Biochemical Pharmacology, Vol. 30, No. 12, pp. 1529–1536, 1981.
Printed in Great Britain.

0006-2952/81/121529-08 $02.00/0

MORPHINE-MEDIATED EFFECTS ON RAT HEPATIC HEME AND CYTOCHROME P-450 *IN VIVO*

ANTAGONISM BY NALOXONE IN THE LIVER*

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(*Received* 26 *August* 1980; *accepted* 5 *December* 1980)

Abstract—Chronic morphine administration to adult male rats has long been known to lower hepatic cytochrome P-450 content and its dependent mixed function oxidase activity. More recently, we found that acute treatment of mature male rats with a dose of morphine higher than that used chronically also reduces their hepatic cytochrome P-450. In the present study, we demonstrate that this acute reduction of cytochrome P-450 in the rat liver is a result of morphine-mediated accelerated turnover (degradation) of its heme moiety and apparently is associated with hepatotoxicity of the drug. These morphine-mediated effects are largely prevented by concomitant administration of naloxone, a morphine antagonist.

Repeated administration of morphine to mature male rats results in reduced hepatic cytochrome P-450 content and associated mixed function oxidase activity [1–4].‡ With a single but higher dose of morphine (45–50 mg/kg, i.p.), an acute fall in cytochrome P-450 content is observed as early as 2 hr after treatment [5]. The cause for this rapid morphine-mediated reduction of hepatic cytochrome P-450 content is not readily apparent.

The hepatic content of cytochrome P-450 is regulated by its turnover, i.e. formation and degradation. Formation of cytochrome P-450 requires synthesis of the apocytochrome and of heme, followed by coupling of these two moieties to assemble the holohemoprotein. Impairment of one or more of the above processes could effectively cause a decrease in the content of cytochrome P-450 in the liver. Alternatively, the observed loss in cytochrome P-450 could be due to accelerated degradation of this hemoprotein(s). Accelerated degradation of cytochrome P-450 appears to precede stimulation of hepatic microsomal heme oxygenase (MHO) [6, 7], the rate-limiting enzyme in heme catabolism. This occurs ostensibly because turnover of the cytochrome leaves its heme moiety unscathed, thereby resulting in a relative excess of unutilized or "free" heme in the liver and consequently in substrate-mediated induction of this enzyme [8, 9]. The activity of this hepatic enzyme is also stimulated in nutritional conditions such as starvation [10] and by several hormones [10, 11] or chemical agents [6, 12–15].

Our observation that the morphine-mediated loss of cytochrome P-450 is associated with stimulation of MHO activity suggests enhanced degradation of heme in the liver [5]. The exact mechanism of such stimulation, however, is unclear. Morphine could enhance MHO activity by a direct mechanism, or indirectly by increasing free hepatic heme, with subsequent substrate-mediated induction of the enzyme. This induction could be due to: (1) over-production of newly synthesized heme coupled with its under-utilization, or (2) intensive drugs-mediated turnover of hemoproteins resulting in an abnormal excess of "free" heme in the liver. For these reasons, the present studies have been designed to investigate whether excessive production of heme or increased turnover of cytochrome P-450 could result in accumulation of "free" heme and thus account for the observed stimulation of hepatic MHO activity in morphine-treated rats.

We have sought also to determine whether morphine-mediated stimulation of MHO activity reflects a specific effect of the drug, preventable by its congener and CNS antagonist naloxone, or is a general toxic effect of the relatively high doses employed.

MATERIALS AND METHODS

Chemicals

NADPH, NADH, glutathione, pyridoxal 5′-phosphate, coenzyme A, hemin hydrochloride, subtilisin (protease, type III), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Co., St. Louis, MO. [4-^{14}C]δ-Aminolevulinic acid (ALA) was obtained from the New England Nuclear Corp., Boston, MA. Morphine sulfate was obtained from Eli Lilly & Co. Indianapolis, IN, and naloxone from Endo Labora-

* This work was carried out by D. Gurantz in partial fulfilment of a Master's Degree in Pharmacology, University of California, San Francisco. Part of the data was published in abstract form in the *Proceedings of the Seventeenth Annual Meeting, Society of Toxicology*, San Francisco, CA, 12–16 March 1978.

