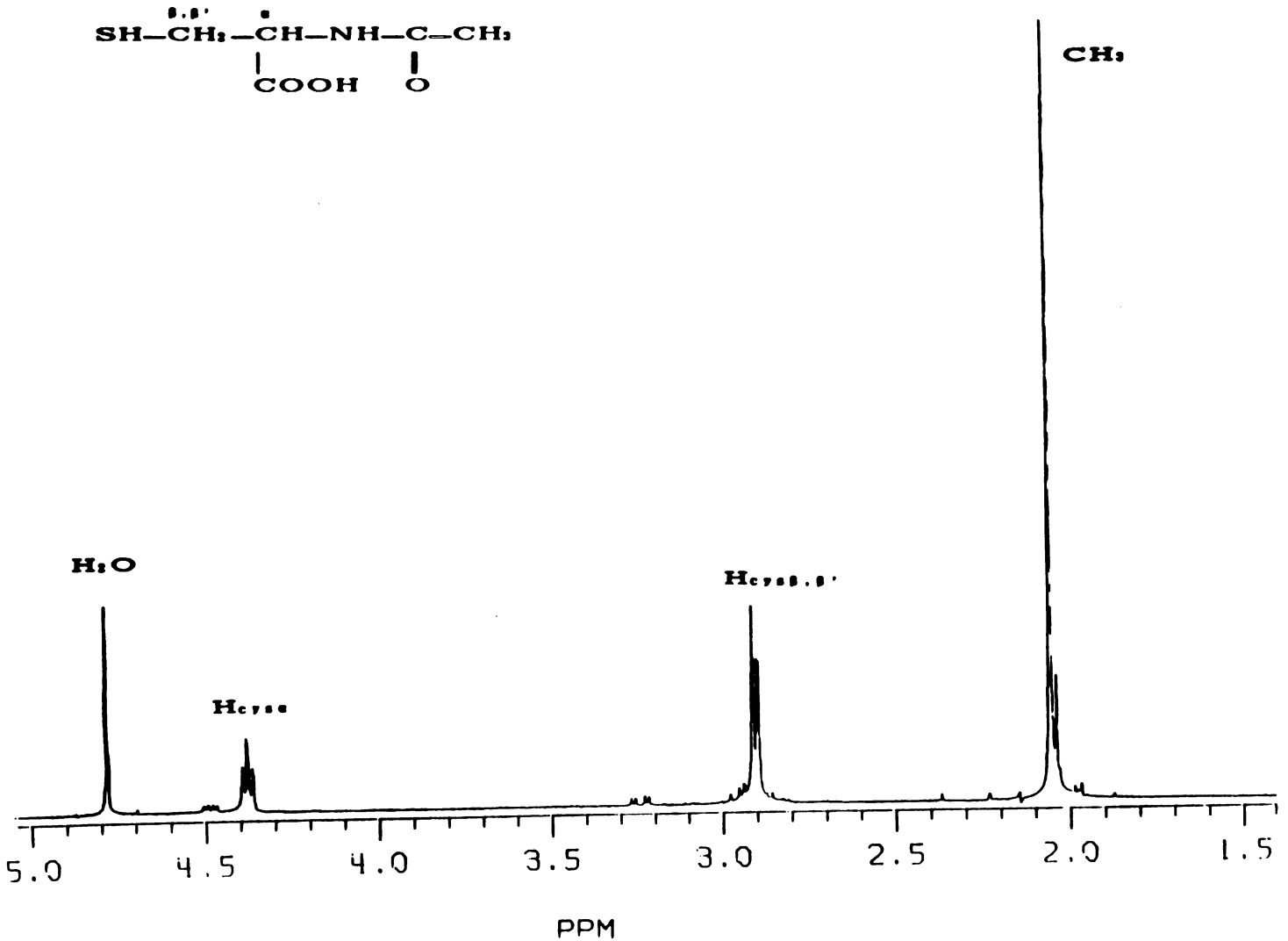
The spectrum of N-acetylcysteine is shown in Figure 5.14. Consistent with the ^{1}H NMR spectrum of cysteine, the triplet near 4.4 ppm was assigned to $H_{cys\,\alpha}$. Irradiation of this triplet collapsed the doublet at 2.9 ppm to a singlet, confirming the coupling of these two signals. The doublet near 2.9 ppm was thus assigned to the $H_{cys\,\beta,\beta'}$ protons. Based on chemical shift, the sharp singlet near 2.05 ppm was assigned to the carbonyl methyl group. Integration of the signal areas confirmed one, two and three protons underlying the signals at 4.4, 2.9 and 2.05 ppm, respectively.

^{1}H NMR spectrum of morphine-N-AcCys adduct

The ^{1}H NMR spectrum for the morphine-N-AcCys conjugate is shown in Figure 5.15. Downfield signals in this spectrum were clearly identical to those in the spectra of morphine and the two morphine adducts. Thus, the downfield doublets at 6.98, 6.78, 5.95 and 5.66 ppm were assigned to H_1 , H_2 , H_7 and H_8 , respectively.

The decreased signal intensity in the region 4.0-5.1 ppm was due to suppression of the H_2O signal. Integration of the signals in this region revealed a single proton underlying each signal. The doublet at 5.05 ppm was assigned to H_5 , and the multiplet at 4.35 ppm was assigned to H_6 .

Figure 5.14 360 MHz ^{1}H NMR spectrum of N-acetylcysteine.


β,β'
SH—CH₂—CH—NH—C—CH₃
COOH
O
CH₃
H₂O
Hcysα
Hcysβ,β'
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
PPM

Based on the spectrum of authentic N-acetylcysteine and on coupling to the $H_{cys\,\beta,\beta'}$ protons, the multiplet at 4.56 ppm was assigned to $H_{cys\,\alpha}$. The doublet of doublets at 3.4 ppm was assigned to $H_{cys\,\beta'}$. Decoupling experiments revealed that $H_{cys\,\beta}$ was one of three protons resonating in the region near 3.0 ppm.

The two signals at 2.24 and 2.0 were assigned to H_{15ax} and H_{15eq} . The carbonyl CH_3 singlet resonated near 2.1 ppm between the two H_{15} protons. In this spectrum, without the overlap of the $H_{glu\,\beta,\beta'}$ signal, the H_{15ax} resonance was unquestionably a triplet of doublets. As in morphine, this multiplicity resulted from geminal coupling with H_{15eq} and vicinal coupling with H_{16ax} and H_{16eq} . This conclusion was confirmed by decoupling experiments. Irradiation of H_{15eq} (2.0 ppm) reduced the H_{15ax} signal to a doublet of doublets. If only one C_{16} proton remained, this signal would have been a simple doublet (Figure 5.16). Thus, the possibility of nucleophilic attack at C_{16} was eliminated.

In morphine, the resonance at 4.21 was assigned to H_9. This proton was coupled only to $H_{10\alpha}$ which resonated as a doublet of doublets. $H_{10\beta}$ was coupled only to its vicinal proton and therefore resonated as a simple doublet. The inability to identify both H_{10} signals, along with confirmation that nucleophilic attachment was not at C_{16} , led to the conclusion that C_{10} was the point of nucleophilic attack. The H_9 signal resonated at nearly the same frequency in all four spectra. The transformation of the H_9 signal from a multiplet in morphine to a singlet in the other spectra suggested that this proton was no longer coupled to $H_{10\alpha}$. The upfield proton was therefore assigned to $H_{10\beta}$.

Figure 5.15 360 MHz ^{1}H NMR spectrum of morphine-N-AcCys adduct.

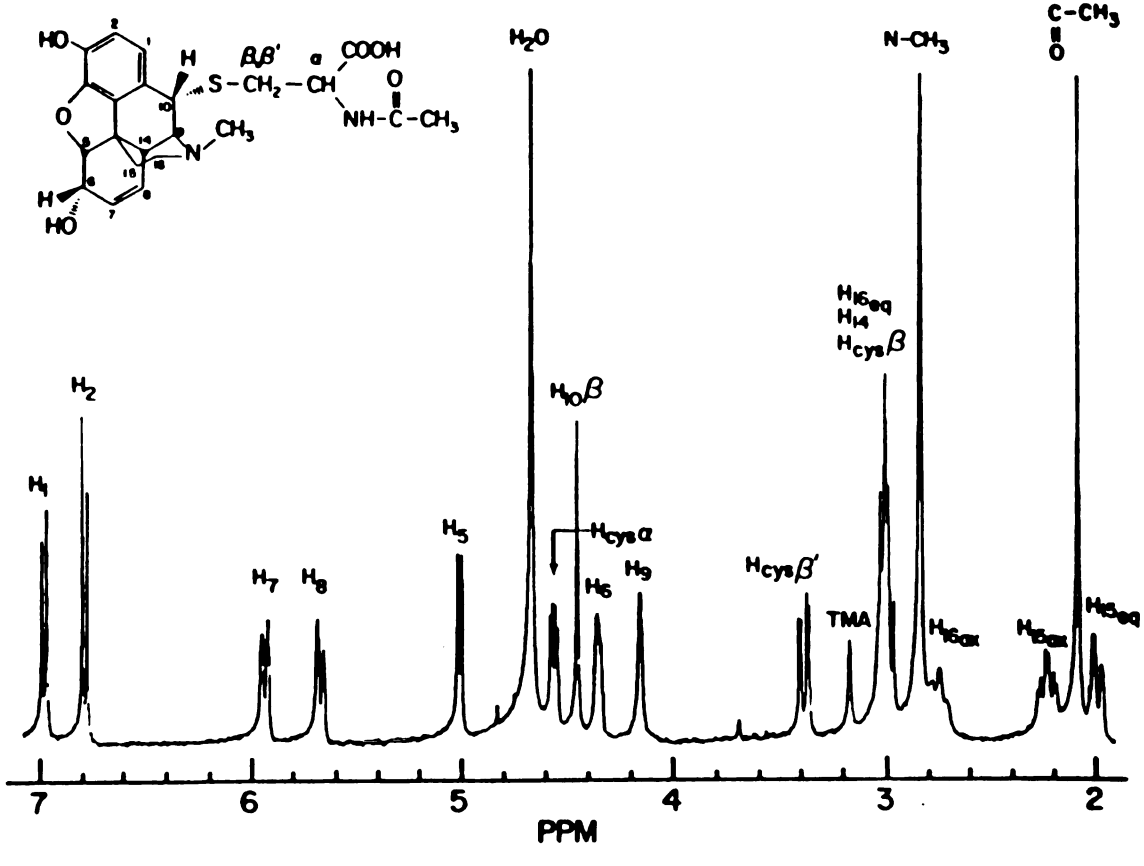
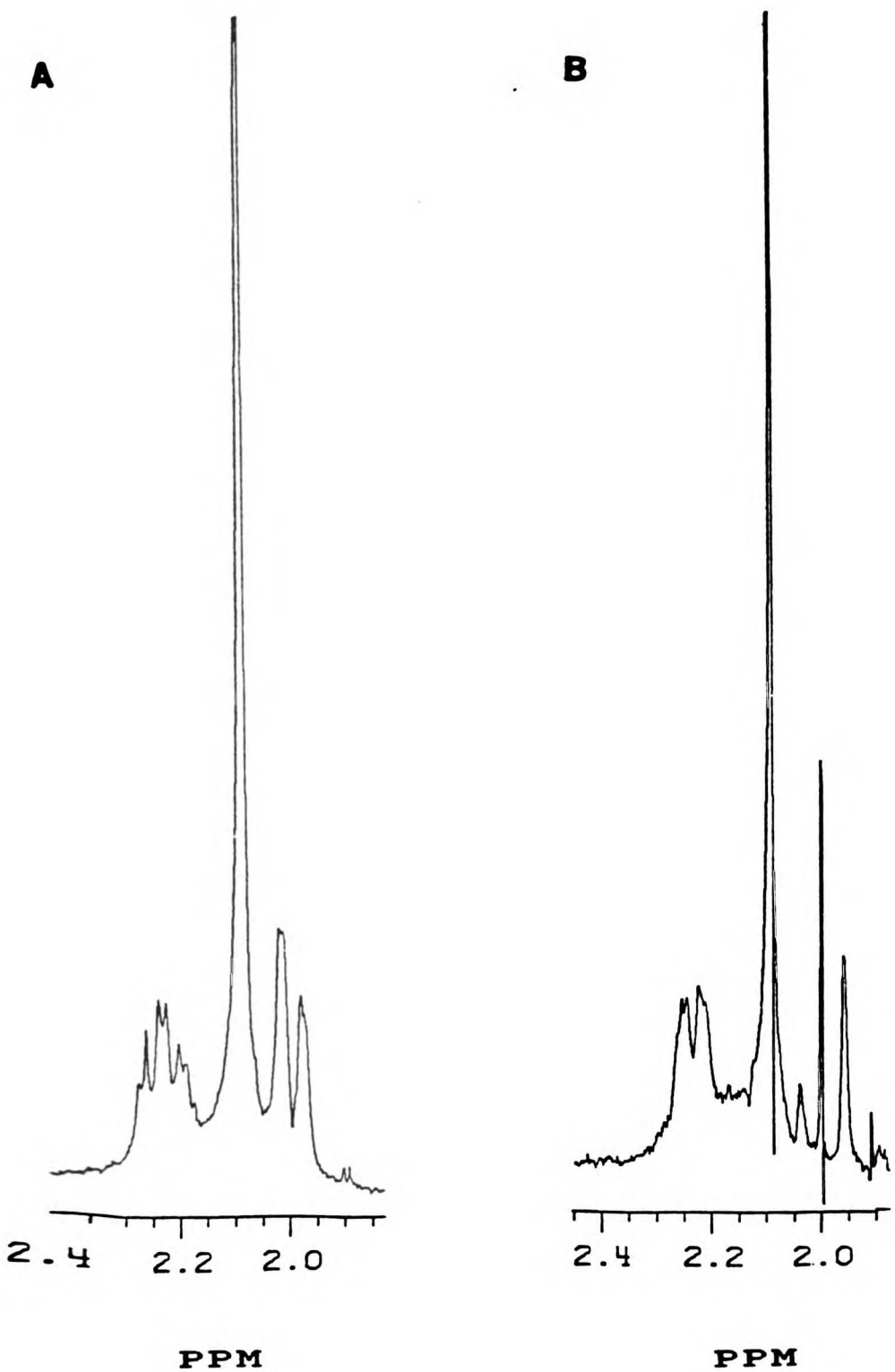

HO
H
S-CH2-CH
ββ'
α
COOH
NH-C-CH3
O
CH3
N
H
HO
H2O
N-CH3
C-CH3
O
H16eq
H14
Hcysβ
H2
H10β
H1
H5
Hcysα
H7
H8
H6
H9
Hcysβ'
TMA
H16ax
H15ax
H15eq
7
6
5
4
3
2
PPM

Figure 5.16 Enlargement of upfield section of ^1H NMR spectrum of morphine-N-AcCys.

(A) Multiplicity of the signals in the range from 1.9 to 2.4 ppm demonstrate the coupling of the H_{15} signals.

(B) Irradiation of signal at 2.0 ppm transformed H_{15ax} to a doublet of doublets.


A
B
2.4
2.2
2.0
2.4
2.2
2.0
PPM
PPM

These conclusions were indeed confirmed by the findings of Dr. Patricia Caldera-Munoz who synthesized and characterized 10α-hydroxycodeine, a model for substitution at C_{10} (Krowech *et al.*, 1986). Comparison of the ^1H NMR spectra of codeine with that of synthetically prepared 10α-hydroxycodeine, indicated that the characteristic $H_{10\alpha}$ and $H_{10\beta}$ signals in the spectrum of codeine (as in morphine, doublet and doublet of doublets, respectively) were absent in the spectrum of 10α-hydroxycodeine. Instead, a sharp singlet shifted downfield from the original H_{10} signals was assigned to $H_{10\beta}$. These findings thus provide a blueprint of the spectral changes associated with substitution at C_{10} and strongly support our structural identification of the morphine adduct as its 10α-N-acetylcysteinyl adduct.

The formation of the 10α-morphine-GSH adduct is believed to occur through the metabolism of morphine to 10-hydroxymorphine. Efforts in other laboratories to isolate this intermediate have not yet been successful.

Proposed mechanism for the formation of the 10α-morphine-GSH adduct

The mechanism of the formation of 10-hydroxymorphine may be via a radical intermediate. It is postulated that such an intermediate is generated by the transfer of a hydrogen atom (or 1 electron and a proton) to activated oxygen bound to the prosthetic heme of cytochrome P-450. This transfer would produce a radical carbon (at C_{10}) as well as a hydroxyl radical bound to the heme iron and would be followed by rapid combination of the carbon intermediate with the activated hydroxyl to form 10-hydroxymorphine. While cytochrome P-450 mediated hydroxylation could occur through a cation intermediate, the formation of a radical intermediate is regarded

as more likely in hydrocarbon hydroxylations (Ortiz de Montellano, 1986).

A proposed scheme for the formation of the morphine adduct is shown in Figure 5.17.

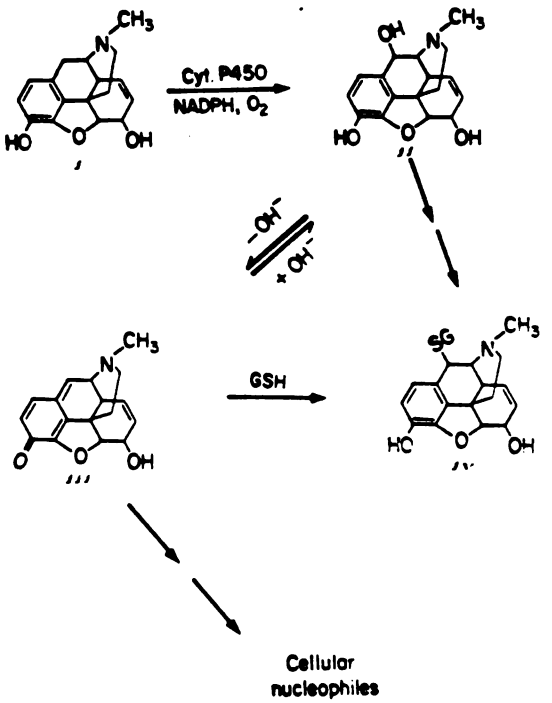


Figure 5.17 Proposed mechanism for the formation of 10α-morphine-GSH

Direct conjugation of 10-hydroxymorphine with GSH is unlikely. It is possible that 10-hydroxymorphine rearranges to form a reactive quinone species (Figure 5.13, *[iii]*), which is then most likely intercepted by GSH to form the 10α-morphine-GSH adduct (Figure 5.13, *[iv]*). In the absence of GSH, the reactive species could bind irreversibly to cellular nucleophiles.

