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TOXIC EFFECTS OF MORPHINE ON HEME DEGRADATION IN RATS.
by

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THESIS

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I. MICROSOMAL HEME OXYGENASE

A. General Introduction

Interest in the mechanism(s) of destruction of senescent erythrocytes with consequent degradation of hemoglobin led to the discovery of various enzymatic systems that convert heme to bile pigments in vitro. Of these systems the microsomal heme oxygenase (MHO) system has been investigated the most because of its probable physiological significance. This system was first identified and characterized by Tenhunen et al. [1, 2].

MHO is localized in the endoplasmic reticulum of spleen, liver and bone marrow cells, the sites of sequestration and destruction of erythrocytes. In the liver, it is found both in the sinusoidal and parenchymal cells which are the major sites for breakdown of senescent erythrocytes and non-erythrocyte hemoglobin, respectively [3, 4, 5]. It is also present in the intestinal mucosa, brain, heart and kidney, although to a much lesser extent [6].

MHO catalyzes the oxidation of heme to equimolar amounts of biliverdin, carbon monoxide and iron, and requires NADPH and molecular oxygen (Fig. 1) [1, 2]. By using partially purified MHO from pig spleen, it has been demonstrated that MHO requires the presence of NADPH-cytochrome-c-reductase [7]. MHO is most active in catalyzing the degradation of free heme or heme loosely attached to protein [1]. The activity is lower when hemoproteins (proteins with heme as prosthetic group) such as methemoglobin and hemoglobin (α and β chains) are the substrates [1]. In contrast, oxyhemoglobin and myoglobin are not catabolized by MHO [1]. By using $^{18}O_2$ and $H_2{}^{18}O$ and mass spectroscopic

analysis, it has been shown that the terminal ring-lactam oxygens of biliverdin and the oxygen atom of the carbon monoxide are derived from molecular oxygen and not from the water molecule [8]. It appears that three molecules of oxygen are being consumed for each mole of biliverdin produced [9]. Similarly to other enzymes that utilize molecular oxygen (e.g., hemoglobin), MHO is inhibited by carbon monoxide [1].

Cleavage of the heme-iron protoporphyrin IX ring could occur at any of the four bridge-carbons: α, β, γ, or δ (Fig. 1), to give the four corresponding isomers of biliverdin. However, it has been demonstrated that MHO catalyzes the breakdown of the heme ring only at the α-methene bridge (Fig. 1), and thus, only the α-isomer of biliverdin is produced [1].

Biliverdin is not the end product of heme degradation *in vivo*, since it is reduced to bilirubin by the enzyme biliverdin reductase, abundantly present in the cytosol of the cell. This enzyme also requires NADPH as an electron donor, but NADH can also serve although much less effectively [10]. Biliverdin reductase exhibits marked preference for the α-isomer of biliverdin [11, 12]. In the enzymatic pathway of heme to biliverdin conversion involving MHO and biliverdin reductase, MHO is the *rate limiting step* [10].

B. Hemoprotein-Heme as Substrate for MHO

Turnover of endogenous hemoproteins may provide heme for MHO. There are two populations of hemoproteins: (a) a major one, consisting mainly of erythrocyte-hemoglobin with a long turnover time (e.g., the life span of human erythrocytes is 120 days [13], and that of rat erythrocytes, 55-65 days [14]), and (b) a minor one, which includes

hemoproteins such as catalase and mitochondrial and microsomal cytochromes, with relatively shorter turnover time (e.g., the cytochrome P-450 half-life time is of the order of 8-48 hours). The turnover of various hemoproteins may be determined by monitoring the degradation products in vivo. Thus the catabolism rate of heme in hemoproteins has been studied in humans as follows: Human volunteers were injected with ^{15}N-glycine, a heme precursor, and stercobilin, one of the pigment products of bilirubin, was measured in the feces [15]. Most of the labeled pigment appeared after 120 days, as expected from the turnover time of the erythrocytes. However, 10-20% of the labeled pigment was detected as early as one day after injection [15]. It was suggested that possible sources for the rapidly emerging labeled pigment were: (a) a unique population of red blood cells with a very short survival time, (b) hemoglobin with a short turnover time, and/or (c) non-erythrocyte heme [15]. As already mentioned, heme is converted to equimolar amounts of bilirubin, carbon monoxide and iron [1]. Thus, the kinetics of heme degradation can also be followed by monitoring bilirubin formation and/or production of labeled carbon monoxide formed after injection of the specific radioisotope heme precursors 4- or 5-^{14}C-delta-aminolevulinic acid (4- or 5-^{14}C-ALA) [16, 24] or ^{14}C-glycine [17]. In such experiments, production of carbon monoxide in the expired air can be detected within minutes of administration of 5-^{14}C-ALA or ^{14}C-glycine [17]. In one study, the ^{14}CO reached a maximum within one to three hours and then declined over a few days in a log linear fashion (Fig. 2). This initial increase has been referred to as the early labeled peak. The early production of carbon monoxide paralleled the

excretion of ^{14}C-bilirubin in the bile [16, 17].

The likelihood that the early labeled peak represents mainly catabolism of hepatic heme is suggested by the following observations: (a) Incorporation of ^{14}C-ALA into hemoproteins other than hemoglobin is particularly high in the liver [18]. In addition, a larger fraction of the label appeared in the early labeled peak when ^{14}C-ALA rather than ^{14}C-glycine is used [16, 18]. Furthermore, production of radioisotopic bilirubin by the isolated perfused rat liver after ^{14}C-ALA administration is comparable in rate and magnitude to the production of bilirubin in the intact rat [19]. (b) ^{14}C-ALA penetrates red blood cells poorly compared to ^{14}C-glycine, consequently it is not incorporated into hemoglobin-heme to the same extent as ^{14}C-glycine [16, 18]. Moreover, changes in erythropoietic activity have little effect on the magnitude and rate of the early formation of bilirubin [16, 20]. These observations indicate that hepatic hemoproteins, rather than hemoglobin, largely contribute towards the early formation of bilirubin. The specific hepatic hemoprotein(s) that serves as a potential source of endogenous heme will be discussed later.