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‡ The reader is also referred to the references cited in Ref. 1.

tories, Inc., Garden City, NY. Assay kits for the determination of glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in serum were also obtained from the Sigma Chemical Co. (catalog No. 55-UV).

Animals and treatment

Male Holtzman rats (210–260 g) were allowed free access to laboratory chow and water and were starved overnight before being killed. Animals were treated with morphine sulfate (45–50 mg/kg, i.p.), naloxone (5 mg/kg, s.c.) or 0.87% NaCl, and were killed at designated times after injection.

Tissue preparations

Liver was perfused *in situ* with isotonic KCl, removed, weighed, and homogenized in 0.1 M Na^+–K^+ phosphate buffer, pH 7.4. Portions of the homogenate were used for preparation of microsomes or the 18,000 *g* supernatant fraction. Liver homogenate was centrifuged at 10,000 *g* for 10 min at 4°, and the resulting supernatant fraction was sedimented at 105,000 *g* for 1 hr at 4°. The microsomal pellet so obtained was suspended in isotonic KCl and resedimented at 105,000 *g* at 4° for 30 min. This pellet was resuspended in 0.1 M Na^+–K^+ phosphate buffer, pH 7.4, to yield "microsomes" at desired protein concentration.

Assay procedures

δ-ALA synthetase activity. Liver homogenate was prepared in 0.9% NaCl–0.1 M Tris buffer, pH 7.4, containing 0.1 mM pyridoxal phosphate [16], and δ-ALA synthetase activity was determined by the method of Strand *et al.* [17].

MHO activity. MHO activity was determined in the 18,000 *g* supernatant fraction of liver homogenate using the method of Tenhunen *et al.* [18] as modified by Correia and Schmid [15].

Tryptophan pyrrolase activity. The enzyme activity was assayed in 20% liver homogenate according to the method of Badawy and Evans [19]. On addition of heme to the reaction mixture, the increase in activity was taken as the measure of reconstitution of the available apoprotein in the liver.

Serum GOT and GPT activities. Animals were anesthetized with ether, and blood was drawn from the descending aorta with an unheparinized syringe. The blood was allowed to sit before sedimenting the red blood cells at 2000 *g* for 10 min, and the serum was collected. GOT and GPT activities were determined in serum of morphine- and/or naloxone-treated and control rats by monitoring the disappearance of NADH from the reaction mixture [20, 21], using the enzyme assay kits and procedures prescribed (Sigma Chemical Co., catalog No. 55-UV).

Determination of cytochrome P-450 heme turnover. Rats were injected with [4-^{14}C]ALA so as to prelabel cytochrome P-450 heme *in vivo*. Eighteen hours later, the animals were given either morphine or 0.87% NaCl and were killed at specified times thereafter. Cytochrome P-450 (CO-binding) particles were prepared from liver microsomes by the subtilisin method of Comai and Gaylor [22], using 0.1 M phosphate buffer, pH 7.4. The microsomal pellet obtained after subtilisin (Sigma "protease", type III) treatment contained cytochrome P-450 almost exclusively, with negligible amounts of cytochrome b_5, indicating almost complete solubilization of the latter cytochrome. Carrier heme was added to aliquots of these preparations, and heme was isolated and crystallized according to the method of Labbe and Nishida [23]. The radioactivity of the isolated heme was determined as described previously [6, 24].

Degradation of cytochrome P-450 in vitro. Washed hepatic microsomes were incubated for 30 min at 37° in the presence of NADPH (0.1 mM) and a NADPH-generating system consisting of glucose-6-phosphate (1.5 mM) and glucose-6-phosphate dehydrogenase (0.6 units/ml), $MgCl_2$ (0.2 mM), phosphate buffer (0.1 M), pH 7.4, and various concentrations of morphine and/or naloxone. Two control reaction mixtures were included, one without NADPH and the other without the drugs. The destruction of cytochrome P-450, if any, was monitored by determining its concentration in aliquots of the mixture drawn at zero time and 30-min intervals [25].

Analytical determinations. Cytochrome P-450 was measured in liver homogenate, microsomes and cytochrome P-450 particles by the CO-reduced minus CO-oxidized difference spectrum [26], and cytochrome b_5 by NADH-reduced minus oxidized difference spectrum [27] using an Aminco DW-2 spectrophotometer. Protein content was assayed by the method of Lowry *et al.* [28], using bovine serum albumin as standard.