6.0 METABOLISM OF MORPHINE TO A REACTIVE INTERMEDIATE IN LIVER MICROSOMES

Introduction

Irreversible binding to microsomal protein and the isolation and characterization of a morphine-GSH adduct from rat liver microsomal incubations of morphine suggested that metabolism to a reactive electrophilic intermediate may contribute to morphine-induced hepatotoxicity in vivo. In this section, the importance of this metabolic pathway relative to the overall metabolism of morphine in liver microsomes is evaluated and discussed. Although liver microsomes largely catalyze morphine-3-glucuronidation, in these studies only cytochrome P-450 dependent metabolism is assessed since that is apparently the pathway that is responsible for activation of morphine to a reactive species.

Metabolism was first studied in liver microsomes from PB-pretreated rats. The formation of morphine-GSH and normorphine was quantitated, and the relative significance of the two metabolites was assessed. Metabolism was also studied in human liver microsomes. Since morphine is usually administered in low doses as an analgesic, the probability of a reactive metabolite causing hepatic damage in humans would be extremely low. Any reactive intermediate formed would be conjugated with GSH without signficantly depleting the nucleophile or resulting in irreversible binding. However, morphine is also used as an anesthetic in cardiac surgery since it causes minimal myocardial depression, and as such, it is administered in relatively large doses. In combination with other GSH depleting drugs, metabolism of morphine to a reactive species might contribute to hepatotoxicity. It was therefore important to

determine if human liver microsomes also metabolized morphine to a GSH adduct.

Species differences, if any, were assessed by comparing morphine metabolism by human, rat and rabbit liver microsomes. Since previous studies on the hepatotoxicity of morphine utilized liver microsomes from PB-pretreated rats, it was of interest to determine the extent of formation of the 10α-morphine-GSH adduct in liver microsomes from untreated rats and to compare metabolism by these microsomes with metabolism by human liver microsomes as well as liver microsomes from PB-pretreated rats. Comparison of the relative formation of the morphine adduct and normorphine in liver microsomes from untreated and PB-pretreated rats would indicate the relative contribution of PB-inducible cytochrome P-450 isozymes to each pathway. Analysis of morphine metabolism by rabbit liver microsomes was also included in these studies which allowed assessment of the relative metabolism of morphine to the 10α-morphine adduct and normorphine in three different species.

Materials and Methods

Metabolism of [^{3}H]morphine by liver microsomes from PB-pretreated rats

For quantitation of in vitro morphine-GSH adduct formation, 2 rats were injected with PB (80 mg/kg, i.p.) for 5 days. Liver microsomes were prepared by the method described in the previous chapter.

Microsomes (2 mg protein/ml) were incubated in phosphate buffer (0.1 M, pH 7.4) with [C_1-^{3}H]morphine (1.0 mM, 0.5 μCi), NADPH (1.0 mM), l-isocitrate (2.5 mM), l-isocitrate dehydrogenase (50 units) and GSH (2.5 mM) in a total volume of 5 ml. The experiment included mixtures which lacked either NADPH or GSH as controls. After a 3 min preincubation period, reactions were initiated by the addition of NADPH and were allowed to proceed in a shaking water bath at 37° C for 15 min. Reactions were terminated by the addition of ice-cold methanol (15 ml) followed by immersion of the reaction flasks in ice. The precipitated protein was removed by centrifugation at 1500 rpm for 10 min, and the supernatant was filtered through a 0.45 μ filter. An aliquot (3 ml) of the filtrate was evaporated under N_2 (to remove methanol) and then lyophilized to dryness.

The residue was dissolved in acetonitrile/H_2O (10:90, v/v) containing 0.5% ammonium acetate (pH 5.5) and chromatographed on an Altex reverse phase C_{18} Ultrasphere ODS (5μ, 10mm i.d. x 250 mm) column at a flow rate of 2 ml/min with the above buffer as the mobile system. The effluent (2 ml) was collected at 1 min increments. Radioactivity was determined in a Beckman L2000 scintillation counter following the addition of 10 ml Aquasol.

The recovery of [^{3}H]morphine was calculated as the ratio of the [^{3}H]morphine recovered after HPLC separation from incubation mixtures which lacked NADPH to the [^{3}H]morphine initially added to the incubate. The average recovery was 70%. The recovery of [^{3}H]normorphine, computed as the ratio of [^{3}H]normorphine formed as determined from HPLC analysis to the formaldehyde liberated as measured by the method of Nash (1953), was found to be 65%. The recovery of [^{3}H]morphine-GSH was assumed to lie within this range also. The figures cited in the text are the uncorrected values.

Metabolism of [^{3}H]morphine by human liver microsomes

Preparation of Microsomes: Human liver tissue was obtained by Dr. Lucy Waskell[3] from normal liver tissue which is excised during the course of the surgical removal of tumors. The liver samples ranged in weight from 2.4 - 14.0 g. Liver samples were stored at -70° C until microsomes were prepared. Preparation of microsomes was normally carried out within one week from the time the liver sample was obtained. The tissue was cut into small pieces and homogenized in 0.25 M sucrose/0.1 M Tris buffer (pH 7.4) using a glass homogenizer fitted with a loose pestle. After filtering the homogenate through two layers of gauze to remove connective tissue, a 15% w/v homogenate was prepared. Such a dilute homogenate was necessitated by the amount of connective tissue in the human liver samples. The homogenate was centrifuged at 9,000 x g at 4° C for 20 min, and the resulting supernatant was then centrifuged at 100,000 x g for 1 h at 4° C. The pellet was resuspended

[3] Veterans Administration Hospital, San Francisco.

in 1.15% KCl, washed free of contaminating hemoglobin, and centrifuged at 100,000 x g for 1 h at 4° C. Typically, incubations were carried out one or two days after preparation of the microsomes. In these cases, the microsomal pellets were stored under a solution of 20% glycerol / 0.25 M sucrose at -70° C.

Microsomal Incubations: For microsomal incubations, the pellet was re-suspended in 1.15% KCl and recentrifuged at 100,00 x g for 1 h at 4° C to remove any remaining glycerol. The pellet was then resuspended in 0.1 M HEPES buffer (pH 7.6), and the protein content was measured by the method of Lowry (1951).

Microsomes (2 mg protein/ml) were incubated in a total volume of 5 ml in 0.1 M HEPES buffer (pH 7.6) with [^{3}H]morphine (1 mM), NADPH (1 mM), l-isocitrate (2.5 mM), l-isocitrate dehydrogenase (50 units) and GSH (2.5 mM) or N-acetylcysteine (7.5 mM) in the presence or absence of NADPH (1 mM). Reactions were initiated by the addition of microsomes and were allowed to proceed for 15 min in a shaking water bath at 37° C. Reactions were terminated by chilling the reaction flasks in ice. Morphine and its metabolites were then extracted from the microsomes by centrifugation at 100,000 x g at 4° C for 1 h.

HPLC Analysis: For HPLC analysis, the 100,000 x g supernatant was passed through a Waters Sep Pak C_{18} cartridge. The cartridge was washed with water (2 ml) and morphine and its metabolites were eluted with methanol (4 ml). The methanol was rotoevaporated to dryness, and the residue was chromatographed on an Altex reverse phase C_{18} Ultrasphere ODS (5μ, 10mm i.d. x 250 mm) column at a flow rate of 2 ml/min with acetonitrile/water (10:90, v/v) containing 0.5% ammonium acetate (pH 5.5) as the mobile phase.

The effluent was collected in 1 ml increments to which 10 ml of Aquasol were added. Radioactivity was monitored in a Beckman Liquid Scintillation counter. The radioactivity of each metabolite was calculated as cpm in the +NADPH sample minus cpm in the -NADPH sample.

In order to rule out the possibility of losses of the [^{3}H]morphine adduct during Sep Pak filtration, the Sep Pak filtrate and 2 ml water wash from the supernatant of microsomal incubations with [^{3}H]morphine and GSH were analyzed. The filtrate and wash were lyophilized to dryness and chromatographed and analyzed by the procedures described above. Neither the [^{3}H]morphine-GSH adduct nor [^{3}H]normorphine was found in these samples.

Comparative Adduct Formation

Liver microsomes from untreated rats were prepared from two rats by the procedures described in the previous chapter. Microsomes from PB-pretreated rats were prepared by the same procedures. Rabbit liver microsomes were obtained from Dr. Anthony J. Trevor. Human liver microsomes were prepared as described above.

Microsomes (2 mg protein/ml) were incubated with [^{3}H]morphine (1.0 mM) and N-acetylcysteine (7.5 mM). Incubation conditions and termination as well as separation of metabolites from microsomes and HPLC analysis were identical to those described above for human microsomal incubations.

Results and Discussion

Metabolism of [^{3}H]Morphine in Rat Liver Microsomes

To examine the formation of morphine-GSH quantitatively, liver microsomes from rats pretreated with PB were incubated in the presence of [^{3}H]morphine (1 mM) and GSH (2.5 mM) and analyzed as described in Materials and Methods. In incubation mixtures lacking NADPH, the only radioactive material recovered during HPLC analysis was [^{3}H]morphine. In the presence of NADPH, two additional radioactive materials were recovered, morphine-GSH and normorphine. The quantitative determination of morphine metabolites in liver microsomes from rats pretreated with PB is shown in Table 6.1.

Table 6.1

Morphine Metabolism by Liver Microsomes from PB-pretreated Rats

Morphine Metabolite	GSH	μmol formed/15 min
Morphine-GSH	+	0.45
Normorphine	+	0.16
Normorphine	-	0.19

Microsomes (2 mg protein/ml) were incubated with [^{3}H]morphine (1.0 mM) and GSH (2.5 mM) for 15 min and analyzed as described in Materials and Methods.

The formation of morphine-GSH was nearly three times as high as that of normorphine. Incubations of [^{3}H]morphine (1.0 mM) in the absence of GSH did not measurably increase the formation of normorphine (0.19 μmol compared to 0.16 μmol). Since the presence or absence of GSH was unrelated to 10α-hydroxy morphine intermediate formation, the absence of the nucleophile did not shunt additional morphine to the normorphine pathway. As will be discussed below, in incubations with liver microsomes from PB-pretreated rats, N-demethylation appeared to be of major significance only when other pathways were saturated.

Metabolism of [^{3}H]Morphine in Human Liver Microsomes

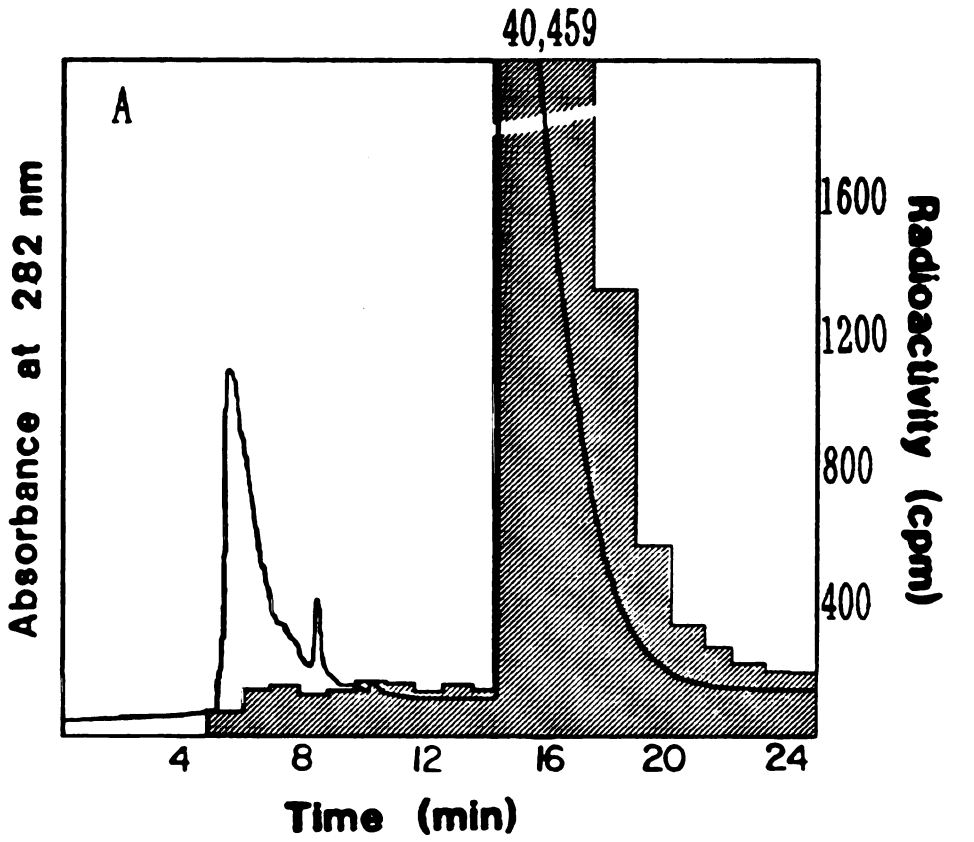
Human liver microsomes were incubated with [^{3}H]morphine (1.0 mM) and GSH or N-acetylcysteine (2.5 mM or 7.5 mM) as described earlier. A HPLC chromatogram derived from an incubation with N-acetylcysteine is shown in Figure 6.1. A [^{3}H]morphine metabolite consistently co-eluted with authentic normorphine (12-13 min) in these incubates thereby indicating that human liver microsomes could N-demethylate morphine. At 9-10 min, a [^{3}H]morphine metabolite eluted with the authentic morphine-N-AcCys adduct.

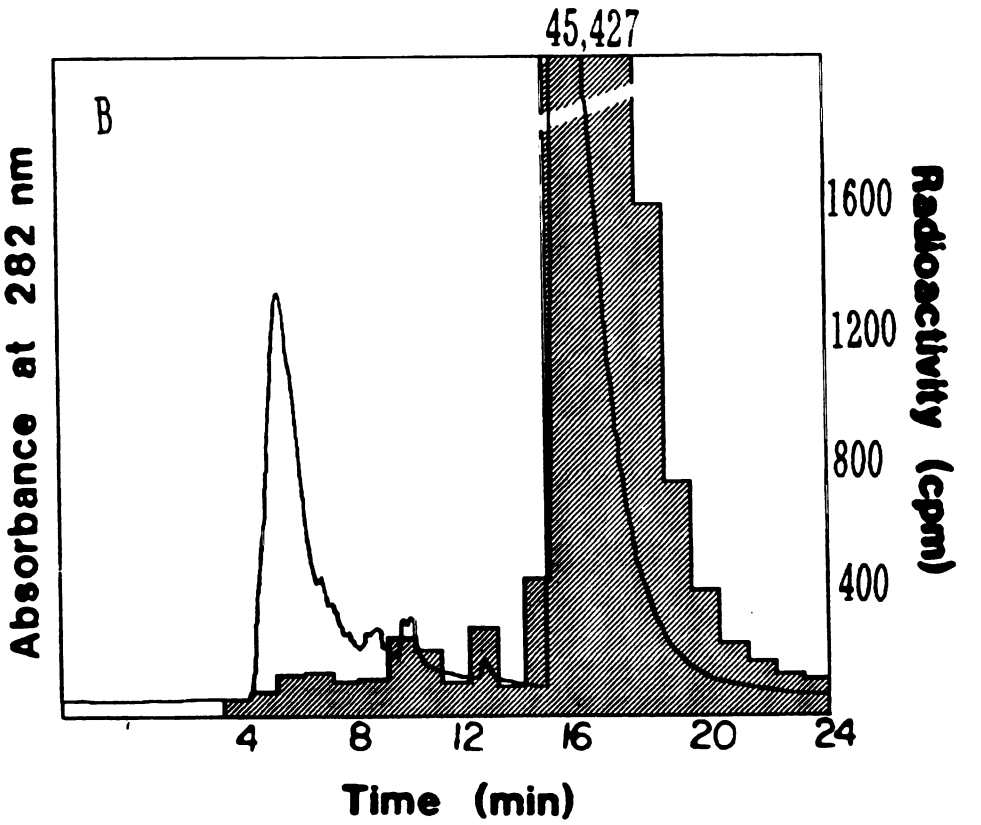
Similarily, in incubations with GSH, a [^{3}H]morphine metabolite eluted with authentic morphine-GSH. Co-elution was observed irrespective of the solvent system employed i.e. methanol/water (20:80, v/v) containing 25mM TMA (pH 6.1) or acetonitrile/water (10:90, v/v) containing 0.5% ammonium acetate (pH 5.5) were used.

Figure 6.1 HPLC profiles of [^{3}H]morphine incubated with human liver microsomes.

(A) Chromatogram from an incubation mixture containing [^{3}H]morphine and N-acetylcysteine in the absence of NADPH.

(B) Chromatogram from a reaction mixture containing [^{3}H]morphine, N-acetylcysteine and NADPH.


40,459
A
Absorbance at 282 nm
1600
1200
800
400
Radioactivity (cpm)
4
8
12
16
20
24
Time (min)


45,427
B
Absorbance at 282 nm
1600
1200
800
400
Radioactivity (cpm)
4
8
12
16
20
24
Time (min)

The formation of a [^{3}H]morphine metabolite which co-eluted with either of the authentic adducts in two different solvent systems clearly demonstrated that human liver microsomes were also capable of metabolizing morphine to a reactive intermediate.

Although very little of morphine was metabolized, all samples were capable of transforming morphine to the morphine-GSH adduct (or morphine-N-AcCys adduct). Additionally, in some of the samples examined, a double radioactive peak eluted in the morphine-GSH (or morphine-NAcCys) window, possibly suggesting metabolism to the 10β-morphine adduct (Figure 6.1B).

Table 6.2 displays results from incubations of [^{3}H]morphine and N-acetylcysteine with human liver microsomes. Interestingly, the amount of morphine adduct was at least equal to but usually greater than the normorphine formed. This was in sharp contrast to morphine metabolism by liver microsomes from untreated rats as discussed below. Table 6.2 includes the total cytochrome P-450 content of each sample. The amount of morphine metabolized did not appear to correlate with the total cytochrome P-450 content as has been previously demonstrated with other xenobiotics (Nelson *et al.*, 1971). Whether a specific cytochrome P-450 isozyme is involved remains to be determined.