C. Regulation of MHO

1. Substrate-Mediated MHO Induction

A variety of hepatic oxygenases such as cytochrome P-450 and tryptophan pyrrolase are substrate inducible [21, 22]. Since it was likely that MHO also shared this property, its activity was examined under a variety of experimental and/or pathological conditions in which heme, free or protein-bound, would prevail in excess.

a. Splenectomy. The spleen is the principal site of sequestration

of senescent red blood cells [23]. Normally, due to the large amount of hemoglobin-heme turnover, the splenic MHO activity is higher than that of the liver [6]. However, splenectomy results in three-fold increase in MHO activity in the liver [6]. This response of hepatic MHO activity suggests a potential compensatory role of the liver in erythrocyte degradation.

b. Hemolysis. Hemolysis in vivo results in the release of abnormally large amounts of free hemoglobin. However, the hemolytic states, such as hemolytic anemia, are accompanied by hyperbilirubinemia rather than hemoglobinemia and methemalbuminemia [23]. This suggests that the capacity of the body to degrade heme is not only adequate but exceeds that for the removal of bilirubin [6].

The relative role of the spleen and the liver in heme degradation was examined in experimental hemolysis, produced by the administration of phenylhydrazine or RBC-antiserum to rats [6]. Due to hypertrophy of the organ, the total capacity of the spleen to convert heme to bilirubin was increased. However, the activity of the splenic MHO was unchanged on the basis of either protein content or tissue weight [6]. In sharp contrast, MHO activity in the liver was markedly elevated [6]. Thus, the liver plays a much greater compensatory role than the spleen in hemolysis.

c. Induction of MHO by heme. Abnormally high levels of heme in the bloodstream resulting from splenectomy or hemolytic anemia most likely account for the observed stimulation of hepatic MHO activity. In support of this hypothesis, administration of heme in the form of either hemoglobin or hematin (methemalbumin) not only elevates MHO activity in the liver [6] but also in the intestinal mucosa [26] and the

kidney [25, 26]. The renal MHO activity is normally low; however, administration of hemoglobin in concentrations exceeding the plasma haptoglobin-binding capacity results in a 30-100 fold stimulation of the renal MHO activity [25, 26]. Furthermore, actinomycin D, puromycin or cyclohexamide prevents this hemoglobin-heme-mediated stimulation of MHO activity in the kidney, indicating that it could be a true induction [25].

2. Hormonal Stimulation

Starvation and hypoglycemia in humans are often accompanied by hyperbilirubinemia [27]. Starvation and/or hypoglycemia are known to cause various hormonal changes [28]. It is likely that these changes result in the overproduction of bilirubin by stimulation of MHO, the rate limiting step in heme degradation. Glucagon release by fasting, hypoglycemia (induced experimentally by insulin or mannose administration), and injection of arginine, as well as administration of glucagon per se, result in induction of hepatic MHO activity in rats [29]. Epinephrine also induces rat liver MHO activity. Concomitant administration of epinephrine and glucagon produces additive effects suggesting separate mechanisms for the hormone-mediated stimulation [29]. It has been shown that bilirubin formed as a result of hormonal stimulation originates from hepatic hemoproteins rather than erythrocyte-hemoglobin [30]. Cyclic adenosine monophosphate (cAMP), the physiological mediator of epinephrine and glucagon action, given exogenously stimulates MHO activity [29]. In contrast, thyroxine, hydrocortisone [29] and prednisone [6] as well as hormonal changes resulting from adrenalectomy or ovariectomy [29] fail to affect MHO activity in the rat liver. Splenic MHO activity is unaffected by any of the above hormonal changes [29].

3. Endotoxin Stimulation

Endotoxins (from Echoli or Salmonella Typhi) added to cell cultures of macrophages actively involved in erythrophagocytosis stimulated MHO activity in these cells [31]. When administered to rats, endotoxin enhanced MHO activity in hepatic parenchymal and sinusoidal cells [31]. Recently, Bissell and Hammaker demonstrated that heme resulting from endotoxin-mediated catabolism of the hemoprotein cytochrome P-450 could account for such stimulation [32].

4. Metal-Mediated Stimulation

Cobalt was the first metal shown to stimulate hepatic MHO activity in rats [33]. It produced a rapid stimulation of the enzyme activity, reaching a maximum (7-8 fold of the basal level) [34] at 16-24 hours after administration [34]. Inhibitors of protein synthesis, such as actinomycin D and puromycin, abolish this cobalt-mediated effect [34]. Furthermore, cobalt added to liver in microsomal preparations in vitro failed to enhance MHO activity [34]. For these reasons, the authors suggested that cobalt acted as an inducer rather than as an activator of the enzyme [34].

Maines and Kappas [35] suggested that this enzyme "induction" was associated with the binding of cobalt to sulfhydryl groups of cellular proteins. Accordingly, sulfhydryl agents such as glutathione (GSH) or cysteine, administered concomitantly with the metal, lowered the cobalt-mediated stimulation of MHO activity [35]. Moreover, this stimulation of the enzyme activity is enhanced when hepatic GSH content is depleted by administration of diethyl maleate [35]. However, sulfhydryl-blocking agents such as p-chlormercuribenzoate and diethylmaleimide added in vitro

fail to affect MHO activity [36]. It was suggested that the cobalt-mediated effect is due to its binding to proteins other than MHO. Thus, cobalt binding to regulatory sulfhydryl proteins that repress MHO could result in derepression of the MHO with consequent enhancement of its activity [36]. Cobalt also stimulates MHO activity in the intestine, brain and kidney, but not in the spleen [36]. Other metals such as Cr, Mn, Fe, Ni, Cu, Zn, Cd, Pb, and Hg also stimulate MHO activity in the liver and other organs [35, 37].