RESULTS

Effect of morphine on hepatic cytochrome P-450 and MHO activity

A single injection of morphine to rats significantly reduced cytochrome P-450 levels as early as 1 hr after injection, with a maximum decrease (30 per cent of basal level) occurring at 6 hr (Fig. 1). Over a similar time period, MHO activity increased rapidly, exhibiting a significant elevation at 2 hr and a 3- to 4-fold stimulation at 8 hr (Fig. 1). Stimulation at this time point appeared to be maximal (unpublished results), hence subsequent experiments were limited to 8 hr.

In contrast to hepatic MHO activity after morphine treatment, hepatic δ-ALA synthetase activity was unchanged during the initial 6 hr but increased significantly at 8 hr (Fig. 2). Failure to detect a significant early rise in the activity of δ-ALA synthetase (the rate-limiting enzyme in the heme synthetic pathway) excluded the possibility that newly synthesized heme could account for the stimulation of MHO activity observed 2 hr after morphine administration (Fig. 2).

Morphine-mediated release of cytochrome P-450 heme in vivo

Morphine treatment resulted in a decrease in hepatic cytochrome P-450 content and in a concomitant stimulation of hepatic MHO activity, raising the possibility that degradation of cytochrome P-450 with release of its heme moiety may have been

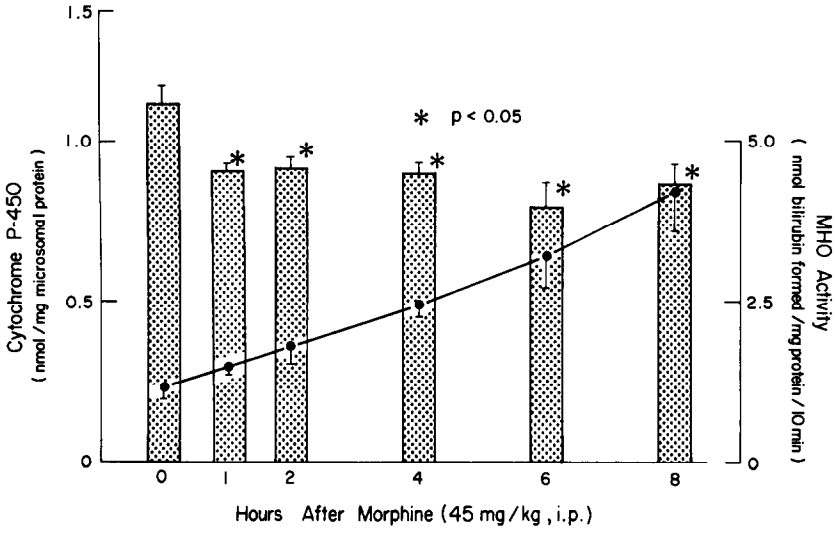

Fig. 1. Rat hepatic cytochrome P-450 content and MHO activity after morphine administration. Animals received a single injection of morphine and were killed at designated times. Values for microsomal cytochrome P-450 (bars) and MHO activity (solid circles) are means ± S.E. of at least four animals. Asterisks indicate significant differences from basal (0 hr) values.

occurring. Hepatic cytochrome P-450 heme turnover, therefore, was determined in control and morphine-treated rats prelabeled with the heme precursor [^{14}C]δ-ALA (see Materials and Methods). In control rats the radioactivity in cytochrome P-450 heme gradually decreased by 25 per cent of zero-time value over a 6-hr period (Table 1). In contrast, in morphine-treated rats cytochrome P-450 ^{14}C-heme-radioactivity rapidly fell by 50 per cent at 1 hr and remained relatively unchanged over the next 5 hr (Table 1). This immediate reduction in heme-radioactivity indicated that morphine treatment elicited a rapid loss of the heme moiety of cytochrome P-450. That during this process the heme moiety actually was liberated "intact" or unscathed was verified independently by monitoring the increase in heme saturation of the cytosolic hemoprotein apotryptophan pyrrolase. Normally, the hepatic content of this hemoprotein (holoenzyme) is only half-saturated with heme and functional. On addition of endogenous or exogenous heme, the other half of the enzyme population (apoprotein) can be functionally reconstituted (heme saturated) to its fully enzymatic potential [29]. Thus, increase in basal activity of tryptophan pyrrolase would be expected to indicate elevation in "free" heme in the liver. Within 40 min of morphine administration, heme saturation of apotryptophan pyrrolase in the liver was increased significantly from 40 to 60 per cent further increasing to 70 per cent within the next 2 hr (Fig. 3). These findings not only indicate that dissociation of cytochrome P-450 heme was accelerated, but that the heme moiety was released "intact," resulting in an increase in available heme in liver.