Table 6.2

Morphine Metabolism by Human Liver Microsomes

Sample	Cytochrome P-450 nmol/mg protein	---- % Total Morphine Recovered ---- Mor-NAcCys	Normorphine
1	0.54	0.7	0.4
2	0.34	0.5	0.4
3	0.17	0.3	0.3
4	0.25	0.7	0.4
5	0.27	0.3	0.3

Human liver microsomes are generally known to have lower oxidative activities than liver microsomes from other species, and thus, the low metabolism was not unexpected. Analysis of the cytochrome P-450 content of 7 samples incubated with either GSH or N-acetylcysteine revealed a range from 0.17 to 0.78 nmol/mg protein with an average of 0.44 nmol/mg protein (Table 6.3).

Table 6.3

Cytochrome P-450 Levels in Human Liver Microsomes

Sample No.	Cytochrome P-450 (nmol/mg protein)
1	0.34
2	0.17
3	0.54
4	0.61
5	0.27
6	0.25
7	0.78

These values fell within the range of cytochrome P-450 values reported by von Bahr *et al.* (1980) and were actually somewhat higher than those reported by Souhaili-El Amri *et al.* (1986).

The results from studies of comparative adduct formation (Table 6.4) were fully consistent with differences in metabolism between rats and humans, and humans and rabbits reported in the literature (Boobis and Davis, 1984; Souhaili-El Amri *et al.*, 1986). This comparison suggested that the relatively low metabolism was not peculiar to these human liver microsomes and that cytochrome P-450 activity was consistent with that of other human samples.

Comparative adduct formation in three animal species

Morphine metabolism by human, rat and rabbit liver microsomes was compared. In addition, both untreated and PB-pretreated rats were used as the source of liver microsomes for metabolic studies. In these studies, [^{3}H]morphine (1.0 mM) was incubated with N-acetylcysteine (7.5 mM) for 15 min. After separation of morphine and its metabolites from the microsomes, and further purification as described in Materials and Methods, the samples were analyzed by HPLC chromatography with acetonitrile/water (10:90, v/v) containing 0.5% ammonium acetate (pH 5.5) as the mobile phase.

The formation of the morphine N-AcCys adduct and normorphine in the different microsomal systems is shown in Table 6.4.

Table 6.4

Comparison of in vitro Morphine Metabolism by Rat, Rabbit and Human Liver Microsomes

Microsomes	% Total Morphine Recovered M-NAC	NM
Rat (PB-pretreated)	6.2	3.6
Rat (Untreated)	1.2	6.3
Rabbit	1.2	2.0
Human 1	0.7	0.4
Human 2	0.3	0.3

Microsomes (2 mg/ml) were incubated in a shaking water bath for 15 min at 37° C and analyzed as described in Materials and Methods. NM = normorphine; M-NAC = morphine-N-AcCys.

As expected, morphine was metabolized the least in incubations catalyzed by human liver microsomes. Given the low metabolism, normal variation and environmental factors, the differences in metabolite formation between the two human samples did not seem significant. Moreover, when compared to the total percent of morphine metabolized by the rat, the percent metabolized by human liver microsomes was consistent with reported values of xenobiotic metabolism by human liver microsomes which were markedly lower than those of the rat (Boobis and Davis, 1984).

The formation of normorphine by untreated rat liver microsomes was over five times greater than that of morphine-N-AcCys. In contrast, the formation of this morphine adduct by human liver microsomes was at least equal if not greater than normorphine formation (Table 6.2). The differences in the relative formation of the two metabolites is exceedingly interesting since literature values of cytochrome P-450

content in humans and untreated rats are very similar, approximately 0.6 nmol/mg protein (Boobis and Davis, 1984). This disparity is most likely due to differences in the composition of cytochrome P-450 isozymes. Furthermore, marked variations in the substrate specificity of a single form of cytochrome P-450 in the two species may also be responsible for observed differences in activity between them (Boobis and Davis, 1984).

Comparison of results from incubations of liver microsomes from untreated and PB-pretreated rats (Table 6.4) underscored the changes in the cytochrome P-450 profile following PB induction. In incubations of liver microsomes from untreated rats, the percent recovered as normorphine was nearly five times that of morphine-N-AcCys. Thus, in untreated rat liver microsomes, the specific cytochrome P-450 isozyme catalyzing the formation of the reactive morphine species may be a small fraction of the total cytochrome P-450 content. Consequently, the amount metabolized to morphine-N-AcCys is relatively small and N-demethylation is the predominant pathway.

PB-pretreatment increased the yield of morphine-N-AcCys from 1.2 to 6.2% but decreased that of normorphine. Induction with PB has been shown to increase specific forms of cytochrome P-450 from 2% to 57% of the total content (Ryan *et al.*, 1982) while repressing others. Clearly, the form of cytochrome P-450 which metabolized morphine to the reactive intermediate appeared to be sizeably increased. Under these circumstances, N-demethylation was much less of a factor in morphine metabolism and the fraction metabolized to normorphine decreased by 50%. PB induction may repress the form of cytochrome P-450 which N-demethylates morphine. The

findings in isolated hepatocytes of untreated and PB-pretreated rats that the normorphine pathway became significant only at high morphine concentrations (2.0 mM) when glucuronidation and GSH conjugation were overwhelmed, and that in hepatocytes of PB-pretreated rats no normorphine was formed at low morphine concentrations (0.2 mM), could be interpreted to suggest that a different cytochrome P-450 isozyme predominates at higher morphine concentrations.

The fraction of morphine metabolized to the morphine-N-AcCys adduct by rabbit liver microsomes was comparable to that metabolized by untreated rat liver microsomes. Metabolism to normorphine, however, was not nearly as significant as in incubations of rat liver microsomes (2.0% compared to 6.3%). Although metabolism to both normorphine and morphine-N-AcCys was greater than that by human liver microsomes, metabolism by rabbit liver microsomes seemed to better approximate morphine metabolism by the human liver samples. Interestingly, as with human liver microsomal incubations, HPLC chromatograms from incubations of rabbit liver microsomes displayed a double peak in the morphine-N-AcCys window (9-10 min). The presence of this doublet again suggested the possibility that both the 10α- and β-congeners of morphine-N-AcCys are formed.

7.0 BILIARY EXCRETION OF MORPHINE-GSH IN UNTREATED RATS

Introduction

The in vitro formation of 10α-morphine-GSH adduct by rat, rabbit and human liver microsomes demonstrated the potential for metabolism to this highly reactive species. However, the predominant metabolite of morphine in vivo is M-3-G. In the presence of a functional UDP-glucuronyltransferase, the enzyme which catalyzes morphine glucuronidation, the extent, if any, of morphine-GSH formation was unknown. This study was carried out to determine if the morphine-GSH adduct was formed in rats in vivo. If so, it was expected, analogously to other GSH conjugates of xenobiotics, to be excreted in the bile. Therefore, the biliary excretion of morphine metabolites following administration of a high dose of morphine (100 mg/kg) was examined.

The biliary excretion of M-3-G into bile has been well documented in studies by Peterson and Fujimoto (1973), Smith *et al.* (1973), Walsh and Levine (1975), and Berman and Fujimoto (1986). After low doses of morphine, M-3-G was found to be extensively excreted into bile. It was estimated that after treatment with a low dose of morphine (5 mg/kg), over 50% of the dose was excreted into bile as M-3-G during the course of a three hour period (Walsh and Levine, 1975). At higher doses (60 mg/kg), however, the recovery of M-3-G in bile was substantially decreased (Berman and Fujimoto, 1986). In the isolated perfused liver (Berman and Fujimoto, 1986), it was shown that at high [^{14}C]morphine doses the biliary recovery of [^{14}C]M-3-G decreased. At the same time, the amount of [^{14}C]morphine in the perfusate increased

while that of [^{14}C]M-3-G in the perfusate was not affected. These findings indicated that the glucuronidation pathway was essentially saturable.

Glutathione S-conjugates of xenobiotics have also been shown to be excreted into bile (Barnhart and Combes, 1978; Degen and Neumann, 1978; Wahllander and Sies, 1979 and Monks *et al.*, 1982). It was expected, then, that if formed in vivo, morphine-GSH would be similarly excreted into bile. Although the full extent of morphine-GSH formation would not be determined from analysis of biliary metabolites, the occurence of in vivo metabolism of morphine to morphine-GSH under basal conditions could be established in the rat.

Materials and Methods

Male Sprague-Dawley rats, weighing between 240-280 g, were used in this study. Each rat was anesthesized with ether, and a midline abdominal incision was made. After exposing the common bile duct, a polyethylene cannula (PE-10) was inserted and tied in place. The incision was then sutured, and the animal was placed in a restraining cage. The rats were allowed to recover for 45 min after the surgical procedure.

Rats were then injected with [^{3}H]morphine sulfate (100 mg/kg, i.p., 5 μCi). Rats were immediately placed in a chamber continuously supplied with 95%O_2/5%CO_2. The O_2 atmosphere was found to prevent death from the respiratory depression induced by the high dose of morphine. Bile samples were collected on ice in 2 ml Eppendorf tubes at 10 min intervals over 30 min. An additional sample was collected from 30-60 min. Preliminary experiments indicated that this collection period would document the excretion pattern of the morphine-GSH adduct as well as record initial M-3-G excretion and the subsequent decrease in the rates of biliary excretion of both metabolites. After collection, bile samples were immediately frozen in a dry ice/ethanol bath and protected from light by aluminum foil until HPLC analysis.

Before HPLC chromatography, the volume of each sample was measured and 120 μl was diluted to 1.0 ml with acetonitrile/H_2O (1:99, v/v) containing 40 mM potassium acetate adjusted to pH 4.7 with acetic acid. A 0.5ml aliquot of this sample was then chromatographed on an Altex reverse phase C_{18} Ultrasphere ODS HPLC

column (5 μ, 5 mm i.d. x 250 mm) with the above buffer as the mobile phase at a flow rate of 1 ml/min. A solution of 12% bile was found to be the highest concentration of bile in which the separation and chromatographic properties of morphine-GSH and M-3-G were retained. Under these conditions, authentic M-3-G and morphine-GSH added to bile collected prior to morphine treatment eluted at 10-12 and 15 min, respectively.

Fractions of the HPLC effluent were collected at 1 ml intervals. After the addition of Aquasol (10 ml), radioactivity was measured in a Beckman Liquid Scintillation counter.

Results and Discussion

HPLC chromatograms of rat bile prior to morphine administration and 20 min after [^{3}H]morphine treatment are shown in Figures 7.1A and 7.1B, respectively. As expected, the predominant metabolite is [^{3}H]M-3-G (10-12 min). A small amount of the [^{3}H]morphine-GSH adduct (15 min) was also detected in bile thereby documenting the in vivo formation of the GSH conjugate. Additionally, a [^{3}H]morphine metabolite, Metabolite A, which was more polar than M-3-G eluted at 8 min (Figure 7.1b).

Figures 7.2 and 7.3 display the cumulative radioactivity of each metabolite at different collection times in two separate studies. Within 10 min after morphine injection, both M-3-G and morphine-GSH were detected in bile. The rate of biliary excretion of M-3-G quite clearly declined by 30 min. The rate of excretion of morphine-GSH also appeared to decrease by 20 to 30 min.

The distribution of [^{3}H]morphine metabolites excreted into bile is shown in Table 7.1. Interestingly, the percentage of the unknown metabolite A increased with time while the percentage of M-3-G and morphine-GSH both decreased.

Figure 7.1 HPLC profiles of rat bile before and after [^{3}H]morphine treatment.

(A) HPLC chromatogram of control rat bile.

(B) HPLC chromatogram of rat bile 10-20 min after morphine treatment (100 mg/kg, i.p.).

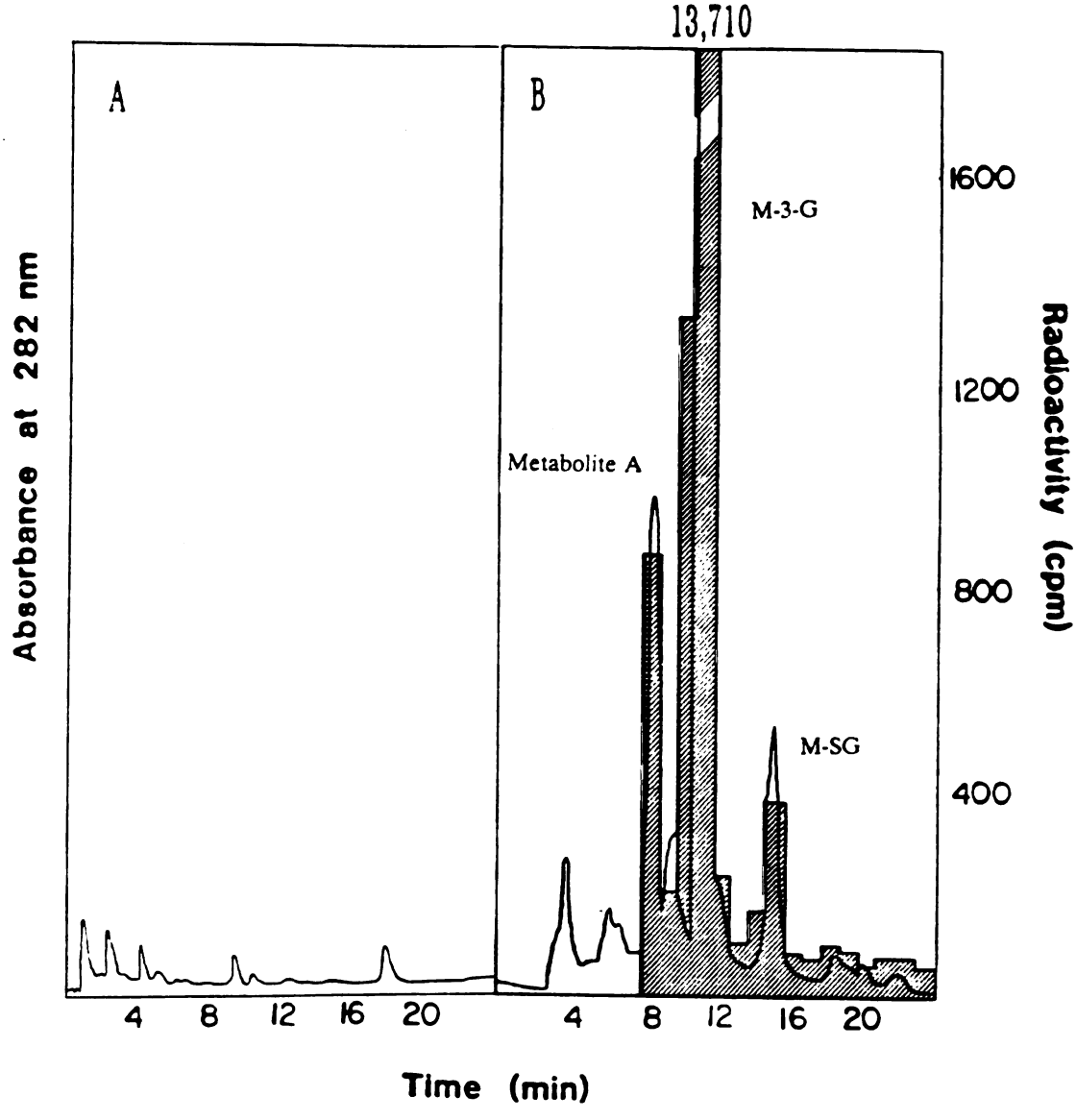

13,710
A
B
M-3-G
Metabolite A
M-SG
1600
1200
800
400
Absorbance at 282 nm
Radioactivity (cpm)
4
8
12
16
20
4
8
12
16
20
Time (min)

Table 7.1

Distribution of [^{3}H]Morphine Metabolites Excreted into Rat Bile

Time Interval After Morphine min	Percentage of Total Metabolites		
	Metabolite A	M-3-G	Morphine-GSH
0-10	1	95	5
10-20	5	92	3
20-30	9	89	2
30-60	12	87	1

Calculations based on [^{3}H] radioactivity of morphine metabolites collected during HPLC analysis. Percentages based on results from experiments with 2 rats.

The nature of this third metabolite (Metabolite A) is unknown. Formation of M-6-G was regarded as unlikely since Rane *et al.* (1985) reported that rat liver microsomes were capable of glucuronidating morphine only at position 3. Yeh *et al.* (1979) however, did report M-6-G as a metabolite in rat urine. On the other hand, MES has never been demonstrated as a metabolite in the rat.

Figures 7.2 and 7.3 (as well as Table 7.1) demonstrate that the time course of the appearance of metabolite A in bile was different from that of M-3-G or morphine-GSH as very little of this metabolite was excreted during the first 10 min after morphine administration. This pattern might be due to slower transport into the bile, slower rate of formation of the metabolite, or perhaps formation of the metabolite in the bile. That this metabolite was not detected in microsomal incubations or in incubations of isolated hepatocytes is indicative of the latter possibility.

Figure 7.2 Cumulative biliary excretion of [^{3}H]morphine metabolites following morphine administration. First study.