5. Selenium Deficiency

Selenium deficiency predisposes rats to enhanced degradation of hepatic heme particularly following administration of the heme inducer, phenobarbital (PB). Normally, increased formation of the hemoprotein cytochrome P-450 is preceded by induction of heme [38]. In selenium deficiency induction of heme and of apocytochrome P-450 by PB are unaffected, but the PB-mediated increase in cytochrome P-450 is impaired [39]. Such impairment possibly results from a defect in the transfer or utilization of the newly induced heme for hemoprotein assembly. Consequently, in PB-treated selenium-deficient rats, a relative excess of heme prevails. Such an excess could, in turn, induce hepatic MHO activity in these rats.

II. CYTOCHROME P-450

Endogenous steroids and/or xenobiotics, such as drugs and endotoxins, are metabolized by the mixed function oxidase system usually to less active, more polar, and easily excretable products [21]. The system is localized in the endoplasmic reticulum of the liver, adrenal, lung, intestine and skin.

Two enzymes play key roles in the mixed function oxidase system: (a) a flavoprotein, cytochrome P-450 reductase and (b) hemoprotein, cytochrome P-450, the terminal oxidase [21, 40, 41].

Cytochrome P-450 is unique in that its substrates are capable of inducing it and thus accelerating their own metabolism and that of the other substrates [21, 40]. Different substrates induce different forms of the cytochrome, each possibly with a specific turnover time. Due to the prevalence of a wide variety of inducers in the environment and diet, cytochrome P-450 is a heterogeneous rather than a homogeneous population of enzyme forms. Consequently, its specific turnover reflects the composite of the turnover of its individual forms. Thus the cytochrome exhibits two major (composite) turnover times: a short one, of 7-10 hours and a longer one of 24-48 hours [42, 43]. These relatively short half-life times make the cytochrome one of the fastest turning over hemoproteins. The fast turnover of the cytochrome, coupled with its high hepatic concentration, could account for its high utilization (70%) of the heme synthesized in the liver [44].

III. RECIPROCAL RELATIONSHIP BETWEEN CYTOCHROME P-450 AND MHO

A reciprocal relationship between hepatic cytochrome P-450 content and MHO activity has been demonstrated in hepatocyte monolayer cultures [45, 46], and in rats following treatment with agents such as endotoxin [31] and cobalt [33]. Thus, following administration of these agents, a decrease in cytochrome P-450 occurs concomitantly with an increase in the MHO activity in rat liver.

Since MHO is a heme-inducible enzyme, it has been postulated that heme derived from degradation of cytochrome P-450 was responsible for the induction of the enzyme [32]. To validate this hypothesis, it has to be demonstrated that after treatment with chemical agents known to stimulate MHO activity: (a) cytochrome P-450 degradation precedes stimulation of MHO activity and (b) cytochrome P-450 heme is catabolized. The hypothesis can be tested in rats prelabeled with a specific radioactive hepatic heme precursor such as ^{14}C-δ-ALA. The specific decrease in the labeled cytochrome P-450 heme can then be monitored in these rats, following treatment with a stimulator of MHO activity, and compared with that in untreated rats.

IV. STIMULATION OF HEPATIC MHO BY MORPHINE

A. Introduction

Sladek et al [47] demonstrated that morphine treatment of mature male rats for four days resulted in a 40% decrease in the hepatic cytochrome P-450 content. This decrease in cytochrome P-450 could be due to inhibited synthesis of the hemoprotein or to its accelerated degradation. Cytochrome P-450 formation requires synthesis of the apocytochrome and heme followed by coupling of these two moieties. Any defect in one or more of the above processes could effectively lower the cytochrome P-450 content. In contrast, an increased degradation rate of the hemoprotein could also account for its observed lower concentration. A decrease in cytochrome P-450 levels is often accompanied by stimulation of MHO activity and enhancement of heme oxidation [32, 33, 39]. Indeed, following morphine treatment of rats for four days, it was found that MHO activity in the liver increased concomitantly with the lowering in cytochrome P-450 level. Studies to be described in the current work attempt to delineate the mechanism of this morphine-mediated stimulation of MHO activity.

Morphine could enhance MHO activity by a direct mechanism or indirectly by increasing hepatic free heme, with subsequent substrate-mediated induction of the enzyme. Experimental and/or pathological conditions resulting in elevated levels of hepatic free heme and in consequent induction of MHO [6] support this hypothesis. Morphine-mediated over-production of newly synthesized heme, coupled with its under-utilization, could effectively produce similar results. In addition, intensive breakdown of hemoproteins can also result in an

abnormally large pool of free heme. Thus, excess heme derived from accelerated turnover of cytochrome P-450 could induce MHO activity and enhance heme degradation. For these reasons, the present studies were designed to investigate whether increased turnover of rat liver cytochrome P-450 or excess production of heme could account for the morphine-mediated stimulation of MHO activity.

B. Materials and Methods

1. Chemicals

NADPH, glutathione, pyridoxal 5'-phosphate, coenzyme A, hemin-hydrochloride, protease (type VI), were obtained from Sigma Chemical Company. ^{14}C-delta-aminolevulinic acid (^{14}C-ALA) was obtained from New England Nuclear Company. Morphine sulfate was obtained from Lilly Co.