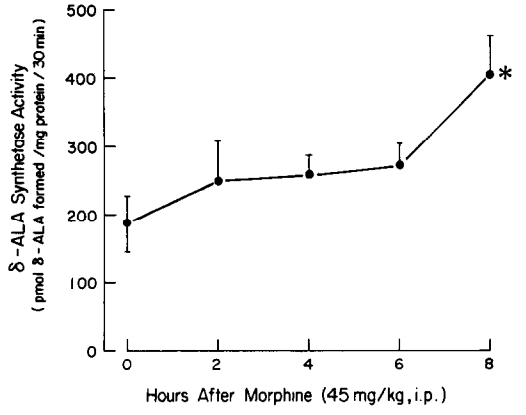

Fig. 2. Rat hepatic δ-ALA synthetase activity after a single injection of morphine. Values for hepatic δ-ALA synthetase activity were obtained from animals treated as in Fig. 1 and are means ± S.E. of at least three animals. An asterisk indicates a significant difference ($P < 0.05$) from basal (0 hr) values.

In vitro *effect of morphine on cytochrome P-450*

The mechanism of the morphine-mediated decrease in cytochrome P-450 content is unknown. Various agents that reduce cytochrome P-450 content *in vivo* also cause its degradation *in vitro* when incubated with hepatic microsomes supplemented with NADPH [30–36]. Drug-induced degradation of cytochrome P-450 *in vitro* can be produced by drug-mediated enhancement of NADPH-dependent microsomal lipid peroxidation or by inactivation of cytochrome P-450 directly or during oxidative metabolism of the drug [25, 30–38]. Whereas the former mechanism is preventable by EDTA, a well known inhibitor of lipid peroxidation, the latter is not and, therefore is believed to be independent of lipid peroxidation [35, 36, 38]. In any event, however, in the absence of EDTA, a destructive agent may be expected to result in additive if not synergistic destruction to that induced by NADPH alone. Because it was possible that hepatic cytochrome P-450 destruction caused by morphine involved either mechanism, we examined the effect of morphine on hepatic microsomal cytochrome P-450 *in vitro*

Table 1. Effect of morphine on ^{14}C-heme content of hepatic cytochrome P-450*

Hours after treatment	Treatment	Cytochrome P-450 ^{14}C-heme (dpm/g liver)
0	Control	40,003
	Morphine	38,766
1	Control	45,497
	Morphine	18,673
2	Control	32,130
	Morphine	16,114
4	Control	30,334
	Morphine	21,767
6	Control	30,717
	Morphine	20,099

* Rats were injected with [^{14}C]ALA 18 hr before morphine or an equal volume of 0·87% NaCl (control) was given. Animals were killed at the designated time following treatment, and the ^{14}C-heme in cytochrome P-450 particles was determined as described in Materials and Methods. Due to the variability in heme utilization (labeling) in rats from experiment to experiment, the results of a typical experiment are presented.

Table 2. Effect of *in vitro* incubation of morphine or naloxone on hepatic mircosomal cytochrome P-450*

Drug	Concn (mM)	Cytochrome P-450 (% decrease)
None		65
Morphine	0.3	27
Morphine	1.0	0
Morphine	2.0	0
Naloxone	0.3	19
Naloxone	1.0	20
Naloxone	2.0	0

* Rat liver microsomes were supplemented with NADPH (0.1 mM) and an NADPH-generating system, and were incubated in the presence or absence of either morphine or naloxone at the concentrations indicated, at 37° for 30 min (Materials and Methods). Microsomal cytochrome P-450 content was determined at 0 hr (0.93 ± 0.09 nmol/mg protein = 100 per cent) and at 30 min following incubation. Values are means of three determinations.