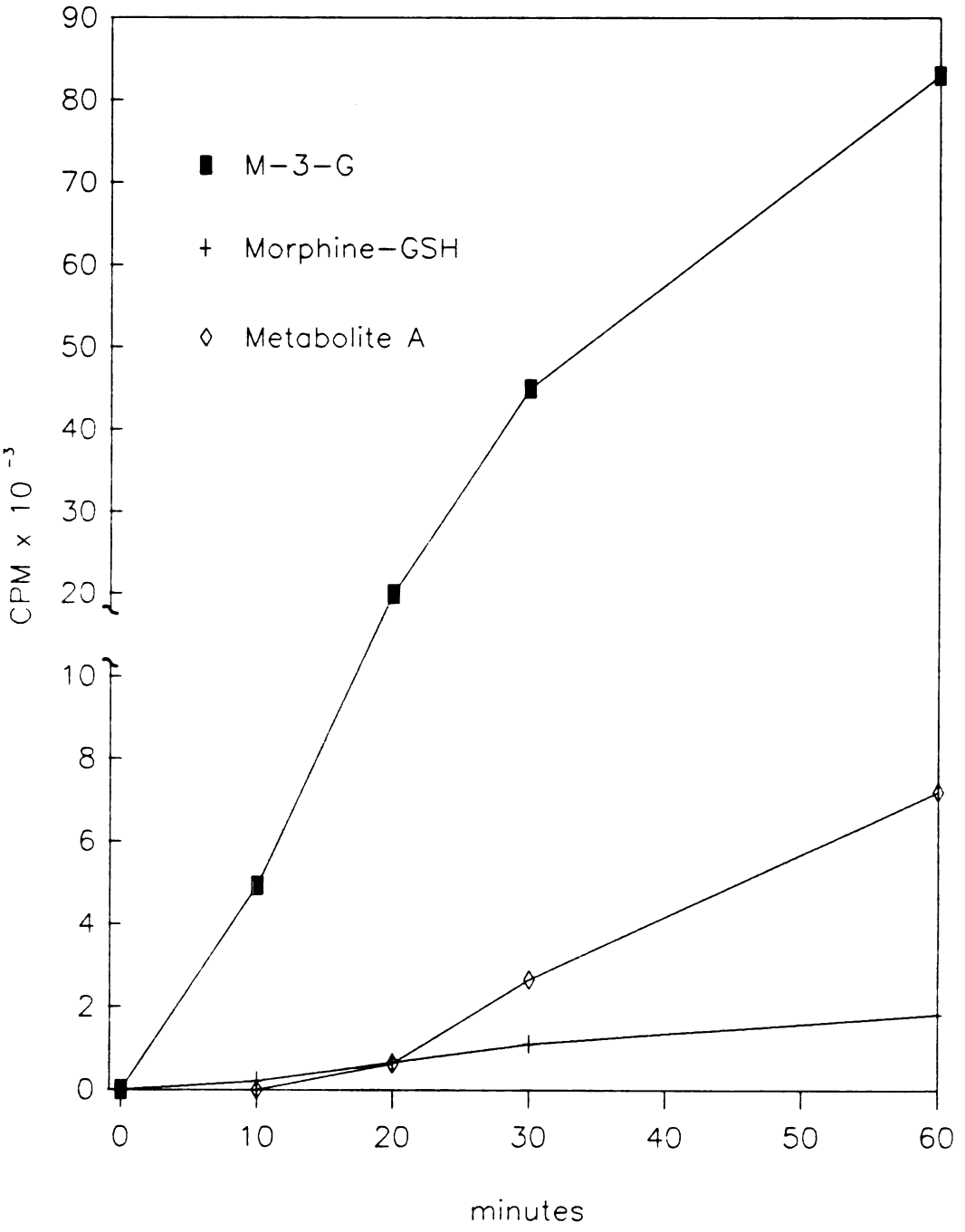
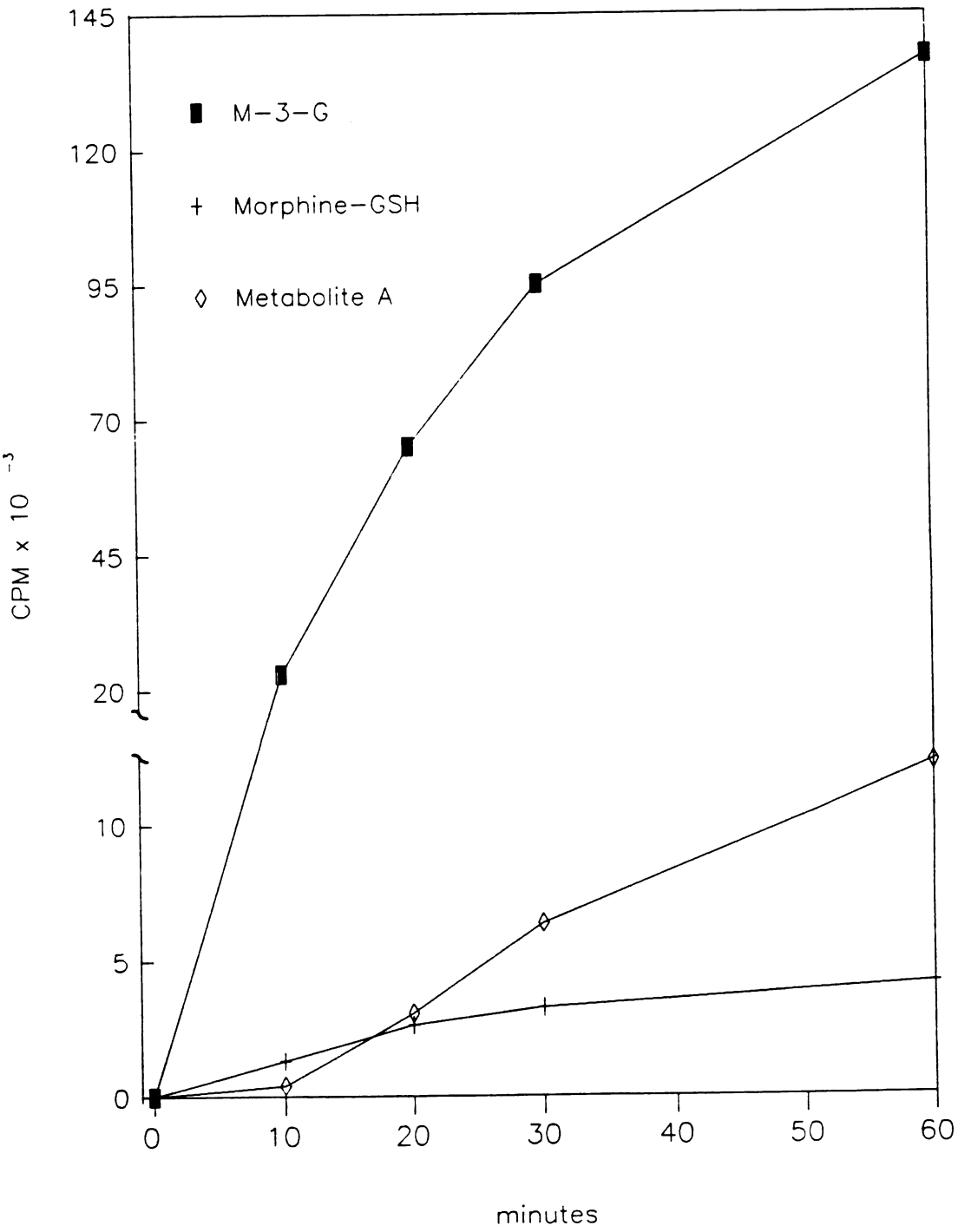

90
80
70
60
50
40
30
20
10
8
6
4
2
0
CPM x 10 -3
M-3-G
Morphine-GSH
Metabolite A
0
10
20
30
40
50
60
minutes

Figure 7.3 Cumulative biliary excretion of [^{3}H]morphine metabolites following morphine administration. Second study.


145
120
95
70
45
20
10
5
0
M–3–G
Morphine–GSH
Metabolite A
CPM x 10 -3
0
10
20
30
40
50
60
minutes

Hydrolysis of a morphine-GSH adduct would be one possibility since τ-glutamyltransferase is present both in the lumen of bile duct cells as well as in the bile canalicular membranes, and intrabiliary hydrolysis of GSH has been established (Ballatori *et al.*, 1986).

Although morphinone-GSH formation was reported in incubations with morphine in freshly isolated rat hepatocytes (Nagamatsu *et al.*, 1986) and although this GSH conjugate (with a MW nearly identical to morphine-GSH), if formed, should be expected to be secreted into bile, none was found. The HPLC characteristics of morphinone-GSH ruled out the possibility that Metabolite A was this conjugate. Similarly, only three [^{3}H]morphine metabolites were observed by HPLC analysis. In addition, monitoring a 20 min time period after [^{3}H]morphine-GSH elution revealed no additional radioactive peaks. These results supported the findings (see next chapter) that no significant morphinone-GSH was detected in freshly isolated rat hepatocytes.

The small amount of morphine-GSH secreted in the bile during the 60 min time frame suggested that although the metabolite was formed, this was a minor pathway in the uninduced rat. Actual comparisons with M-3-G formation, however, were not possible since this study examined only biliary excretion. In liver perfusion studies, Berman and Fujimoto (1986) showed that under conditions of increased M-3-G formation (treatment with trans-stilbene oxide) the morphine glucuronide excretion into the bile decreased while secretion into the caval perfusate increased.

The relative extent to which morphine-GSH is secreted into bile (canalicular excretion) and into plasma (sinusoidal excretion) at high doses of morphine is

unknown. Akerboom *et al.* (1982) have shown competition between GSSG and GSH conjugates of 1-chloro-2,4-dinitrobenzene for transport into bile. Although morphine is not known to increase biliary GSSG output, increased GSH secretion into bile as well as caval perfusate of the isolated perfused liver has been found (Correia, Hill and Burk, unpublished observations). Whether the morphine-GSH adduct utilizes this same transport system and whether competition for transport with GSH interferes with morphine-GSH secretion is not known.

8.0 MORPHINE METABOLISM AND MORPHINE-INDUCED GSH DEPLETION IN FRESHLY ISOLATED RAT HEPATOCYTES

Introduction

While studies with rat liver microsomes demonstrated that the reactive morphine intermediate was a significant metabolite in vitro, studies of the biliary metabolites of morphine suggested that morphine-GSH was of minor importance to morphine metabolism in vivo. The conflicting results may have been due to a non-functional UDP-glucuronyltransferase in liver microsomes. However, the rat bile fistula model does not permit simultaneous assessment of sinusoidal and biliary excretion of morphine-GSH thereby preventing a complete evaluation of in vivo metabolism of morphine. The overall metabolism of morphine and morphine-induced GSH depletion were therefore investigated in freshly isolated rat hepatocytes. The use of isolated rat hepatocytes enabled us to simultaneously monitor hepatic morphine-GSH formation and GSH depletion at various time intervals during the incubation. A further advantage of the system was that the concentrations of both enzymes involved in morphine metabolism were similar to those in the intact animal since the hepatocytes were used while fresh over only short periods of time. Additionally, central nervous system involvement in GSH depletion and hepatic damage could be excluded from the study. The elimination of this factor was essential in revealing the importance of the hepatic metabolism of morphine to morphine-induced hepatotoxicity.

Morphine metabolism and GSH depletion were examined in hepatocytes of both untreated and PB-pretreated rats. Specifically, the relative importance of the morphine-GSH adduct to overall morphine metabolism and the relationship between

formation of this morphine adduct to GSH depletion were investigated at high (2.0 mM) and low (0.2 mM) morphine concentrations. The relationship between GSH depletion, covalent binding and GSH-adduct formation was also examined.

Formation of the morphine-GSH adduct and GSH depletion were also explored in the presence and absence of the GSH precursors cysteine and methionine. It was previously reported that the presence of cysteine and methionine in the incubation medium prevented gradual GSH depletion in hepatocytes over time (Thor *et al.*, 1979; Aw *et al.*, 1984). Additionally, Thor *et al.* (1978) reported that in hepatocyte incubations with bromobenzene, GSH levels did not decrease below 30% basal level when incubated in a medium containing these amino acids. Thus, it was important to determine what effect the presence of these amino acids would have on morphine-GSH formation and GSH depletion.

To further elucidate the mechanism of morphine-induced GSH depletion, the structural characteristics necessary for the acute GSH decline were examined by monitoring the GSH depletion provoked by various morphine analogs. In addition, the involvement of cytochrome P-450 was examined by investigating the requirement for O_2 in GSH depletion and by coincubating the hepatocytes with the cytochrome P-450 inhibitor benzimidazole.

Materials and Methods

Preparation of hepatocytes

Solutions: All buffers were gassed with 95%0_2, 5% CO_2 and warmed to 37° C. Before perfusion, the pH was adjusted to 7.4. Four solutions were used during the perfusion and incubation in a modification of the method of Moldeus *et al.* (1978). Solution A, used as the initial perfusion medium, was a modified Hank's solution [NaCl (8.0 g), KCl (0.4 g), $MgSO_4 \cdot 7H_20$ (0.2 g), Na_2HPO_4 (0.048 g), KH_2PO_4 (0.06 g), $NaCO_3$ (2.19 g) in a volume of 1 liter] containing 2% albumin (BSA, fraction V, Sigma Chemical Co.) and 0.5 mM EGTA. Following initial clearing, the liver was perfused with Solution B, the same modified Hank's buffer containing 0.12% collagenase (Type I, Sigma Chemical Company) and 4mM Ca^{2+}. After cell disruption was observed, the liver was immersed in Solution C, a Krebs-Henseleit buffer [NaCl (6.9 g), KCl (0.36 g), KH_2PO_4 (0.13 g), $MgSO_4 \cdot 7H_20$ (0.295 g), $CaCl_2 \cdot 2H_20$ (0.426 g), $NaHCO_3$ (2.0 g) in a volume of 1 liter] containing 2% albumin and 12.5 mM HEPES buffer. Solution D, used as the incubation buffer, consisted of the same Krebs-Henseleit buffer supplemented with 2% albumin, 12.5 mM HEPES, and Waymouth medium. The Waymouth medium was included in the solution both with and without cysteine and methionine. When cysteine and methionine were included, the concentrations of these amino acids were 0.5 mM and 1.0 mM, respectively.

Perfusion Procedure: Fed male Sprague-Dawley rats (220-270 g) were used throughout these studies. The rats were anesthetized with ether and the liver was perfused in situ. After opening the peritoneal cavity, 500 units (in 0.1 ml) heparin was

injected into the lower vena cava. The portal vein was then cannulated and the liver slowly perfused with Solution A (250 ml). Cannulation of the superior vena cava afforded outflow from the liver. Once the two cannulas were secured, the inferior vena cava was tied off thus forcing circulation through the liver alone. The flow rate of the perfusion pump was slowly increased to 15 ml/min. Blood cleared immediately and after five to seven min, perfusion with 200 ml of Solution B (collagenase) was initiated and continued for 7-12 min until slight evidence of cell break-up was visible.

At this point, the liver was immediately removed from the rat and immersed in a 250 ml beaker containing Solution C (100 ml). The liver capsule was cut open and the cells were dispersed by gentle prodding. The suspension was then filtered into a 125 ml Ehrlenmeyer flask through eight layers of cotton gauze. The hepatocyte pellet was formed after standing for 2-3 min at room temperature. The supernatant was aspirated and the cells were then resuspended in Solution C and allowed to repellet.

The hepatocyte pellet was then resuspended in 15 ml of Solution D. Cells were counted using a hemocytometer and a light microscope. The average yield of hepatocytes was 300 x 10^6. Before in vitro incubations, cell viability was routinely assessed by the trypan blue exclusion test (Moldeus *et al.*, 1978). Only cells in which trypan blue exclusion was over 90% (92-98%) were used.

Hepatocyte Incubations

Hepatocyte incubations (final volume, 2.5 ml unless otherwise noted) contained 2 x 10^6 cells/ml, [^{3}H] morphine (0.2 or 2.0 mM morphine,) and Solution D. Immediately after hepatocytes were isolated and counted, incubations were initiated with the addition of hepatocytes to the reaction vial. Samples were incubated for 15 or 30 min at 37° C in a shaking water bath. Due to the brevity of the experiments, 0_2 was not bubbled into the reaction flasks.

Reactions were terminated by the addition of 25% trichloroacetic acid (0.625 ml/2.5 ml incubation vial). The samples were transferred to conical test tubes, vortexed and allowed to sit on ice for 30 min. The mixture was sonicated to disrupt cells and then sedimented by centrifugation at 1500 rpm for 10 min. GSH was routinely measured by the method of Ellman (1959).

In incubations involving hepatocytes from PB-pretreated rats, rats were pretreated as before with PB (80 mg/kg/day i.p.) for 3 days.

When structural analogs of morphine were tested, compounds were first dissolved in Solution D, and the pH was adjusted before the addition of hepatocytes.

In experiments involving benzimidazole, hepatocytes were preincubated with benzimidazole (2 or 5 mM) for 5 min; reactions were initiated with the addition of morphine. When the requirement for 0_2 was examined, half of the reaction vials were incubated under a constant stream of N_2.

In experiments where irreversible binding of the drug to the cellular protein was analyzed, the incubations were terminated in the normal manner. After centrifugation at 1500 rpm for 10 min, the supernatant was removed and the resulting pellet

was processed by the method of Rollins and Buckpitt (1979).

HPLC Analysis of Cellular Metabolism of Morphine

A fraction of the acidified supernatant was used for HPLC analysis. Sixty μl of the supernatant was diluted with 250 μl of buffer (2.5% acetonitrile, 40 mM KOAc at pH 4.7) or a combination of buffer with standards. 250 μl of this solution was then chromatographed on an analytical Altex reverse phase C_{18} Ultrasphere ODS column with the above described buffer as the mobile phase at a flow rate of 1 ml/min.

The presence of metabolites M-3-G, morphine-GSH adduct and normorphine were confirmed by radioactivity and authentic standards. M-3-G was purchased from Sigma Chemical Company. Normorphine was a gift of Dr. Sidney Nelson, University of Washington. The morphine-GSH adduct was prepared and isolated as previously reported for the morphine-N-AcCys adduct (Krowech *et al.*, 1986). The retention time of this morphine metabolite was identical to that of morphine-GSH adduct which was previously identified by FABMS and [^{1}H]NMR. Additionally, the retention time was identical to the morphine-GSH adduct when chromatographed in a solvent system consisting of 20% methanol, 25 mM tetramethylammonium hydroxide (TMA) at pH 6.1. It was therefore judged to be authentic morphine-GSH adduct. Morphinone and the morphinone-GSH adduct were synthesized by Dr. Patricia Caldera-Munoz by the methods of Rapoport *et al.* (1957) and Nagamatsu *et al.* (1982), respectively.

Metabolites were collected in 0.5 ml increments, dissolved in 10 ml Aquasol, and counted in a Beckman Liquid Scintillation counter.

Recovery of [^{3}H]Morphine

The recovery of [^{3}H]morphine was measured as follows: After the normal procedures (trichloroacetic acid addition, sonication and centrifugation) for recovering morphine metabolites were carried out, hepatocyte pellets were resuspended in 2 ml Krebs-Henseleit buffer to which 2 ml MeOH were added. This solution was sonicated and centrifuged for 10 min at 1500 rpm. An aliquot of the resulting supernatant was analyzed by HPLC as described above. The pellet was washed a second time with this mixture and then twice with ether. After each washing, an aliquot was removed to count radioactivity. Finally, radioactivity in the remaining pellet was counted.