2. Animals

Mature male Holtzman rats (210-260 g) were allowed free access to laboratory chow and water. Prior to every experiment the rats were starved overnight.

3. Treatment

Rats were injected intraperitoneally with morphine sulfate in saline (45 mg/Kg) and sacrificed at specified time intervals after injection. When "CO-particles" were prepared 5 μCi of ^{14}C-ALA was given to the rats by tail vein injection, 18 hours prior to morphine treatment.

4. Tissue Preparations

Livers were perfused in situ with isotonic KCl, removed, and homogenized in 0.1M Na^+-K^+-phosphate buffer, pH 7.4. Portions of the

homogenate were used for the different assays.

Microsomes. Liver homogenate was sedimented at 10,000 g for 10 minutes at 4°C, and the supernatant centrifuged at 105,000 g for an hour at 4°C. The pellet so obtained was resuspended in isotonic KCl and resedimented at 105,000 g at 4°C for 30 minutes. This pellet was resuspended in 0.1M Na^+-K^+-phosphate buffer, pH 7.4, at the desired protein concentration.

Cytochrome P-450 particles. Cytochrome P-450 particles were prepared by using the subtilisin method of Comai and Gaylor [48] as follows: Microsomes were prepared in 0.1M phosphate buffer, pH 7.4, containing 10mM nicotinamide and 2mM glutathione. Glycerol (20% v/v) was added to the microsomal suspension (10-12 mg protein/ml). Following addition of subtilisin (Sigma "protease" type VII, 10 µg/mg microsomal protein) the microsomal suspension was incubated for 15 hours at 4°C with agitation and then sedimented for 1 hour at 105,000 g. The pellet so obtained was washed once in order to remove the subtilisin and resuspended in phosphate buffer, pH 7.4. The microsomal pellet obtained after subtilisin treatment contained cytochrome P-450 almost exclusively with negligible amounts of cytochrome b_5, indicating almost complete solubilization of the latter.

5. Analytical Determinations

MHO activity. Liver homogenate was sedimented at 10,000 g for 10 minutes at 4°C. The supernatant consisting of microsomes and cytosol was used to measure MHO activity. The method of Tenhunen et al [1] as modified by Correia and Schmid [24] was employed. Methemalbumin (25:1, heme:albumin) was used as a substrate, since it was found that heme

loosely associated with protein is the best substrate for the enzymatic assay [1].

The final reaction mixture consisted of: methemalbumin, 4mM; phosphate buffer, 0.1M, pH 7.4; liver supernatant, 1.5-2.0 mg/ml. NADPH (0.5mM) was used to initiate the reaction, and the formation of bilirubin at 37°C was monitored at 468 nm by difference spectrophotometry in an Aminco DW-2 spectrophotometer. The extinction coefficient of bilirubin employed was 60 $mM^{-1}cm^{-1}$. The activity of MHO was expressed as n moles bilirubin formed per mg protein per 10 minutes.

Cytochrome P-450 and b_5. Cytochromes P-450 and b_5 were measured in homogenate, microsomes and cytochrome P-450 particles, by difference spectrophotometry in an Aminco DW-2 spectrophotometer. Cytochrome P-450 was determined by the CO-reduced minus CO-difference spectrum described by Raj and Estabrook [49]. Cytochrome b_5 was estimated by the NADH reduction as described by Omura and Sato [50].

Protein. Protein content was assayed by the method of Lowry et al [51] using bovine serum albumin as the standard.

^{14}C-heme. ^{14}C-heme was measured in liver homogenate, microsomes and CO particles of rats previously injected with a radioactive heme precursor. A measured amount of carrier heme was added to the tissue samples. The carrier consisted of rat erythrocyte-hemoglobin. The erythrocytes were washed and suspended in equal volume of isotonic saline. The concentration of hemoglobin was determined with the Hycel Cyanmethemoglobin Reagent (Hycel) and the heme concentration was thus calculated. One ml of the carrier containing about 6 mg of heme was mixed with one or one-half ml of the tissue sample.

Heme was isolated according to the method of Labbe and Nishida [52].

The method involved precipitation of protein and extraction of the heme in a boiling solution of $SrCl_2$ in acetic acid/acetone followed by crystallization of the extracted heme. After sequential washes with 50% acetic acid, water, ethyl alcohol and ether, the heme crystals were dried under vacuum overnight. A weighed amount of the dried crystal (about 0.5 mg) was dissolved in 1N NaOH (0.2 ml) and the solution was then bleached with 30% hydrogen peroxide (0.2 ml). After the scintillation fluid (Dimilume-30 Packard I Instrument Company, Inc., Santa Clara, CA) was added, the radioactivity was determined in a Beckman LS-250 spectrometer.

The total radioactivity (DPM) of each sample was calculated by knowing the amount of heme initially added, the weight of the isolated tissue heme and its radioactivity (CPM). The amount of tissue heme in the sample is negligible in comparison with the amount of carrier heme added and, therefore, was not taken into consideration.

Microsomal lipid peroxidation. Microsomal lipid peroxidation has been incriminated in the degradation of cytochrome P-450 [53, 54]. Microsomal lipid peroxidation is catalyzed by the microsomal flavoprotein, NADPH-cytochrome-c-reductase, and requires molecular oxygen and NADPH. This enzymatic reaction can be monitored in various ways [55]. Of these, we chose to follow the production of malonaldehyde, one of the lipid peroxidation products.