(Table 2), having deliberately excluded EDTA from the incubation mixture. In the absence of morphine, 65 per cent of cytochrome P-450 initially present in the incubation mixture was lost due to NADPH. This loss was preventable by coincubation with EDTA (1 to 1.5 mM) and therefore, can be ascribed to NADPH-dependent lipid peroxidative degradation, in agreement with our previous findings [35] and those of others [25, 37, 38].

In discordance with any of the plausible mechanisms for cytochrome P-450 destruction considered above, however, morphine (0.3 mM) not only significantly reduced NADPH-dependent breakdown of cytochrome P-450, but at higher concentrations (1.0 and 2.0 mM) completely prevented it. Moreover, since the reaction mixture satisfied all the *in vitro* requirements for the mixed function oxidase system, it would have adequately supported the metabolism of morphine. Thus, the effects, if any, of morphine metabolites on cytochrome P-450 would have been detected in the same reaction mixture.

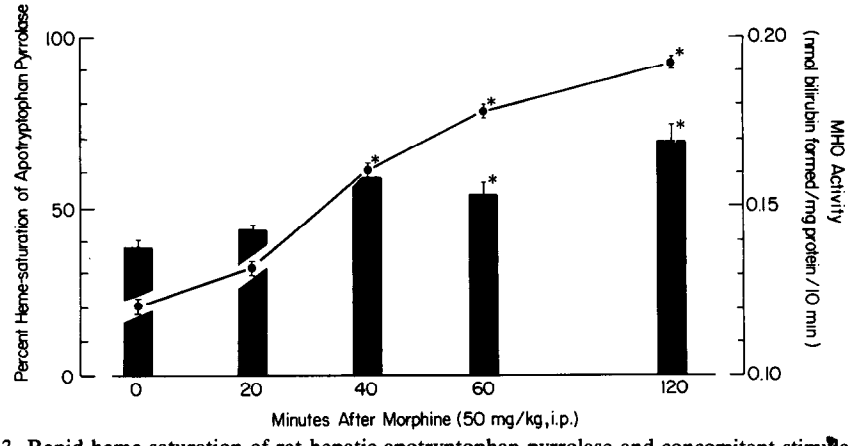

Fig. 3. Rapid heme saturation of rat hepatic apotryptophan pyrrolase and concomitant stimulation of MHO activity following morphine. Rats were given a single injection of morphine and killed at the times indicated. Percentage saturation of apotryptophan pyrrolase was calculated from the activity obtained after addition of heme *in vitro* × 100 (solid bars). MHO activity is illustrated by solid circles. Values are means ± S.E. of at least three animals. Asterisks indicate significant differences ($P < 0.01$) from basal (0 hr) values.

Inhibition of morphine-mediated stimulation of MHO

Naloxone administration to rats failed to affect cytochrome P-450 levels (unpublished observations) and hepatic MHO activity (Fig. 4). When injected repeatedly during the first 3 hr of morphine administration, however, it dramatically blocked morphine-mediated stimulation of MHO activity (Fig. 4). Similar results were obtained when naloxone treatment was extended over the entire 6-hr period (unpublished observations). Furthermore, naloxone (1–2 mM), when incubated with hepatic microsomes supplemented with NADPH *in vitro*, abolished the NADPH-dependent degradation of microsomal cytochrome P-450.

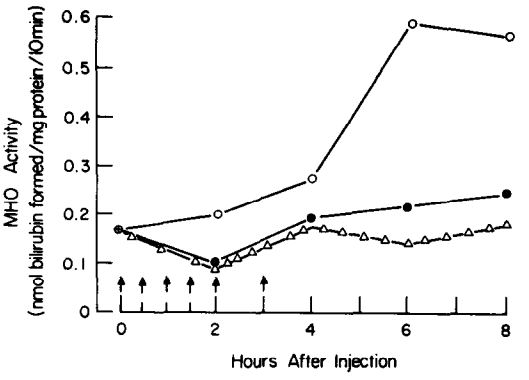

Fig. 4. Inhibition of morphine-mediated stimulation of rat hepatic MHO activity by naloxone. Rats were given a single injection of morphine (50 mg/kg, i.p.) at 0 hr (○) or of naloxone (5 mg/kg, s.c.) alone at the times indicated by the arrows (△) or of morphine (50 mg/kg, i.p.) at time 0 hr concomitantly with and followed by naloxone (5 mg/kg, s.c.) at the times indicated by the arrows (●). Animals were killed at the times designated. Values are means of at least three individual animals.