Results from these studies indicated that [^{3}H]morphine metabolites were only partially recovered by the normal procedures used to extract the metabolites. The MeOH/Krebs-Henseleit buffer wash yielded significant increases in metabolites. The increases in recovery of M-3-G and morphine-GSH were identical (37%). The increase in normorphine was slightly higher (41%). Subsequent washes yielded only minimal radioactivity. For purposes of stoichiometric comparisons of metabolites to GSH depletion, recovery adjusted values for all three metabolites are used throughout.

Results and Discussion

GSH depletion in freshly isolated rat hepatocytes

Hepatocyte incubations with morphine (2 mM) resulted in a profound depletion of GSH. Initial GSH levels were 59.1± 15.2 nmol/10^6 cells. This GSH level, considered to be replete, was lowered to 75% of basal values during the course of a 30 min incubation. In contrast, in the presence of morphine (2 mM), GSH levels dropped by at least 75% within 15 min and reached zero after 30 min (Figure 8.1). This depletion was clearly concentration-dependent. At morphine concentrations of 0.2 mM, GSH levels dropped to approximately 60% of control values over a 30 min period.

When GSH levels were measured at 5 min intervals after morphine (2 mM), most of the depletion occurred within the first 5 min of the incubation, i.e. GSH levels were reduced to 34% of the initial values and to 12% by 10 min (Figure 8.2).

Morphine metabolism and the role of morphine-GSH

To explore the importance of morphine-GSH and the relationship of this conjugate to GSH depletion, morphine metabolism was studied at both high and low concentrations. Freshly isolated hepatocytes were incubated with [^{3}H]morphine and processed as described in Materials and Methods. Aliquots of acid treated hepatocyte supernatant were analyzed by HPLC on a C_{18} Ultrasphere ODS column with a mobile

Figure 8.1 GSH depletion in freshly isolated rat hepatocytes incubated with 0.2 mM or 2.0 mM morphine. One prototype experiment of the three conducted.

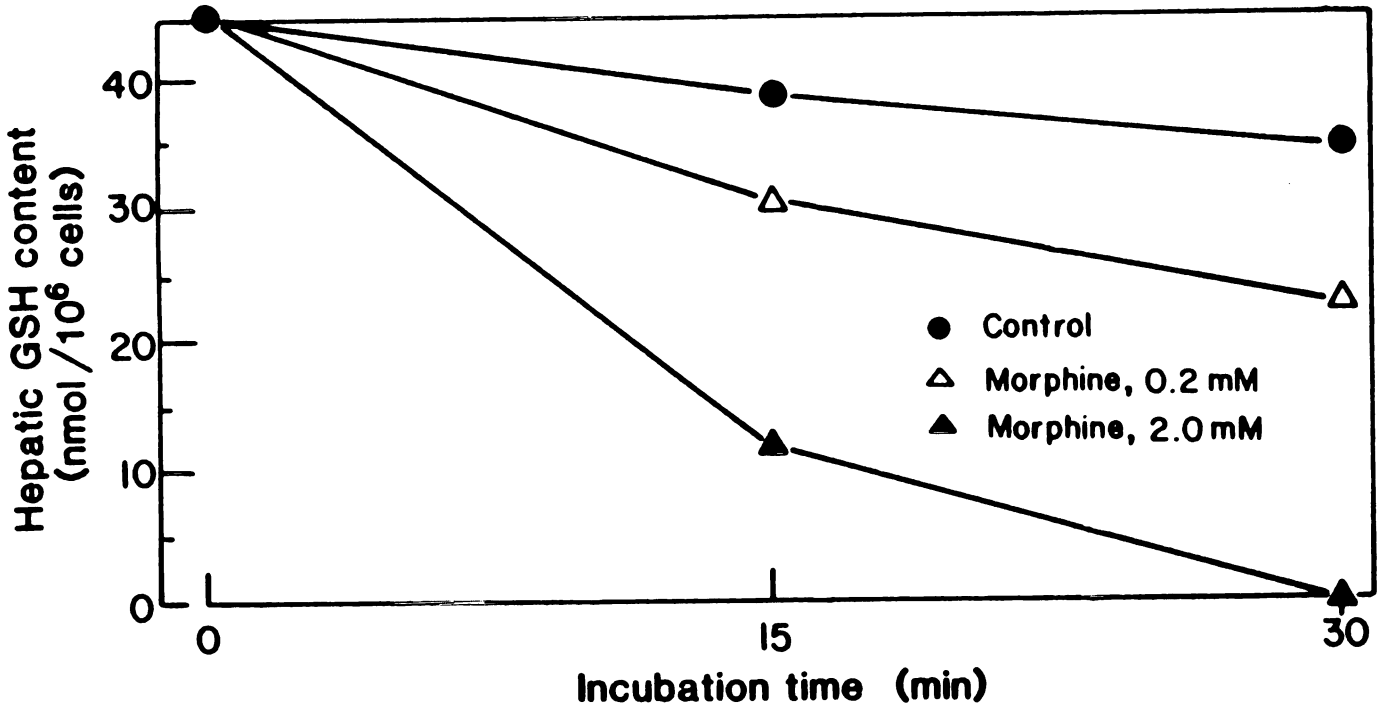
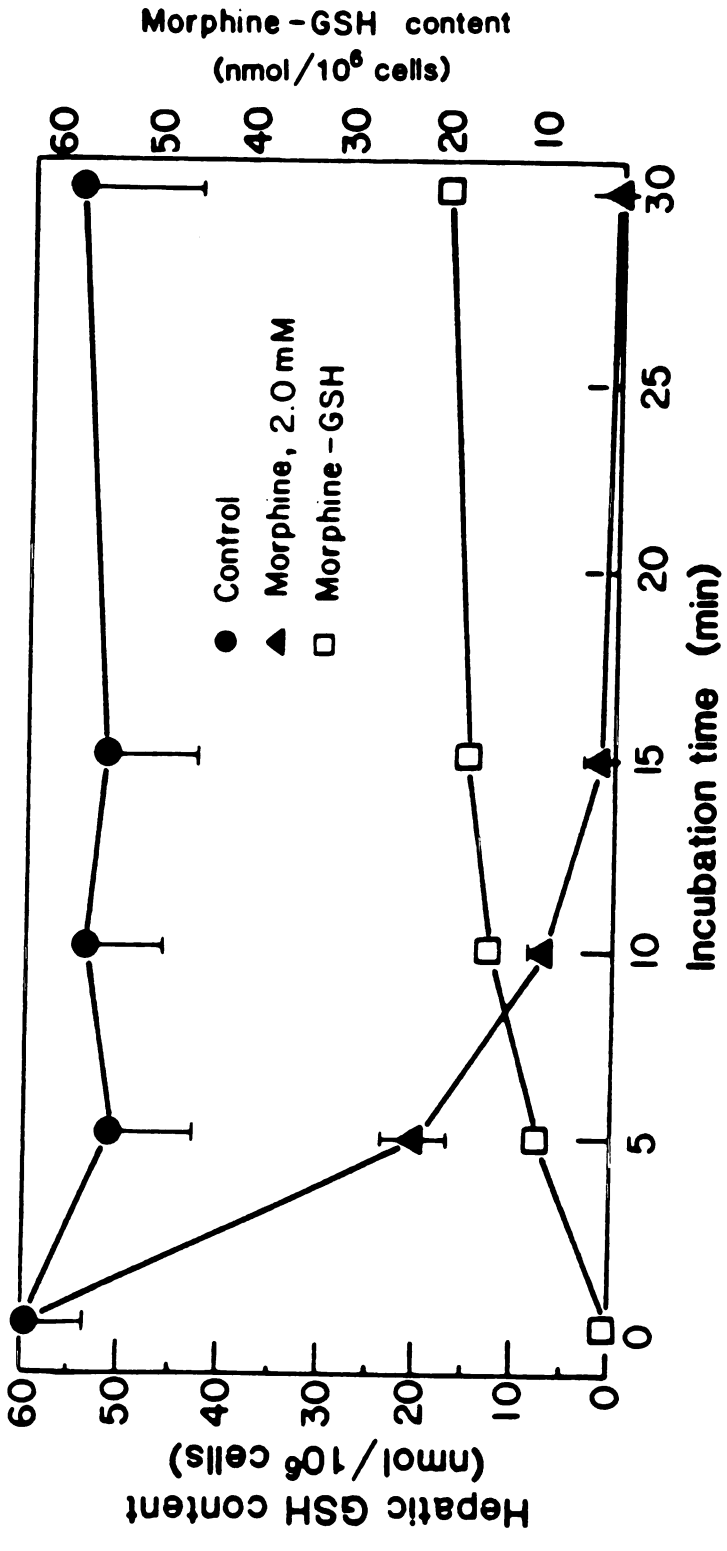

Hepatic GSH content
(nmol/10^6 cells)
40
30
20
10
0
0
15
30
Incubation time (min)
Control
Morphine, 0.2 mM
Morphine, 2.0 mM

Figure 8.2 GSH depletion and morphine-GSH formation in hepatocytes incubated with 2.0 mM morphine. Hepatocytes were supplemented with 0.5 mM cysteine and 1.0 mM methionine. GSH values are mean ± S.D. of 3 experiments.


Morphine-GSH content
(nmol/10⁶ cells)
60
50
40
30
20
10
Control
Morphine, 2.0 mM
Morphine-GSH
0
5
10
15
20
25
30
Incubation time (min)
Hepatic GSH content
(nmol/10⁶ cells)

phase of aqueous KOAc (40 mM) containing 2.5% acetonitrile at pH 4.7. The three major metabolites recovered were [^{3}H]M-3-G, [^{3}H]morphine-GSH, and [^{3}H]normorphine. At a flow rate of 1 ml/min, they eluted at approximately 7.5, 9.5, and 18 min, respectively (Figure 8.3).

At low concentrations of morphine (0.2 mM), M-3-G was clearly the major metabolite. Normorphine and the morphine-GSH adduct were consistent albeit minor metabolites (Figure 8.4A, Table 8.1).

Table 8.1

Comparison of Morphine(M) Metabolites Generated in Hepatocyte Incubations
nmol/10^6 cells/30 min

	[M]	M-3-G	Morphine-GSH	Normorphine
Untreated				
(n=3)	0.2mM	28.7± 5.6	4.1± 1.7	12.4± 2.0
(n=7)	2.0mM	37.4± 9.5	19.0± 4.6*	59.0± 16.9**
PB-pretreated				
(n=3)	0.2mM	44.6± 4.2	12.6± 1.2	0
(n=3)	2.0mM	68.2± 7.2	50.6± 9.6**	49.8± 2.5***

Significant differences from corresponding morphine (0.2 mM) treated hepatocytes: *P < 0.01, **P < 0.025, ***P < 0.005.

At high concentrations of morphine (2 mM), the metabolic profile shifted as normorphine became the predominant metabolite. While the amount of M-3-G increased marginally at high concentrations, there was a greater than 4-fold increase in normorphine and the morphine-GSH adduct. The preference for morphine glucuronidation at low concentrations appears to render all other pathways minimal. At high concentrations, glucuronidation may become overwhelmed, and N-demethylation to

Figure 8.3 HPLC profiles of hepatocyte incubations with morphine (2.0 mM).

(A) HPLC chromatogram immediately after addition of hepatocytes (t = 0).

(B) Metabolic profile after 15 min incubation.

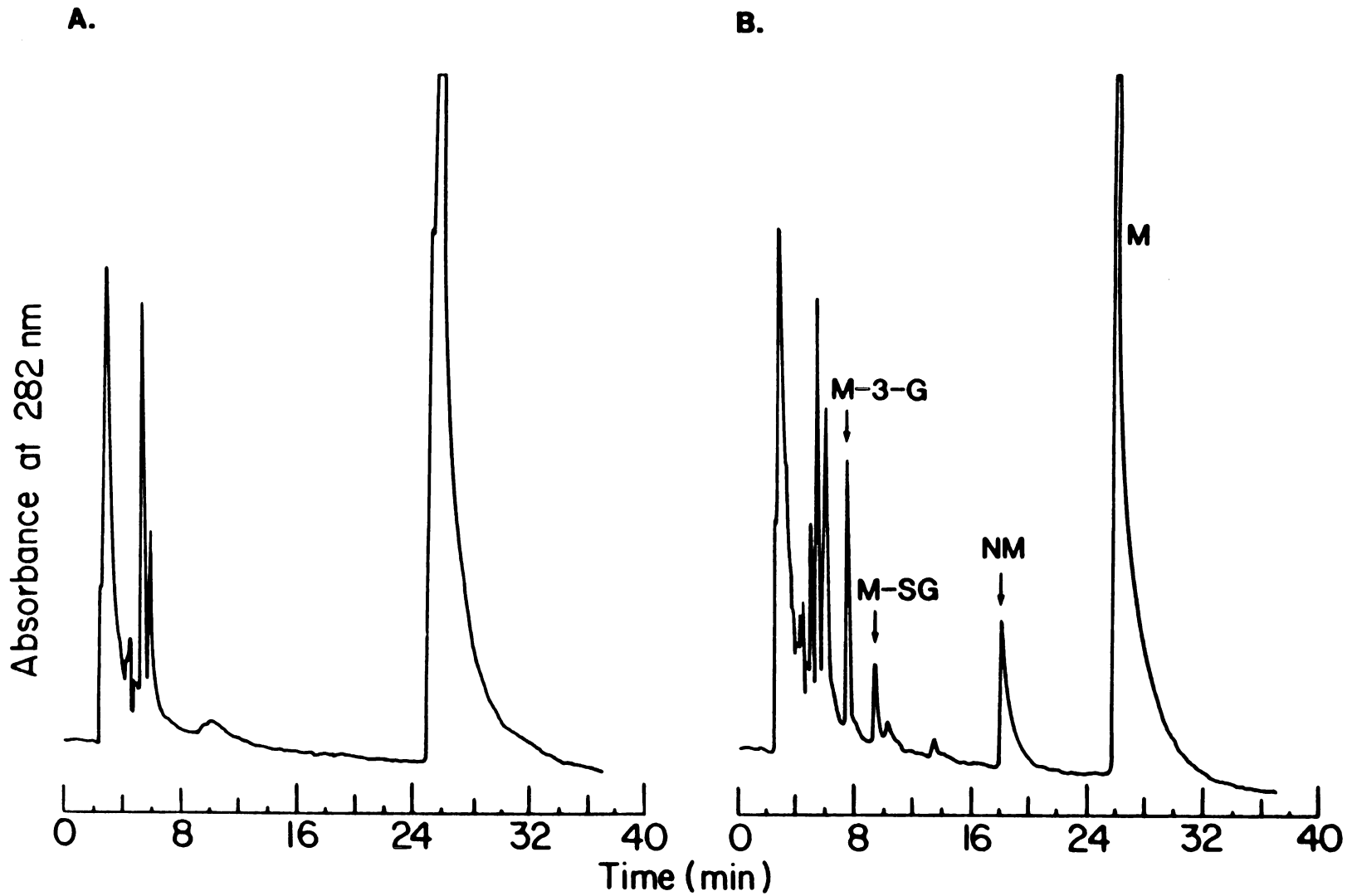
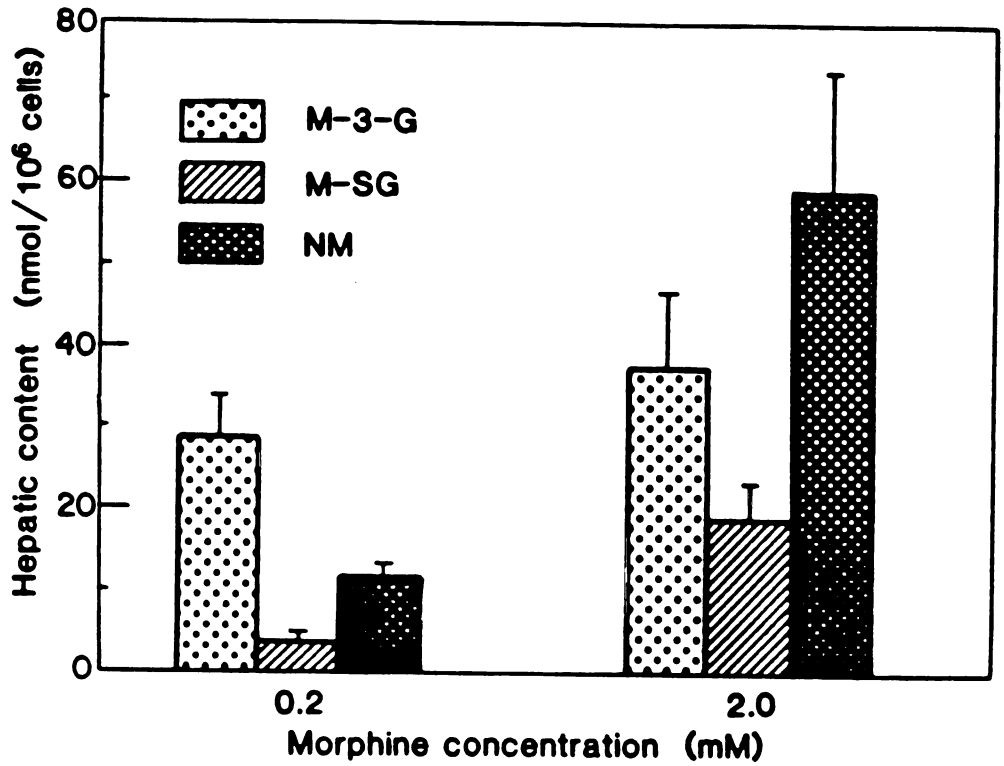
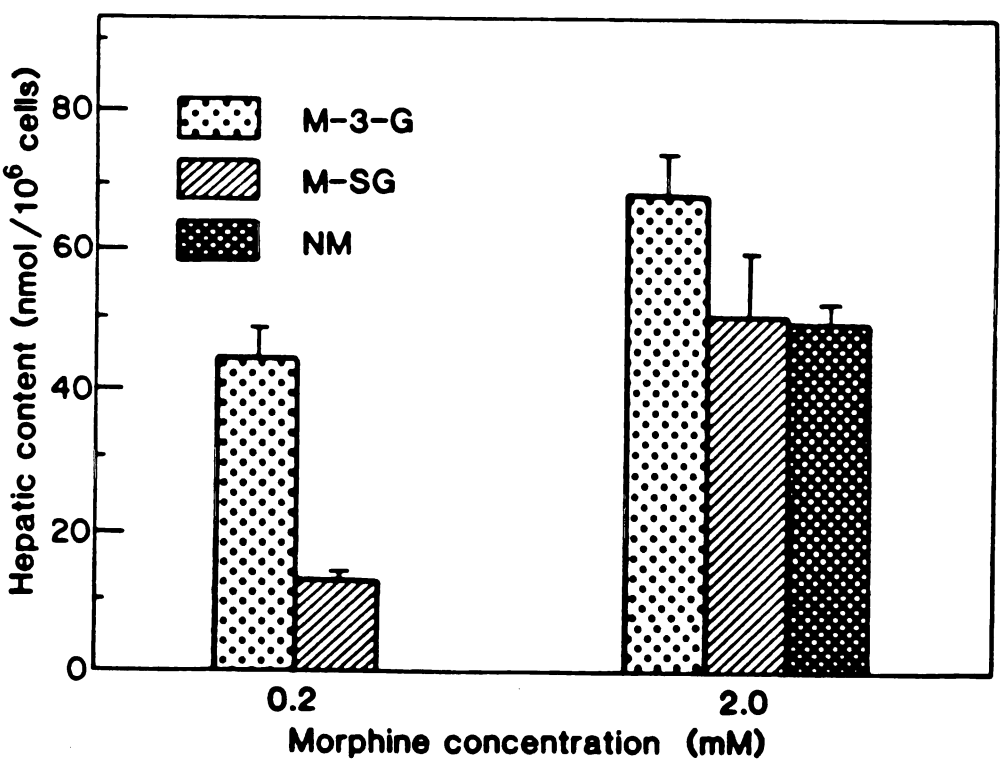

A.
B.
Absorbance at 282 nm
M-3-G
M-SG
NM
M
0
8
16
24
32
40
Time (min)

Figure 8.4A Comparison of metabolic profile at low and high concentrations of morphine in 30 min incubations. Values are means ± S.D. of at least three incubations.