Washed microsomes were incubated in the presence of NADPH (0.1mM), NADPH-generating system: glucose-6-phosphate (1.5mM) and glucose-6-phosphate dehydrogenase (0.6 units/ml), $MgCl_2$ (2mM), phosphate buffer (0.1M), pH 7.4, and morphine or naloxone (0.3mM). Control reaction mixtures excluding NADPH, morphine or naloxone were included. Aliquots

(3 ml) of the mixture were drawn at different times during the incubation. The cytochrome P-450 level and malonaldehyde were assayed in each sample. The latter was measured as follows. Proteins in the reaction mixture were precipitated with an equal volume of TCA (20%). Aliquots of the supernatants were then reacted with thiobarbituric acid (67%) and the color intensities were measured at 535 nm.

Since the reaction mixtures met all the requirements for the mixed function oxidase, it could adequately support the metabolism of morphine and naloxone. Thus, the effects of such metabolites could be followed in the same reaction mixture.

ALA-synthetase (ALAS) activity. Liver homogenate (10% w/v) was prepared in 0.1mM pyridoxal phosphate-9% NaCl-0.01M Tris buffer, pH 7.4. For measuring ALAS activity, the radiochemical microassay of Strand et al [56], was employed. The assay essentially consisted of two parts. The first part involved incubation of the homogenate at 37 ^{o}C with ^{14}C-succinate, a precursor of ALA. In the second part, the ALA so formed was isolated from the other constituents of the reaction mixture by using three sequential ion-exchange columns. The calculation of the amount of ALA formed in the reaction was based on: (a) the specific activity of ^{14}C-succinic acid initially added to the reaction mixture (Since one molecule of succinic acid yields one molecule of ALA, the specific activity of the succinic acid was the same as that of the ALA formed) and (b) the amount of labeled ALA formed. The ALAS activity is expressed as p moles ALA formed/mg protein/30 minutes.

C. Results

1. The Effect of Morphine on Cytochrome P-450

Morphine treatment (45 mg/Kg) intraperitoneally for four days produced a 30% decrease in the cytochrome P-450 level in the rat liver. These results confirmed previous studies carried out with a lower dose of morphine (20 mg/Kg) [47]. In the present study, morphine at 20 mg/Kg failed to lower cytochrome P-450. For this reason, a higher dose, i.e., 45 mg/Kg was used in all subsequent experiments. Doses higher than 45 mg/Kg, although yielding more pronounced effects, could not be administered due to high mortality of the animals at these doses.

2. Morphine-Mediated Stimulation of MHO Activity

Following morphine treatment of rats for four days, the decrease in hepatic cytochrome P-450 level was paralleled by an elevation in MHO activity in the liver. In subsequent experiments this effect was shown to occur at earlier time periods and with a single injection of morphine. Table 1 shows the changes in liver cytochrome P-450 content at 1, 2, 4, 6 and 8 hours after morphine treatment. A slight but significant decrease in cytochrome P-450 was observed at 1 hour with a maximum decrease observed at 6 hours following morphine administration. In contrast to the effect on P-450, morphine significantly stimulated MHO activity as early as 2 hours increasing to four-fold of the basal level at 8 hours following its administration. The experimental period was limited to the initial 8 hour interval of morphine treatment in subsequent studies.

3. Effect of Morphine on Cytochrome P-450-Heme Turnover

The turnover of hepatic cytochrome P-450-heme was monitored in cytochrome P-450 particles from morphine-treated rats prelabeled with

^{14}C-ALA, a heme precursor. Due to intergroup variations in labelling, the results of three individual experiments could not be pooled and, therefore, are documented separately (Table 2).

The radioactive content of cytochrome P-450-heme decreased by 34% and 32%, respectively, 6 hours following administration of morphine (Table 2, Experiments 2 and 3). The average half-life for cytochrome P-450 in morphine-treated rats, calculated on the basis of the data in Table 2, is 8 hours. This value corresponds to the fast turnover time of cytochrome P-450. However, contrary to expectation, this value failed to significantly differ from that of control (untreated) rats obtained in the present study (Table 2) and in other studies in which untreated rats were used [42, 43].

4. In Vitro Effect of Morphine and Naloxone on Lipid Peroxidation and Cytochrome P-450

Degradation of microsomal cytochrome P-450 *in vitro* in the presence of NADPH and molecular oxygen is associated with microsomal lipid peroxidation [53]. The flavoprotein NADPH cytochrome-c-reductase plays a key role in the peroxidation of microsomal membrane lipids. This flavoprotein is also a component of the cytochrome P-450 dependent drug metabolizing system.

A variety of xenobiotics (substrates of cytochrome P-450), particularly allyl compounds, all substrates of cytochrome P-450, were found to stimulate lipid peroxidation *in vitro* and to degrade cytochrome P-450 *in vivo* [53, 54]. Since administration of morphine lowered cytochrome P-450 *in vivo*, we investigated whether the decrease resulted from morphine-mediated stimulation of lipid peroxidation. For these reasons,

we studied the effect of morphine and its allyl congener naloxone on lipid peroxidation *in vitro*.

In vitro morphine (0.3mM) and naloxone (0.3mM) decreased the degradation of cytochrome P-450 and impaired lipid peroxidation (Table 3). Furthermore, despite its allyl-nature, naloxone protected microsomes from lipid peroxidation and decrease of cytochrome P-450 to a greater extent than morphine. These observations can be explained as follows: Both Lipid peroxidation and metabolism of morphine and naloxone require NADPH-cytochrome-c-reductase as an electron donor. Thus, in the presence of the drug, its metabolism could conceivably compete with the lipid peroxidative reaction for reducing equivalents from NADPH.