Hepatotoxic effect of morphine

Accelerated turnover of cytochrome P-450 heme in the morphine-treated rat liver could conceivably represent an acute toxic manifestation of the relatively high dose of morphine administered in our studies. Serum GOT and GPT activities were therefore examined as indices of hepatotoxicity following morphine administration [21]. Consonant with a hepatotoxic effect, the serum activities of GOT and GPT were increased significantly in the morphine-treated rats at 2 and 5 hr after its administration (Table 3).

Naloxone, although ineffective *per se*, when administered in combination with morphine significantly prevented the morphine-mediated enhancement of serum GOT activity and completely blocked the increase in serum GPT activity (Table 3). These results suggest that naloxone indeed antagonized the hepatotoxic action of morphine.

DISCUSSION

Chronic treatment of mature male rats with morphine (20 mg/kg, i.p., daily for 4 days) reportedly results in a significant decrease in hepatic microsomal cytochrome P-450 levels and in a parallel decrease in hexobarbital metabolism *in vivo* [1]. In marked contrast to mature male rats, similar treatment of immature male rats or of mature and immature female rats has failed to depress their hepatic cytochrome P-450 content and cytochrome P-450-associated parameters in drug metabolism [1, 3, 39–41]. Because such failures can be largely reversed by administration of testosterone to these rats and because it can be demonstrated in developing male rats treated with estrogens, androgenic hormones have been implicated in the impairment of cytochrome P-450 caused by chronic morphine treatment [1, 3, 39–41]. Rat hepatic cytochrome P-450 and its dependent enzymes are apparently subject to regulation not only by androgens but also by neurohumoral factors controlled by the hypothalamic–pituitary–adrenal axis [1, 3, 41]. Because morphine has been shown to activate this axis [1, 42–47], it has been suggested that a concerted interaction of these neurohumoral factors, as well as of androgenic hormones, may be critical in the regulation of morphine-mediated impairment of hepatic cytochrome P-450 and its associated enzymes in rats chronically treated with morphine [1].

Table 3. Effect of morphine and/or naloxone on rat serum GOT and GPT activities*

Drug	Hours after treatment	Serum activity (Karmen units/ml) GOT	GPT
None (control)		93 ± 4.7	27 ± 3.3
Morphine	2	209 ± 51†	59 ± 15†
	5	316 ± 68‡§	51 ± 9.8†§
Naloxone	2	109 ± 10	27 ± 1.8
	5	90 ± 7.5	21 ± 1.5
Morphine + naloxone	2	178 ± 27†	28 ± 5.8
	5	107 ± 16§	23 ± 2.0§

* Rats were treated with morphine and/or naloxone exactly as in Fig. 4, and were killed at 2 or 5 hr following treatment. Controls received an equal volume of 0.87% NaCl and were killed 5 hr later. Serum GOT and GPT activities were determined as described in Materials and Methods; values are means ± S.E. of at least three animals.

† Significantly different from control, $P < 0.05$.

‡ Significantly different from control, $P < 0.01$.

§ Significant difference between these values, $P < 0.05$.

Chronic morphine administration to mice of either sex also fails to lower their hepatic cytochrome P-450 content and associated drug metabolism [41]. Indeed, a single i.p. injection of morphine (30 mg/kg) to male mice was found to modestly increase both their hepatic cytochrome P-450 content and NADPH–cytochrome *c* reductase activity within 3 hr of administration [45]. Nondiscriminatory failure to impair hepatic cytochrome P-450 and its dependent enzymes of chronic morphine treatment in mice of either sex is consonant with the reported lack of sex-dependent differences in cytochrome P-450-associated drug metabolism in mice, in contrast to rats [41, 46, 47].