Figure 8.4B Comparison of metabolic profile at low and high concentrations of morphine in 30 min incubations of hepatocytes of PB-treated rats. Values are means ± S.D. of three experiments.


80
60
40
20
0
Hepatic content (nmol/10⁶ cells)
M-3-G
M-SG
NM
0.2
2.0
Morphine concentration (mM)

A


80
60
40
20
0
Hepatic content (nmol/10⁶ cells)
M-3-G
M-SG
NM
0.2
2.0
Morphine concentration (mM)

B

normorphine and GSH conjugation, therefore, become increasingly important. The capacity for glucuronidation may be limited by the availability of UDPGA, the co-substrate for glucuronidation, as has been suggested for that of acetaminophen (Moldeus *et al.*, 1979; Price and Jollow, 1984).

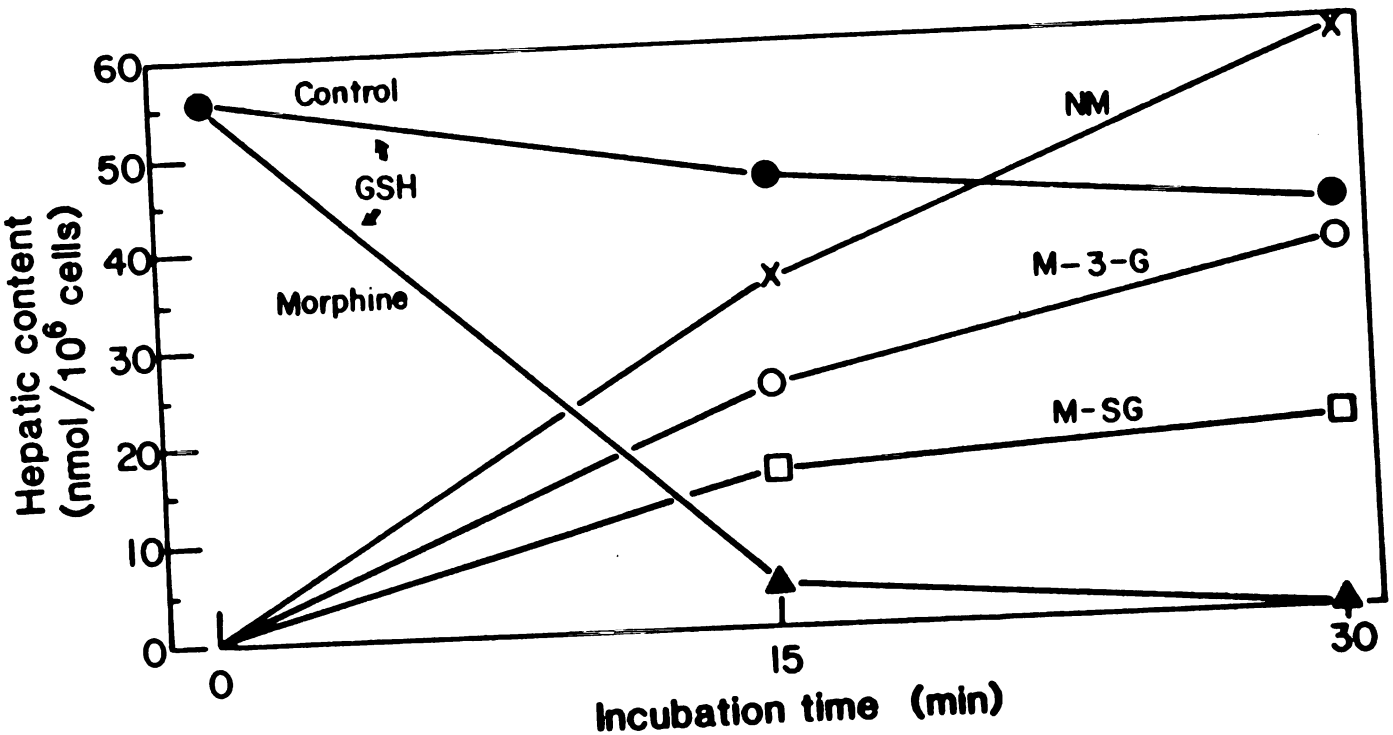
The formation of morphine metabolites at 15 and 30 min and concomitant GSH depletion are shown in Figures 8.5 and 8.6A. Morphine-GSH was found to account for approximately 50% of the GSH loss.

Morphine metabolism and GSH depletion in hepatocytes of PB-pretreated rats

In hepatocytes from PB-pretreated rats, GSH levels were 90% depleted by 15 min and almost completely exhausted 30 min after incubation with morphine (2 mM) (Figure 8.7). Basal GSH levels in PB-pretreated rat hepatocytes were approximately 60% higher than those in untreated rat hepatocytes. Since levels of GSH transferase are known to be increased 40-60% by PB (Jakoby, 1978), increased levels of GSH may be related to this induction. Whatever the reason for elevated GSH, these experiments do suggest that morphine depletes GSH to zero regardless of the initial GSH level.

As expected, hepatocytes from PB-pretreated rats metabolized far greater amounts of morphine since PB induces both enzymes involved in its metabolism (cytochrome P-450 and UDP-glucuronyltransferase). At low concentrations of morphine, PB-pretreated rat hepatocytes formed 50% more M-3-G and 200% more morphine-GSH than untreated hepatocytes (Table 8.1).

Figure 8.5 GSH depletion and formation of morphine metabolites in hepatocytes incubated with 2 mM morphine. Values are means ± S.D. of at least three experiments.


Hepatic content (nmol/10^6 cells)
60
50
40
30
20
10
0
Control
GSH
Morphine
NM
M-3-G
M-SG
0
15
30
Incubation time (min)

However, the metabolic profile did change in these hepatocytes (Figure 8.4B). At low concentrations of morphine, no normorphine was formed, again suggesting that the normorphine pathway was activated only when the amount of morphine stressed the capacity for glucuronidation. At high concentrations of morphine, the formation of morphine-GSH was significantly increased. At 15 min, the amount of adduct formed was equivalent to that of M-3-G formed (Figure 8.6B). This suggests that at least initially, the GSH conjugation may be as important as glucuronidation at high morphine concentrations.

Time course of morphine-GSH adduct formation

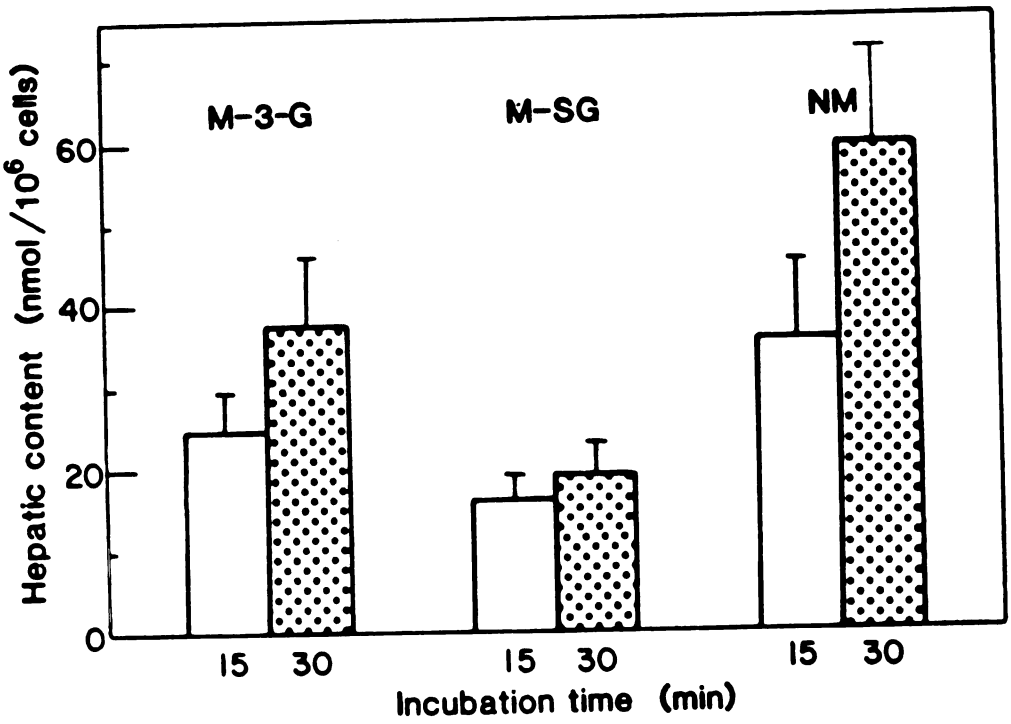
Formation of the morphine-GSH adduct was found to be nearly complete by 15 min and in fact, began to slow down after 5 min in parallel with GSH depletion (Figure 8.2). In PB-pretreated rats, this was clearly the case: No further GSH-adduct was formed after 15 min (Figure 8.6B). In contrast, glucuronidation and in particular, N-demethylation continued to increase between 15 and 30 min.

Effect of cysteine and methionine on hepatic GSH depletion and morphine-GSH adduct formation

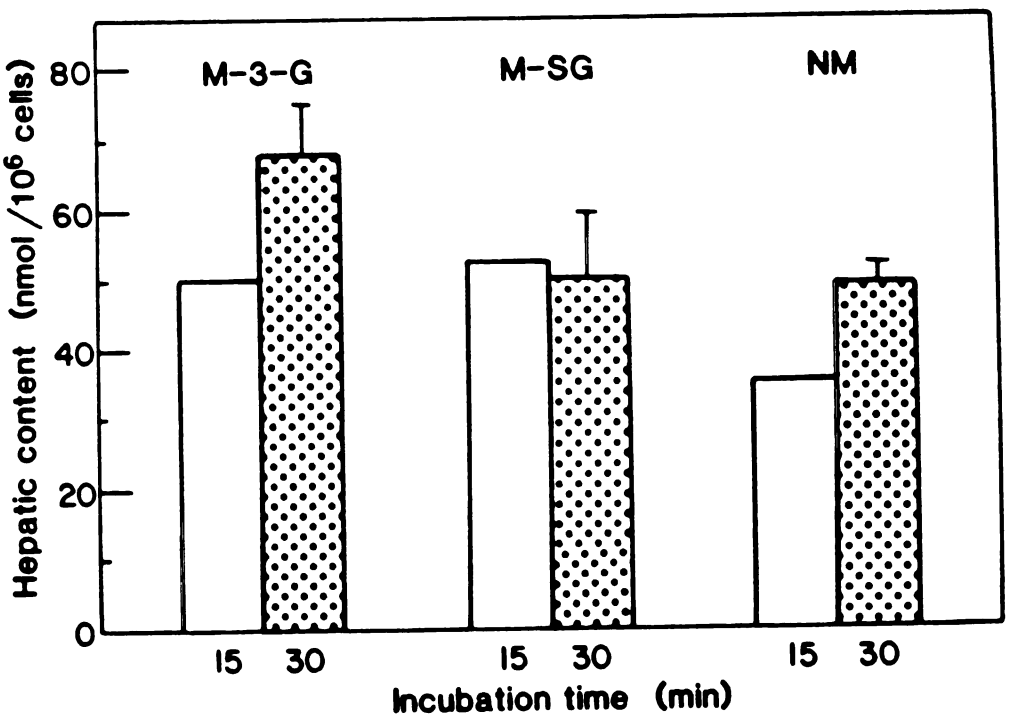
It was important to determine if the formation of the adduct and/or the GSH depletion could be affected by supplementation of the incubation medium with the GSH-precursors, cysteine and methionine. Aw *et al.* (1984) had found that in the presence of methionine (1.0 mM), GSH efflux was inhibited by 70% over a 1 h period. Similarly, Thor *et al.* (1979) reported that the steady GSH decrease in hepatocytes incubated in a medium lacking cysteine or methionine could be reversed and the GSH

Figure 8.6A Formation of morphine metabolites at 15 and 30 min in hepatocytes incubated with 2 mM morphine. Values are means ± S.D. of at least three experiments.

Figure 8.6B Formation of morphine metabolites at 15 and 30 min in hepatocytes of PB-treated rats incubated with 2.0 mM morphine. 15 min values are means of two experiments. 30 min values are means ± S.D. of three experiments.


M-3-G
M-SG
NM
Hepatic content (nmol/10^6 cells)
0
20
40
60
15 30
15 30
15 30
Incubation time (min)

A


M-3-G
M-SG
NM
Hepatic content (nmol/10^6 cells)
0
20
40
60
80
15 30
15 30
15 30
Incubation time (min)

B

Figure 8.7 GSH depletion in hepatocytes of PB-treated rats incubated with 2.0 mM morphine.