5. Effect of Morphine on Heme Synthesis

MHO activity can conceivably be stimulated by a relative excess of heme resulting from increased synthesis, coupled with its under-utilization. For these reasons, we investigated whether the morphine-mediated stimulation of MHO activity involved synthesis of heme. The first and the rate-limiting step in the pathway of heme synthesis is the formation of δ-Aminolevulinic acid (ALA) catalyzed by δ-Aminolevulinic acid-synthetase (ALAS) [57, 58]. ALAS is under feedback inhibition by the end product heme, and is inducible by drugs such as phenobarbital [59]. The effect of morphine on heme synthesis was examined by monitoring ALAS activity (Table 4). Significant increase in ALAS activity was observed only at 8 hours after morphine treatment. Direct inducers of ALAS such as phenobarbital usually cause a maximal elevation of the enzyme activity within much shorter time intervals [38]. Moreover, in the present studies the stimulation of the ALAS activity followed (rather than

preceded) the stimulation of MHO activity and subsequent degradation and lowering of intracellular heme. Consequently, these observations suggest that this increase in the enzyme activity is probably due to derepression of the enzyme rather than to its induction.

D. Discussion

The above observations indicate that morphine produces a rapid stimulation of hepatic MHO activity in rats. The mechanism of this effect is unclear. In an attempt to elucidate this morphine-mediated effect, it was investigated whether accelerated turnover of cytochrome P-450 and the release of its heme could account for the stimulation of MHO activity (mechanism schematically illustrated in Fig. 3a). Although cytochrome P-450 content was lowered following morphine administration, we failed to observe any significant difference in cytochrome P-450-heme turnover between untreated and morphine-treated rats. Thus, the hypothesis that morphine stimulated MHO activity by accelerating cytochrome P-450-heme turnover could not be substantiated in the present study. Further work will be required to clarify this finding.

Alternatively, the morphine-mediated decrease in cytochrome P-450 content, in the absence of any alteration of its turnover, may be explained as follows: Metabolites of a variety of drug substances such as amphetamines [62] and methylenedioxyphenyl derivatives [63] have been shown to bind cytochrome P-450 and, on chemical reduction of such cytochrome P-450 metabolites complexes, to yield spectral absorption maxima of either 430 or 455 nm [62, 63]. Furthermore, such formation has been shown to inhibit the binding of CO to the cytochrome and, consequently, to interfere in its spectral determination [64].

In vivo formation of such inhibitory complexes by morphine metabolites could theoretically account for the partial decrease in cytochrome P-450 content observed in Table 1. However, the observations in Table 3 fail to support such a hypothesis, since the presence of morphine and/or its metabolites in the reaction mixture increased rather than decreased microsomal cytochrome P-450 content. Furthermore, difference spectrophotometry of a reaction mixture containing microsomes reduced with NADPH, morphine and morphine metabolites, versus a mixture containing an equivalent of reduced microsomes in the absence of morphine or its metabolites, yielded no detectable spectral absorption between 400 and 500 nm, indicating the absence of any inhibitory metabolite-cytochrome P-450 complexes. Thus, the reduction in cytochrome P-450 content cannot be attributed to inhibitory complex formation.

Other possible mechanisms for morphine-mediated stimulation of hepatic MHO activity include: (a) Morphine-mediated induction of heme resulting in an elevation of intracellular heme pools and consequent stimulation of MHO activity. This possibility was excluded on the basis that the stimulation of heme synthesis (ALAS activity) occurred much later than that of MHO activity, very likely as a result of the increased oxidation of heme. (b) Intensive breakdown of RBC with release of hemoglobin-heme followed by MHO induction. It is unlikely that this is the mechanism, because RBC breakdown results in a much more delayed response of hepatic MHO [6] than that observed in the current study. (c) A direct induction or an activation by morphine of hepatic MHO in the liver by a mechanism which is independent of heme (Fig. 3b). Existence of this mechanism has not yet been proved for any MHO stimulator.

The present study involved only rats, whereas the effect of morphine on hepatic MHO activity in other species is still unknown. In mice, at doses slightly higher than those employed in this study, a decrease in hepatic cytochrome P-450 level was observed [60]. It can be speculated that in mice morphine also could stimulate hepatic MHO activity. The importance of these findings when extrapolated to humans remains to be determined. Most agents known to stimulate MHO, such as cobalt, are potential toxins. In contrast, morphine is widely used not only clinically but also as a drug of abuse. Furthermore, morphine is present in high concentrations in the bloodstream of heroin addicts, since heroin is rapidly metabolized to morphine in vivo [61]. In humans, if chronically elevated blood levels of morphine were to stimulate hepatic MHO activity, then the following clinical features could be expected: (a) hyperbilirubinemia and/or (b) breakdown of cytochrome P-450 with consequent lowering of drug metabolism. However, the above symptoms are not typical of heroin addicts.

Assessment of the morphine-dependent effect on human heme metabolism is complicated by multiple drug intake. Barbiturates are often ingested along with heroin. Thus, the effect of morphine on heme catabolism may be masked by concomitantly ingested barbiturates which are known stimulators of heme synthesis. Although the above mentioned clinical features are not readily apparent, they should not be overlooked in the diagnosis of addicts.

Table 1

Effect of Morphine on Hepatic Cytochrome P-450 Content and MHO Activity

Treatment (HR)	N	Cytochrome P-450*	N	Heme Oxygenase Activity**
0	8	1.13 ± 0.15	8	0.12 ± 0.04
1	4	0.92 ± 0.05[b]	4	0.15 ± 0.01[c]
2	9	0.92 ± 0.10[a]	9	0.18 ± 0.07[b]
4	8	0.90 ± 0.10[a]	7	0.24 ± 0.03[a]
6	7	0.79 ± 0.20[a]	7	0.32 ± 0.13[a]
8	5	0.86 ± 0.16[b]	5	0.42 ± 0.13[a]

* n mole/mg microsomal protein.

** n mole Bilirubin formed/mg protein/10 min.

N = number of rats in each experiment.