In the present studies, however, the acute effects of a single but higher dose of morphine (45–50 mg/kg, i.p.) on rat hepatic cytochrome P-450 were examined. The findings described earlier (see Results) clearly indicate that acute morphine treatment of male adult rats elicited a prompt decrease in hepatic cytochrome P-450 levels. That the decrease in cytochrome P-450 content was not due to reduced heme formation is suggested by the failure of morphine to alter the activity of the rate-limiting enzyme in heme synthesis, δ-ALA synthetase, during the initial 6 hr of treatment. Furthermore, should impairment have occurred at any other heme synthetic step, then the resultant decrease in hepatic heme would have been expected to enhance δ-ALA synthetase activity by derepression of the enzyme [48–50].

In contrast to its failure to affect hepatic heme formation, morphine markedly enhanced heme degradation in the liver. This is ostensibly evident from the rapid and profound stimulation of hepatic MHO activity (Fig. 1). Stimulated MHO activity has been associated with excessive concentrations of "free" or "uncommitted" heme in the so-called free heme pool in the liver [6, 24, 51–53]. Such excess "free" heme may result from limited availability of heme-utilizing apoproteins [24] or from accelerated turnover of cytochrome P-450 heme in the liver [6, 54]. Indeed, consistent with the latter possibility, morphine caused a rapid acceleration of cytochrome P-450 heme turnover (Table 1). Furthermore, that this morphine-mediated acceleration of cytochrome P-450 heme turnover resulted in a relative excess of "intact" heme in the "free" heme pool is evidenced by increased saturation of apotryptophan pyrrolase with endogenous heme (Fig. 3), observed within 2 hr of morphine administration. Although increased heme saturation of tryptophan pyrrolase after morphine administration has been reported previously [55], its mechanism has remained unclear. On the basis of the temporal profile of accelerated cytochrome P-450 heme turnover and increased apotryptophan pyrrolase saturation after morphine observed in the present studies, we now suggest a causal relationship between these two variables. A similar relationship between these hepatic variables has been reported previously in endotoxin-treated rats [56].

Additionally, despite increased heme saturation of apotryptophan pyrrolase, accumulation of heme liberated from morphine-mediated acceleration of cytochrome P-450 turnover apparently had compromised the heme-binding capacity of the liver. Disposal of such excessive heme was instituted by its induction of hepatic MHO, the rate-limiting enzyme in the oxidative metabolism of heme.

A variety of chemical agents, including allylisopropylacetamide, secobarbital, fluroxene, ethynylestradiol, or carbon disulfide and other sulfur-containing chemicals, which destroy cytochrome P-450 *in vivo* and *in vitro*, incur such destruction during their cytochrome P-450-mediated biotransformation to a reactive metabolic species [30–36, 54]. Covalent binding of this newly generated species to the heme or apocytochrome moiety of the cytochrome [34, 54, 57] results in inactivation of cytochrome P-450. Unlike such drugs, however, morphine, while reducing hepatic cytochrome P-450 content *in vivo*, failed to destroy cytochrome P-450 *in vitro* when incubated with hepatic microsomes supplemented with NADPH (Table 2). Indeed, morphine, when included in the reaction mixture, significantly protected cytochrome P-450 from the NADPH-dependent lipid peroxidative degradation. This protection may be ascribed to successful diversion of NADPH-reducing equivalents into oxidative metabolism of morphine rather than concurrent microsomal lipid peroxidation as has been observed with other drugs [58]. Alternatively, morphine binding to cytochrome P-450 may yield a stable complex, less susceptible to lipid peroxidative degradation.

In view of the relatively large doses of morphine administered, it is possible that direct hepatotoxicity played a role in the *in vivo* destruction of cytochrome P-450. Indeed, we observed that morphine administration rapidly caused leakage of the intracellular enzymes GOT and GPT into the plasma (Table 3). Notably, the morphine antagonist naloxone, when administered in combination with morphine at the customary 1:10 molar ratio, effectively prevented this effect. Although antagonistic effects of naloxone have been well documented in the CNS and the peripheral nervous system [59], no such demonstration had ever been reported for naloxone in the liver. The partial reversal of morphine-mediated reduction of hepatic mixed function oxidase activity in rats given nalorphine, another allyl morphine congener, was perhaps the only other recorded instance [2]. Subsequent to our presentation of these novel findings in poster form [5], however, a report essentially confirming the antagonistic effects of naloxone on morphine-mediated increases in the activities of mouse serum GOT and GPT was published [60]. Additionally, these authors demonstrated that the hepatotoxic effects of morphine were dose-dependent and manifested only during the initial 12–24 hr of morphine pellet implantation. Because these effects were drastically reduced, if not abolished, with continuous morphine treatment, it was suggested that they were a reflection of acute hepatotoxicity [60]. Furthermore, failure of morphine to elicit such effects in hypophysectomized mice led the authors to postulate that the observed hepatotoxicity was brought about by activation of the pituitary–adrenal axis and, therefore, represented a central effect of the drug [60]. However, since the liver is apparently innervated parasympathetically [61], the hepatotoxic effects of morphine and their antagon-