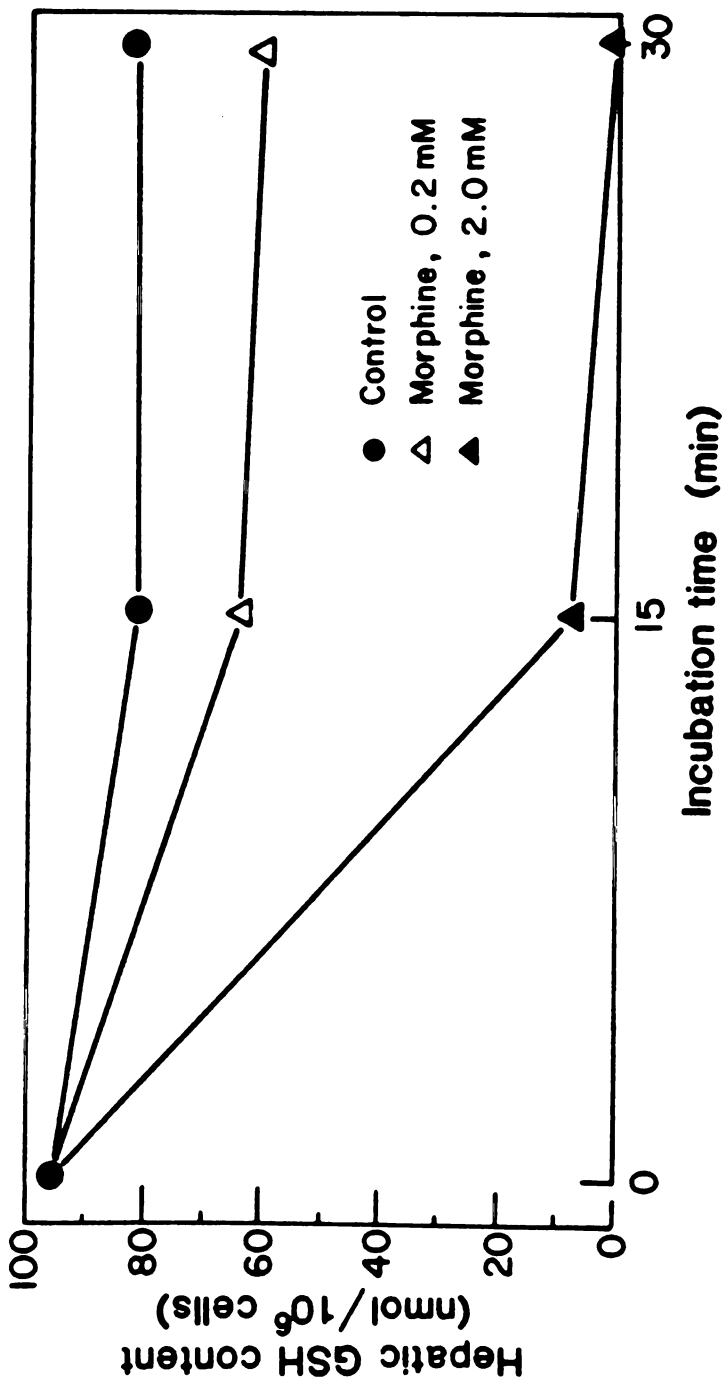
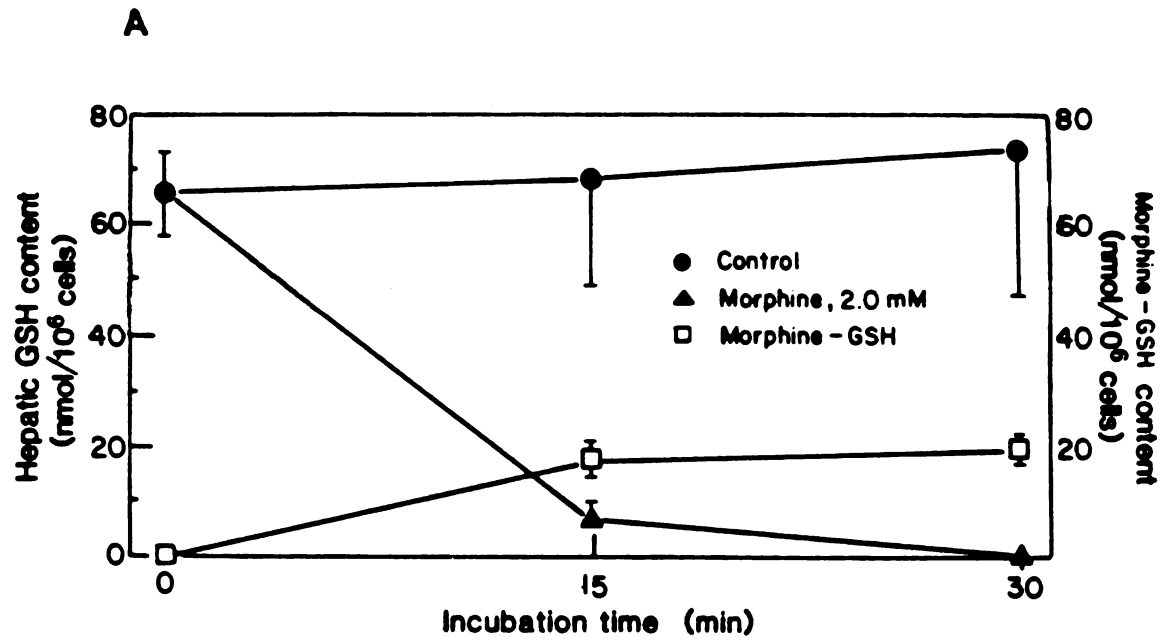
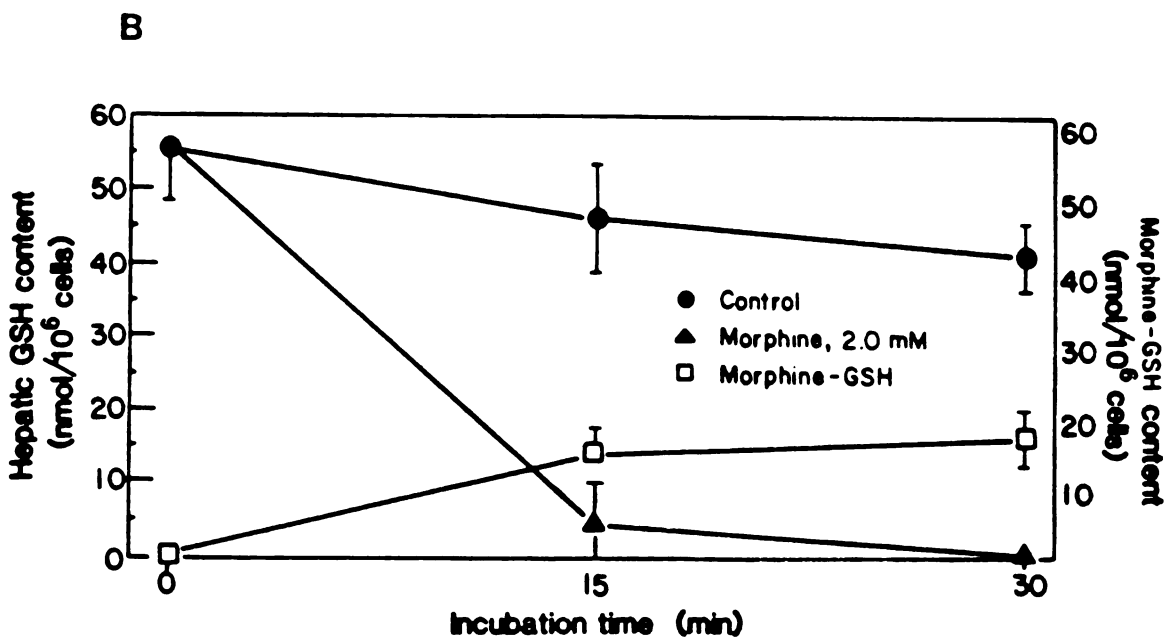

Hepatic GSH content
(nmol/10⁶ cells)
100
80
60
40
20
0
0
15
30
Incubation time (min)
Control
Morphine, 0.2 mM
Morphine, 2.0 mM

Figure 8.8A GSH loss and morphine-GSH adduct formation in hepatocytes incubated with 2.0 mM morphine in the presence of cysteine (0.5 mM) and methionine (1.0 mM). Values are means ± S.D. of three experiments.

Figure 8.8B GSH loss and morphine-GSH adduct formation in the absence of cysteine and methionine. Values are means ± S.D. of three experiments.


A
Hepatic GSH content (nmol/10^6 cells)
Morphine-GSH content (nmol/10^6 cells)
Control
Morphine, 2.0 mM
Morphine-GSH
Incubation time (min)


B
Hepatic GSH content (nmol/10^6 cells)
Morphine-GSH content (nmol/10^6 cells)
Control
Morphine, 2.0 mM
Morphine-GSH
Incubation time (min)

levels even slightly increased when these amino acids were added to the incubation mixture. This was indeed found to be the case in the present studies in control cells (Figures 8.8A and 8.8B). However, the availability of cysteine and methionine for GSH synthesis and the presence of methionine to prevent GSH efflux had no effect on the dramatic morphine-mediated GSH depletion or the formation of morphine-GSH. Thus, the damage produced by incubation with morphine (2 mM) was apparently so rapid that increased GSH synthesis or inhibition of normal GSH efflux was inconsequential.

Covalent binding of highly reactive morphine intermediate(s)

Since GSH was essentially depleted by 15 min, it seemed likely that the amount of adduct formed was limited by existing GSH levels. It was important, therefore, to consider whether under those conditions, the electrophilic morphine intermediate(s) that normally bind to GSH would in its absence bind to protein thiols. Such a possibility is indicated by the fact that incubation of rat liver microsomes with [^{3}H]morphine results in covalent binding to microsomal protein (Correia, 1984*a*), and this irreversible binding is largely attenuated by coincubation with GSH, presumably by preferential binding of the reactive intermediate(s) to GSH.

In the present studies, the amount of covalent binding increased as GSH levels decreased. Unfortunately, the rapid GSH depletion induced by morphine prevented a direct correlation of covalent binding at incremental stages of GSH depletion. However, the rate of covalent binding appeared to be slowest in the first 5 min of the incubation, the interval of time during which most of the GSH was being depleted

(Figure 8.9). As expected, irreversible binding of [^{3}H]morphine to cell protein progressively increased from 15 to 30 min, at which time GSH was essentially depleted and the formation of the [^{3}H]morphine-GSH adduct had levelled off.

Because conceivably the formation of reactive intermediate(s) could be increased by shunting metabolism away from the glucuronidation pathway, dextrorphan, a known competitive inhibitor of morphine-glucuronidation in rabbit liver microsomes (Sanchez *et al.*, 1978), and a potential competitive inhibitor of N-demethylation (Mannering and Takemori, 1958) was coincubated with [^{3}H]morphine (Figure 8.10). This approach was expected to drive an excess of [^{3}H]morphine into the pathway leading to the reactive intermediate thereby increasing its irreversible binding to the protein. Covalent binding in the presence of [^{3}H]morphine (2 mM) averaged 6.7± 0.8 nmol/mg protein (n=4) during the course of a 30 min-incubation. In coincubations of [^{3}H]morphine and dextrorphan (1.5 mM), not only was the covalent binding nearly abolished but the rate of GSH depletion was markedly reduced (Figure 8.10).

It is possibile that dextrorphan limits the uptake of morphine to hepatocytes. The fact that morphine metabolism to M-3-G was essentially unchanged at dextrorphan concentrations of 1.5 mM (Table 8.2) may argue against this premise. Glucuronidation in the presence of dextrorphan does indicate that unlike its rabbit liver microsomal counterpart, dextrorphan was not a good inhibitor of rat liver microsomal glucuronidation.

Figure 8.9 GSH loss and irreversible binding in hepatocytes incubated with [^{3}H]Morphine (2.0 mM). First study.

[3H]-Morphine bound
(nmol/mg protein)
8
6
4
2
0
30
25
20
15
10
5
0
Incubation time (min)
Control
Morphine, 2.0 mM
[3H]-Morphine bound
Hepatic GSH content
(nmol/10^6 cells)
80
60
40
20
0

Figure 8.10 GSH depletion and irreversible binding of [^{3}H]Morphine in the presence and absence of dextrorphan. Second study.

[3H]-Morphine bound
(nmol/mg protein)
8
6
4
2
0
30
25
20
15
10
5
0
Incubation time (min)
Hepatic GSH content
(nmol/10^6 cells)
80
60
40
20
0
Control
Morphine
3H-bound (M)
Dextrorphan
M+D
3H-bound (M+D)

Table 8.2

Effect of Dextrorphan on Morphine Metabolism

	M-3-G	M-GSH*	NM**	Irreversible binding
		nmol/10^6 cells		nmol/mg protein
Morphine	37.4± 9.5	19.0± 4.6	59.0± 16.9	6.7± 0.8
Morphine + Dextrorphan	33.0	10.1	28.5	1.2

*M-GSH = morphine-GSH; **NM = normorphine.

Competition of dextrorphan with morphine in the metabolic pathway leading to the morphine-GSH adduct may explain its inhibition of morphine-GSH adduct formation as well as morphine-mediated covalent binding (Table 8.2) If dextrorphan were also metabolized to a reactive intermediate in hepatocytes, less [^{3}H]morphine would have been metabolized via this pathway, and thus less binding would have occurred. Lowered GSH levels in hepatocyte incubations of dextrorphan alone and attenuation of massive GSH depletion in coincubations of morphine and dextrorphan support this hypothesis (Figure 8.10).

GSH depletion provoked by morphine analogs

In order to examine the possible structural requirements of morphine for its induction of hepatic GSH depletion, several morphine analogs were examined in hepatocyte incubations in the presence or absence of morphine. GSH depletion by two analogs, ethylmorphine and levorphanol, was monitored at 5 min intervals (Figures 8.11A and 8.11B). By themselves, these analogs also depleted hepatic GSH content

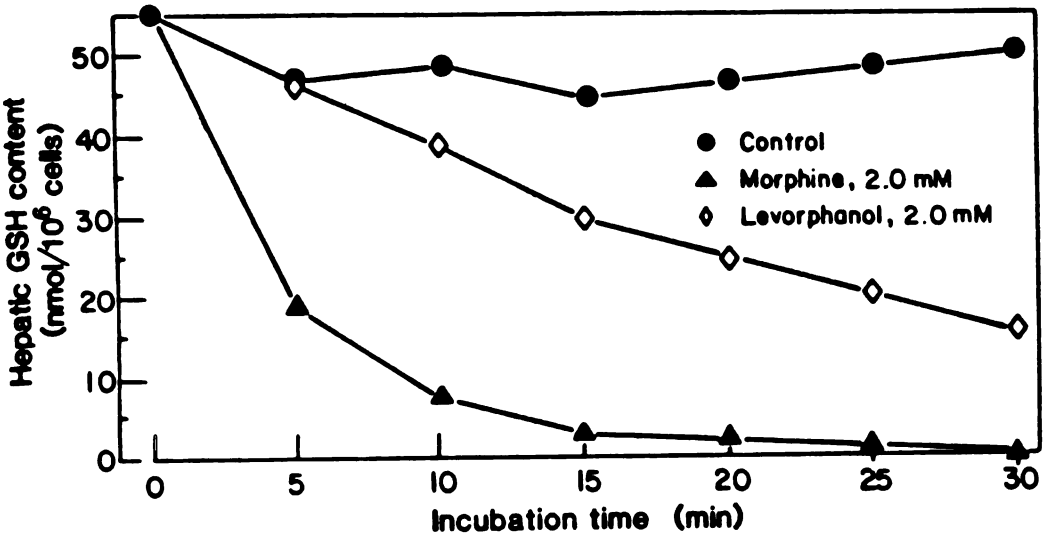
over 30 min. However, in common with dextrorphan, their depletion of GSH was gradual, in contrast to the rapid drop associated with morphine-induced GSH depletion.

When the potential to deplete hepatic GSH of various structural analogs was compared with that of morphine, no compound was found to deplete GSH quite as acutely and profoundly as morphine (Figure 8.12A). Most analogs showed a response of approximately 50% of the morphine-induced GSH depletion. Of the analogs tested, normorphine affected GSH levels the least whereas nalorphine caused the most severe depletion. In coincubations with morphine, normorphine did not affect morphine-induced GSH depletion whereas dextrorphan and naloxone both prevented this depletion (Figure 8.12B).

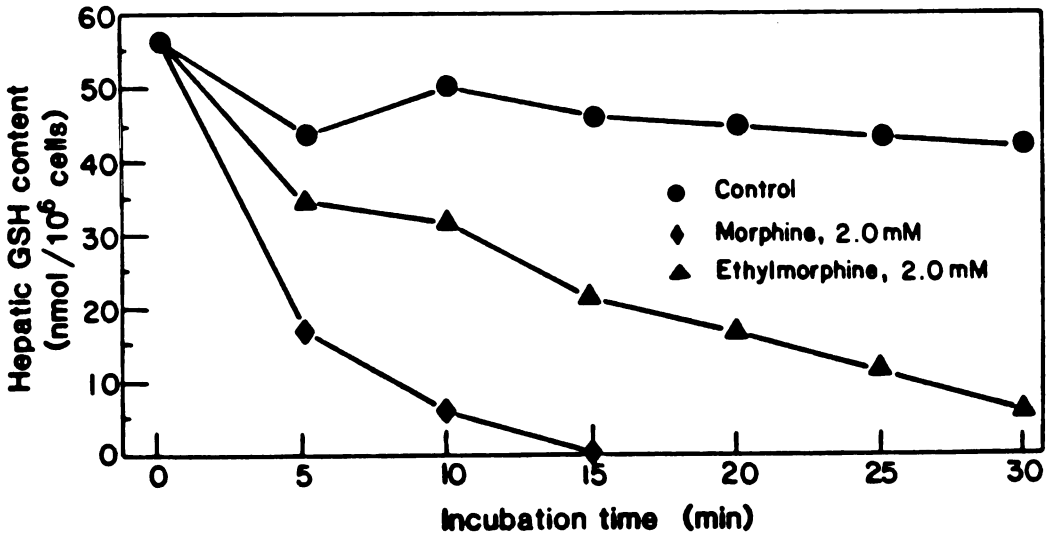
The inability of normorphine to enter the hepatocytes might explain its limited effect on GSH levels. If, however, normorphine should enter the cells, then the N-alkyl group would appear to be critical for GSH depletion. Although the depletion caused by nalorphine (N_{17}-allyl, but otherwise identical in structure to morphine) would appear to support this possibility, ethylmorphine, which has an N_{17}-methyl group and is N-demethylated to a far greater extent than morphine, is found to induce far less GSH depletion. It is possible that the presence of the O-ethyl group at C_3 may mechanistically obstruct this effect. Partial GSH depletion induced by dextrorphan and levorphanol suggest that an element critical for toxicity is missing in these isomers (i.e. the absence of C_6-OH, C_7-C_8 double bond, or the oxygen bridge between C_4 and C_5). Whether the mechanism for the GSH depletion is a membrane effect or a result of metabolism is unclear from the pattern established above.

Figure 8.11A GSH depletion induced by morphine (2.0 mM) and by levorphanol (2.0 mM).

Figure 8.11B GSH depletion induced by morphine (2.0 mM) and ethylmorphine (2.0 mM).


Hepatic GSH content (nmol/10^6 cells)
50
40
30
20
10
0
0
5
10
15
20
25
30
Incubation time (min)
Control
Morphine, 2.0 mM
Levorphanol, 2.0 mM

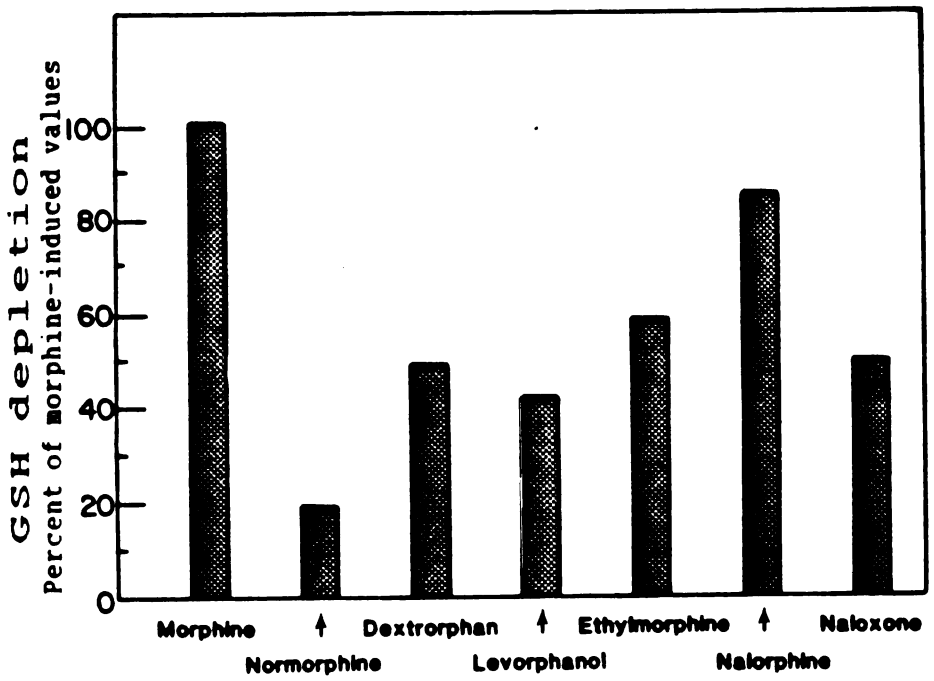
A


Hepatic GSH content (nmol/10^6 cells)
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Incubation time (min)
Control
Morphine, 2.0 mM
Ethylmorphine, 2.0 mM

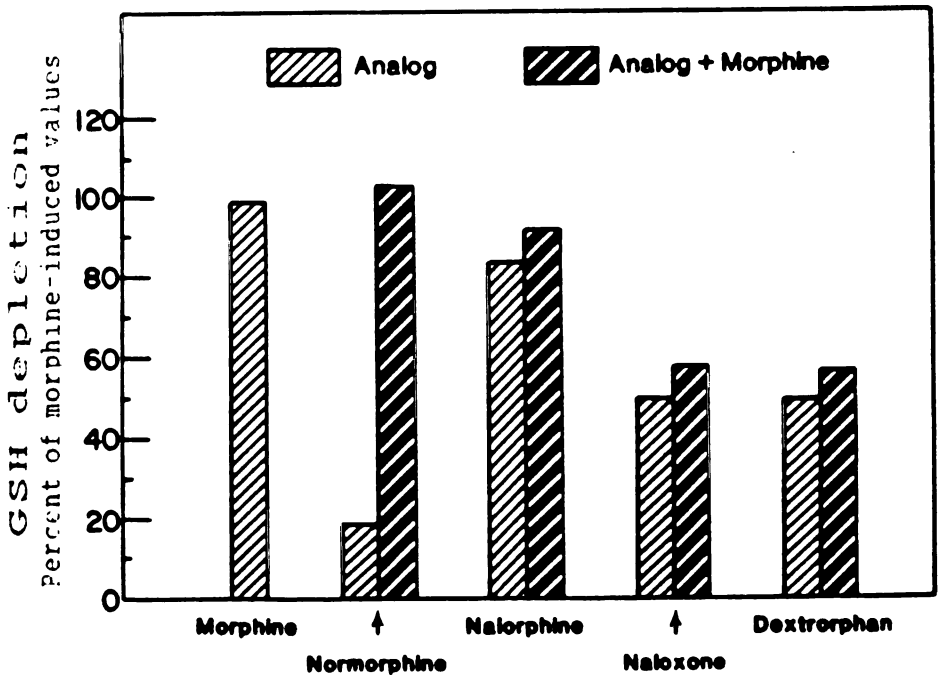
B

Figure 8.12A GSH depletion induced by morphine and morphine analogs in 15 min incubations. Concentration of all analogs was 2.0 mM except dextrorphan which was 1.5 mM.