Statistical difference from control values:

a $p < 0.005$
b $p < 0.05$
c not significant

Rats were treated with morphine sulfate (45 mg/Kg) intraperitoneally at 0 HR and sacrificed at the time points indicated.

Table 2

Effect of Morphine on the ^{14}C-Heme Content of Cytochrome P-450

Sacrifice Time (HR)	Control* DPM**		Morphine-Treated* DPM**					
	N	Experiment 1	N	Experiment 2	N	Experiment 3	N	Experiment 4
0	1	42,195	2	16,967	2	51,201		---
1	1	42,587	2	16,227	1	44,806	1	34,993
2		---	1	13,779	2	39,998	1	27,396
4	1	29,119		---	2	35,358	1	24,434
6	1	24,837	3	11,230	1	34,832	1	19,501
		$t_{1/2}$= 7 HR		$t_{1/2}$= 8.9 HR		$t_{1/2}$= 9 HR		$t_{1/2}$= 6 HR

*Morphine Sulfate (45 mg/Kg i.p.) administered to rats at 0 HR; control rats received saline.

**DPM due to ^{14}C-labeled cytochrome P-450 heme/g liver.

^{14}C-Heme content of cytochrome P-450 was determined in control and morphine-treated rats [Methods].

Table 3

Lipid Peroxidative Effects of Morphine and Naloxone on Microsomal Cytochrome P-450 and Malonaldehyde Formation

Additions	Microsomal Cytochrome P-450 (% decrease from initial concentration)	Malonaldehyde Formation (% of control)
None	66	100
Morphine (0.3 mM)	36	31
Naloxone (0.3 mM)	20	41

Rat liver microsomes were incubated in the presence of NADPH (0.1 mM) and in the presence or absence of morphine or naloxone for 60 minutes at 37°C, as described [Materials and Methods]. Cytochrome P-450 content was determined at 0 (100%) and 60 minutes following incubation. Malonaldehyde formed concomitantly in these incubation mixtures was also determined. The amount of malonaldehyde produced in the reaction mixture in the absence of either drug was taken as 100%.

Table 4

Effects of Morphine on MHO and ALAS Activities

Treatment (HR)	N	Heme Oxygenase Activity*	N	ALAS Activity**
0	8	0.12 ± 0.04	4	103.3 ± 39.2
1	4	0.15 ± 0.01[c]		---
2	9	0.18 ± 0.07[b]	3	248.5 ± 106.3[c]
4	7	0.24 ± 0.03[a]	5	257.1 ± 65.5[c]
6	7	0.32 ± 0.13[a]	5	269.9 ± 72.9[c]
8	5	0.42 ± 0.13[a]	5	403.1 ± 138.5[b]

* n moles Bilirubin formed/mg protein/10 min.

** P moles ALA formed/mg protein/30 min.

N = number of rats in each experiment.

Statistical difference from control values:

a $p < 0.005$
b $p < 0.05$
c not significant

Rats were treated with morphine (45 mg/Kg) intraperitoneally at 0 HR and sacrificed at the time points indicated.