ism by naloxone could involve neurochemical receptors in the liver. Thus, it remains to be determined whether naloxone-mediated antagonism of morphine is elicited in the CNS, or in the liver at as yet unraveled neurochemical receptor sites, at an active site of a hepatic enzyme, or even at the hepatocellular membrane. Indeed, a saturable uptake process in isolated rat hepatocytes has been demonstrated for morphine and nalorphine [62]. At equimolar concentrations, the rate of nalorphine uptake exceeded that of morphine, apparently due to its relatively higher lipophilicity. Unfortunately, these elegant studies provided no indication of any competitive interaction between these drugs when presented concomitantly to the hepatocytes [62]. Presumably, such competitive uptake could also explain the antagonistic effects of morphine and naloxone observed in our studies. However, since in the current studies the dose of morphine administered *intraperitoneally* was 10-fold higher than the concomitant dose of naloxone injected *subcutaneously*, morphine uptake by the liver would be favoured largely over that of naloxone. Thus, it is unclear whether the antagonism between morphine and naloxone occurs in the CNS or in the liver.

Nevertheless, consonant with its blockade of morphine-mediated hepatotoxicity, naloxone very significantly inhibited the stimulation of hepatic MHO activity evoked by morphine (Fig. 4). This effect, however, could be due to a mechanism which is unrelated to classical antagonism of the two drugs. Naloxone, like allylisopropylacetamide and secobarbital, possesses an allyl group. Allylic functionalities are critical in the metabolic inactivation (alkylation) of cytochrome P-450 heme to its corresponding covalent drug–heme adduct formed during oxidative metabolism of these drugs [30, 31, 34, 35]. Thus, *in vivo*, naloxone could destroy cytochrome P-450 by converting its heme to a naloxone–heme adduct. This conversion would essentially divert heme from hepatic heme oxygénase and bilirubin formation and thereby significantly reduce hepatic MHO activity in rats given naloxone and morphine. This rationale for the mechanism of naloxone action can be refuted on the following grounds: (1) naloxone administration to rats failed to lower their hepatic cytochrome P-450 content (unpublished observations); (2) naloxone incubation *in vitro* with hepatic microsomes supplemented with NADPH prevented cytochrome P-450 destruction (Table 2); and (3) naloxone substantially reduced the hepatotoxic potential of morphine (Table 3).

These observations argue for a specific naloxone–morphine antagonism in the liver. The exact mechanism of this novel finding is unclear. It has been recently observed, however, that morphine depletes hepatic glutathione, and that the narcotic antagonists naltrexone (R. C. James and D. R. Goodman, personal communication) and naloxone (M. A. Correia, unpublished observations) block this depletion. Therefore, it is tempting to speculate that the observed antagonism between morphine and naloxone might be merely chemical in nature and occur at the active site of an enzyme converting morphine to a reactive hepatotoxic metabolite. Thus, substantial metabolic activation of morphine to a reactive intermediate would be critically required for expression of its hepatotoxicity and consequent reduction of hepatic cytochrome P-450. Accordingly, the failure of morphine to reduce hepatic cytochrome P-450 and its associated enzymes in immature male rats, in mature and immature female rats and in mice of either sex [1, 3, 41], or in hypophysectomized mice [60], could be intrinsically related to the inherently lower rates of drug metabolism in these animals as compared with those in male rats. We are currently examining this intriguing possibility.

Acknowledgements—The authors are grateful for the kind technical assistance provided by Mr. Steve Olson and Ms. Mildred Kendrick in some parts of this study. They also thank Dr. D. M. Bissell for critical review of the manuscript, and Ms. Kathleen McNamara for very expert secretarial assistance. This work was supported by NIH Grant P50 AM 18520.

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