Figure 8.12B GSH depletion induced by morphine analogs in the presence and absence of morphine. Hepatocytes were incubated for 15 min with morphine (2.0 mM) and/or analog (2.0 mM except 1.5 mM dextrorphan).


GSH depletion
Percent of morphine-induced values
100
80
60
40
20
0
Morphine
Normorphine
Dextrorphan
Levorphanol
Ethylmorphine
Naloxone
Nalorphine

A


GSH depletion
Percent of morphine-induced values
Analog
Analog + Morphine
120
100
80
60
40
20
0
Morphine
Normorphine
Nalorphine
Naloxone
Dextrorphan

B

Requirement for O_2 in GSH depletion

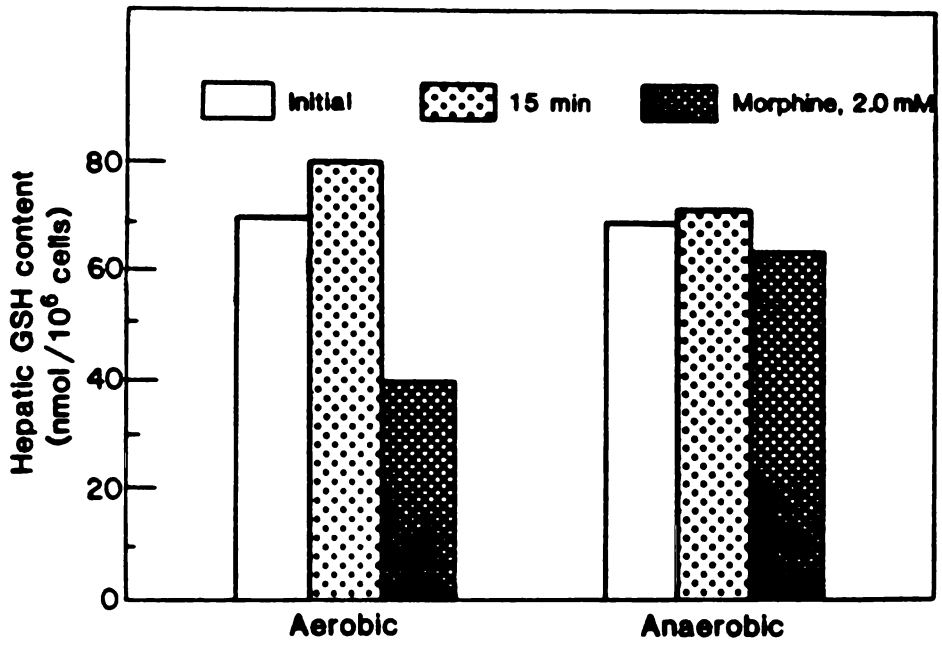
To further examine GSH depletion, the requirement for 0_2 was tested. Figure 8.13A shows the result of morphine incubations in the presence and absence of 0_2. Under anaerobic conditions, morphine-induced GSH depletion is nearly abolished.

Involvement of Cytochrome P-450 in GSH depletion

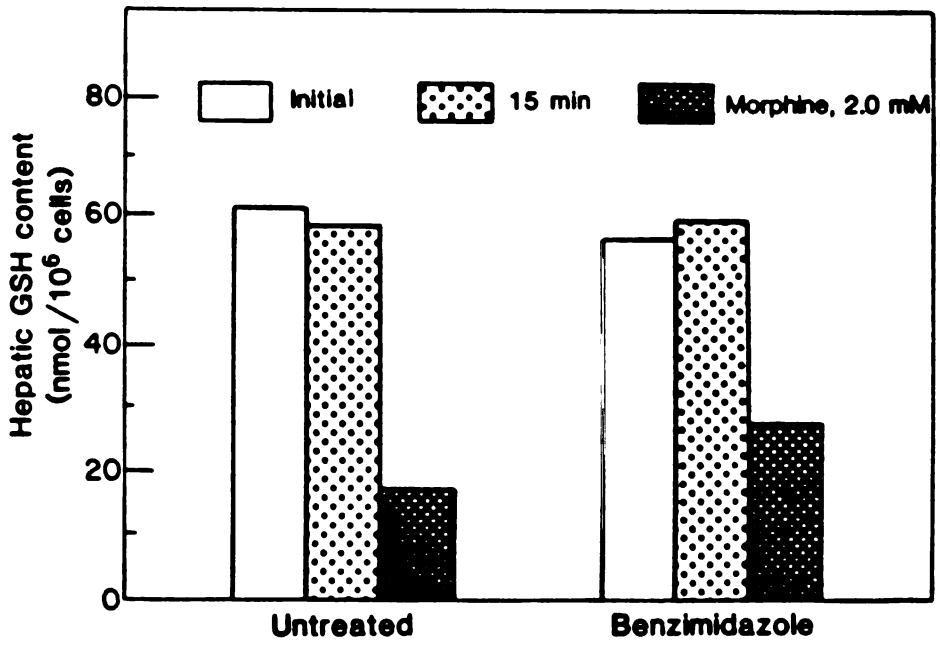
The requirement for O_2 is consistent with, but by no means establishes, the involvement of the cytochrome P-450 monooxygenase system in GSH depletion. To further explore the involvement of cytochrome P-450, GSH levels were monitored in incubations which contained the cytochrome P-450 inhibitor benzimidazole. In incubations of benzimidazole alone, no decrease of GSH was observed. In coincubations with morphine, benzimidazole only partially inhibited GSH depletion (Figure 8.13B). In one experiment, morphine (2 mM) alone depleted GSH to 30% of control values, and upon coincubation with benzimidazole (5 mM), GSH depletion was attenuated from 30 to 50% of control values (Figure 8.13B). Coincubations with benzimidazole, however, significantly inhibited morphine metabolism to the morphine-GSH adduct and normorphine (approximately 50-60% decrease in metabolites), thereby confirming the participation of cytochrome P-450 in such metabolism. Furthermore, its lack of effect on morphine-3-glucuronidation confirmed that benzimidazole affects only cytochrome P-450 pathways (Figure 14). Thus, while benzimidazole inhibited formation of the morphine-GSH adduct by approximately 50%, its inhibition of hepatic GSH depletion was modest (less than 20%). This result confirms the results reported earlier, namely that formation of the morphine-GSH

Figure 8.13A GSH depletion in hepatocytes incubated with morphine (2.0 mM) under aerobic and anaerobic conditions.

Figure 8.13B GSH depletion in untreated or benzimidazole treated hepatocytes. Treated hepatocytes were pre-incubated with benzimidazole (5.0 mM) for 5 min. One prototype experiment of three individual ones that were conducted.

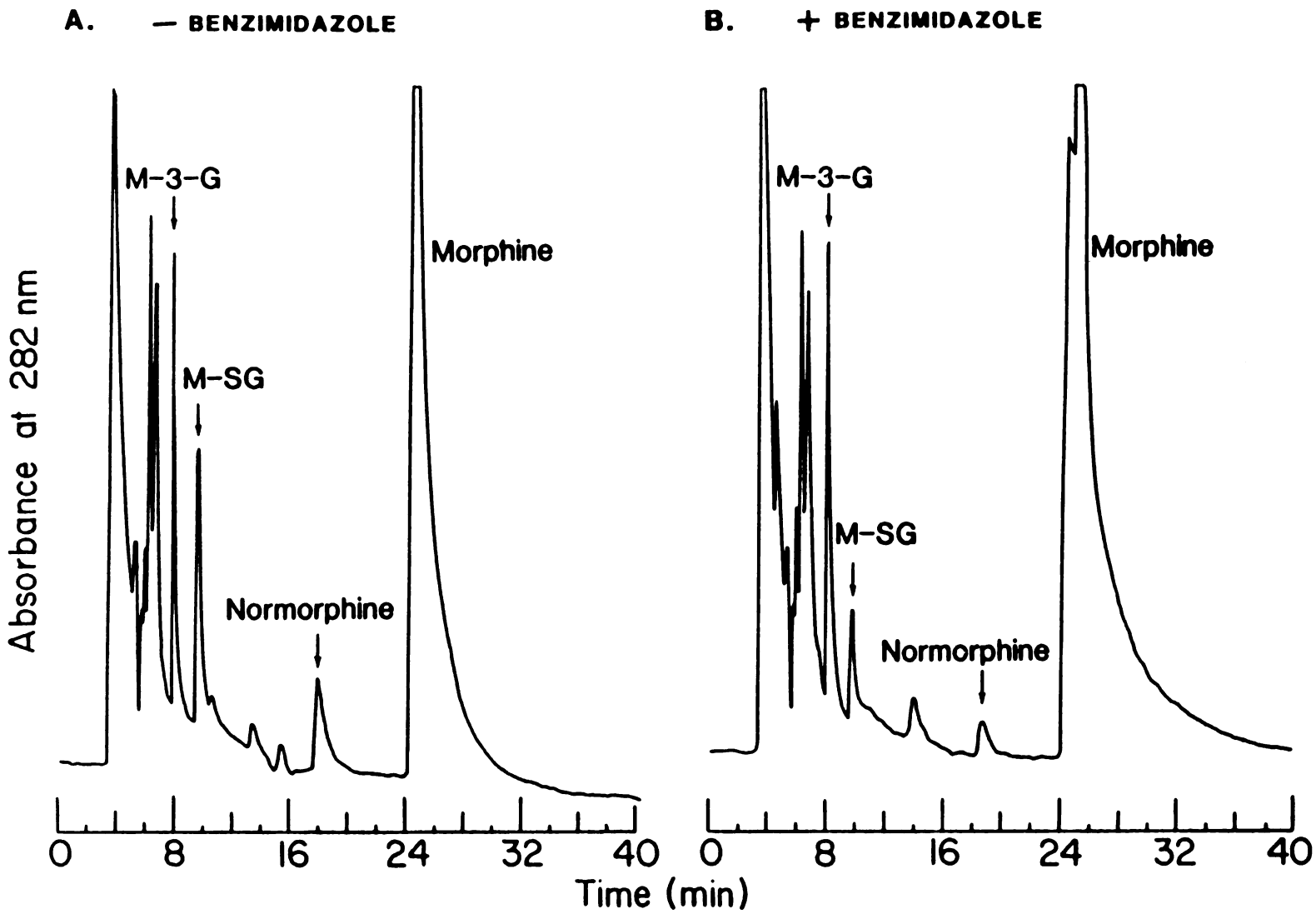

Initial
15 min
Morphine, 2.0 mM
Hepatic GSH content (nmol/10^6 cells)
80
60
40
20
0
Aerobic
Anaerobic

A


Initial
15 min
Morphine, 2.0 mM
Hepatic GSH content (nmol/10^6 cells)
80
60
40
20
0
Untreated
Benzimidazole

B

Figure 8.14 HPLC profiles of hepatocytes incubated for 15 min with morphine (2.0 mM) in the presence and absence of benzimidazole (5.0 mM).


A. — BENZIMIDAZOLE
B. + BENZIMIDAZOLE
M-3-G
M-SG
Normorphine
Morphine
Absorbance at 282 nm
0
8
16
24
32
40
Time (min)

adduct does not account for all of the GSH loss. This finding, of course, does not preclude the involvement of a different cytochrome-P-450 species not inhibitable by benzimidazole in the remaining GSH depletion.

Relationship between GSH depletion and Morphine-GSH adduct recovery

The possibility that a substantial amount of GSH loss (not accountable by morphine-GSH adduct formation) resulted from oxidation to GSSG during the incubation was considered. It was also possible that an increase in GSH efflux and subsequent oxidation to GSSG in the medium could partially account for the GSH loss. In either event, this GSH loss would not have been detected by the method of Ellman (1959). In samples analyzed by the method of Reed *et al.* (1980), where both GSH and GSSG of the incubate are monitored, GSSG levels in morphine (2 mM) treated hepatocytes were not found to be increased over controls[4] (Table 8.3).

[4] Samples analyzed by Dr. David Ross, School of Public Health, UC Berkeley.

Table 8.3

Comparison of GSH and GSSG Levels
in Hepatocytes Incubated with Morphine (2 mM)

Sample	Sampling time (min)	GSH	GSSG
		nmol/10^6 cells	
Control	0	70	24
		69.5	26.5
Control	15	58	38
Morphine(2mM)	15	9	13
		11.5	23.5

Freshly isolated hepatocytes, 2 x 10^6 cells/ml, were incubated with morphine (2 mM) in a shaking water bath at 37° C for 15 min. An aliquot of the hepatocyte incubation mixture was removed and analyzed for GSH/GSSG content according to the method of Reed *et al.* (1980).

This result suggests that the unaccounted for GSH may be present in the form of an additional GSH adduct.

One such possible adduct may be morphinone-GSH. Nagamatsu *et al.* (1986) have suggested that morphine induced GSH depletion is due to the formation of an adduct formed by the Michael addition of GSH to the C_8 position of morphinone, which is generated from morphine by the cytosolic enzyme 6-β-dehydrogenase. However, no morphinone-GSH adduct was found in cells incubated with morphine. Furthermore, failure to detect such an adduct was not due to its decomposition during the assay because authentic morphinone-GSH standard is found to be quantitatively stable in the acidic conditions in which the metabolites were analyzed.

In HPLC tracings of morphine (2 mM) incubated hepatocyte supernatants, two small but consistent peaks were noted. These peaks contained [^{3}H]morphine and thus appear to be [^{3}H]morphine metabolites. These metabolites eluted at 10 and 13 min

(Figure 3) and contained 3.9± 1.2 and 7.9± 2.3 nmol [^{3}H]morphine/10^6 cells, respectively. These metabolites were found to be increased in PB-pretreated rat hepatocytes (Table 8.4).

Table 8.4

Unidentified Metabolites in Untreated and PB-pretreated Rat Hepatocytes

	Peak 10 at min	Peak 13 at min
	nmol/10^6 cells	
Untreated (n=7)	3.9± 1.2	7.9± 2.3
PB-pretreated (n=3)	5.2± 0.1	9.6± 1.2

Freshly isolated hepatocytes, 2 x 10^6 cells/ml, were incubated for 30 min in a shaking water bath at 37° C. Aliquots of supernatants from acid treated incubates were analyzed by HPLC as described in Materials and Methods.

If, in both PB-pretreated and untreated rat hepatocytes, the observed [^{3}H]morphine metabolites eluting in HPLC peaks at 10 and 13 min were indeed GSH adducts, then together with the morphine-GSH adduct this would constitute approximatley 80% of the hepatic GSH loss.

The metabolite which elutes at 10 min may well be the 10-β-S-glutathionylmorphine since it elutes immediately after the 10α-morphine-GSH conjugate. This peak appears to be affected by benzimidazole while the peak eluting at 13 min apparently is not. In PB pretreated rat hepatocytes, this metabolite, like the 10α-morphine-GSH adduct, is maximally formed by 15 min.

9.0 SUMMARY AND CONCLUSION

The identification of 10α-N-acetylcysteinylmorphine by ^{1}H NMR spectroscopy confirmed a new metabolic pathway in morphine metabolism: oxidation at the benzylic C_{10} carbon. The characterization of this adduct established a potential link between the cytochrome P-450 mediated metabolism of morphine and the long observed morphine-induced hepatotoxicity.

Metabolism of morphine to the 10α-morphine-GSH was demonstrated in human and rat liver microsomes, in rat bile and in freshly isolated rat hepatocytes. Metabolism to the reactive morphine species was thus shown to be an in vivo as well as in vitro metabolic pathway. In human liver microsomes, the pathway leading to this GSH adduct was found to be at least as important as N-demethylation in vitro. The identification of this metabolite in human liver microsomes attests to the potential for morphine-induced hepatotoxicity if morphine should be given in high doses in combination with other GSH depleting xenobiotics. In hepatocytes from both PB-pretreated and untreated rats, the metabolite was most significant at high morphine concentrations. This dramatic increase appeared to occur when glucuronidation was overwhelmed.

In isolated hepatocytes, incubations with morphine resulted in a profound dose-dependent depletion of GSH. Significant depletion occurred within 5 min and was essentially complete by 15 min. The pattern of GSH depletion paralleled that of 10α-morphine-GSH formation. GSH depletion was also associated with an increase in covalent binding. The inverse relationship between covalent binding and GSH levels

suggests that in the absence of GSH, the reactive morphine species may bind to other tissue nucleophiles.

Together these findings suggest that cytochrome P-450 dependent activation of morphine at the benzylic C_{10}-position is a viable metabolic pathway both in vivo and in vitro which contributes, at least in part, to the observed morphine-mediated hepatic GSH depletion and liver damage.

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