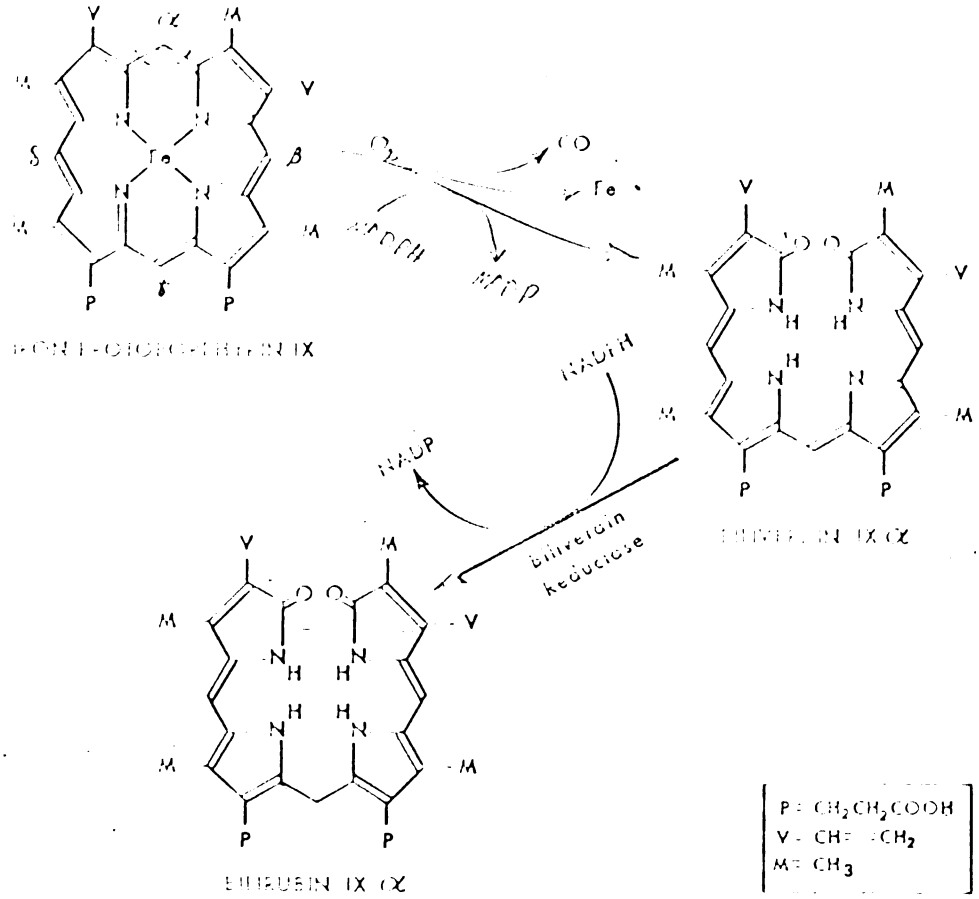

Fig. 1: Pathway of Heme Degradation

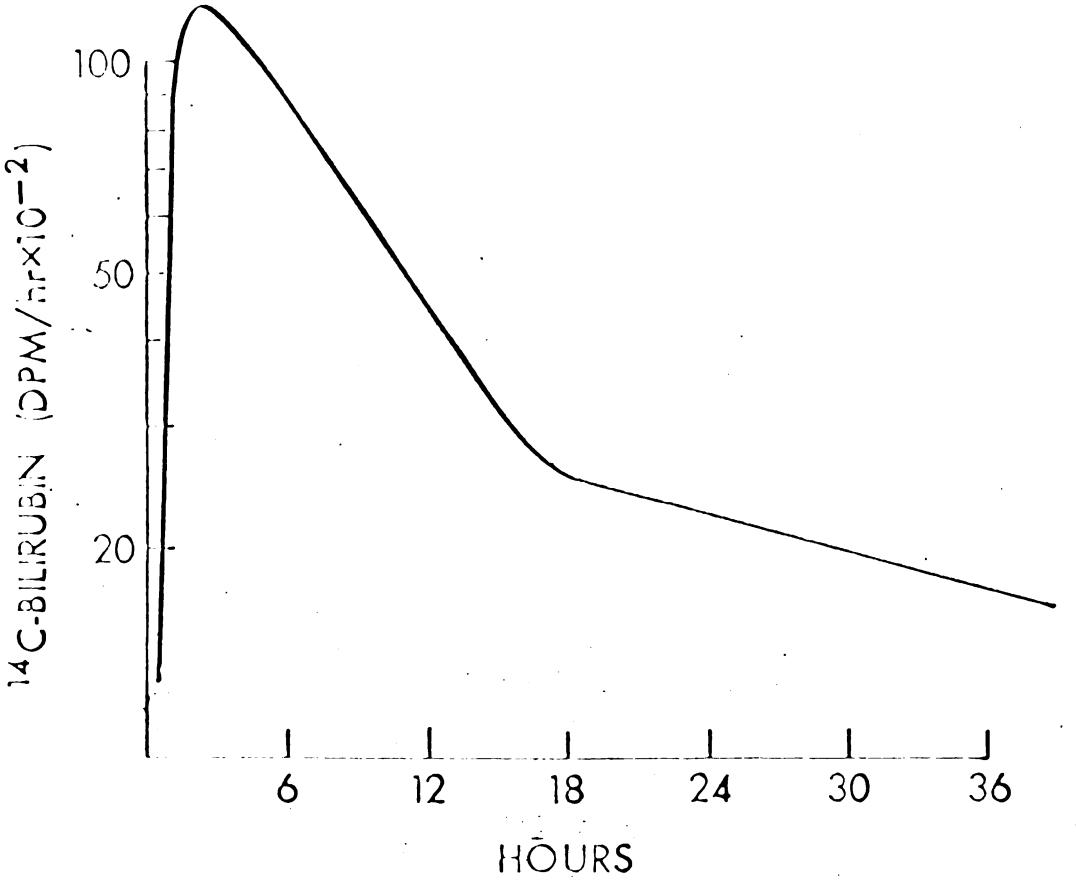

Fig. 2: Formation of [^{14}C] bilirubin in bile after administration of [2-^{14}C] glycine. (Copied with permission from Bissell, D. M.: Formation and Elimination of Bilirubin. Gastroenterology 69: 519-538, 1975.)

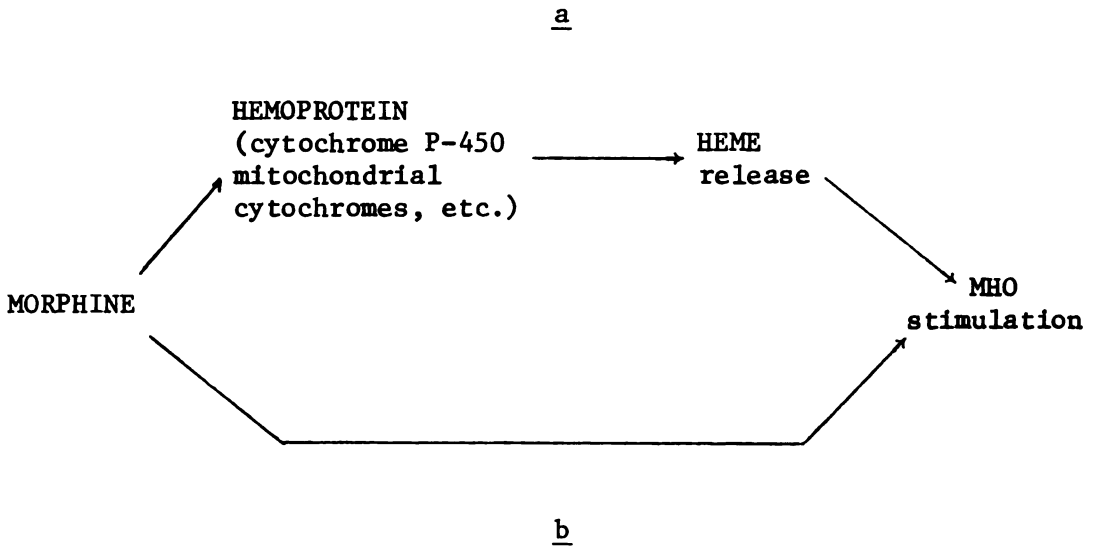

Fig. 3: The hypothetical mechanisms for morphine-mediated MHO stimulation.

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