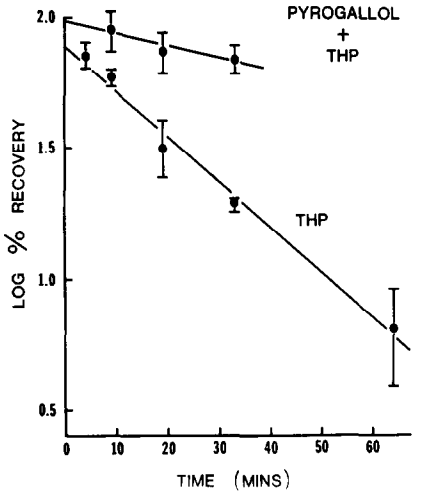

Fig. 2. Log of the percentage injected THP ± S.E.M. recovered at different times after the intraventricular injection of 10.0 μg THP, with (N = 8) and without (N = 18) prior intraperitoneal administration of 250 mg/kg of pyrogallol.

mg/kg i.p. of the catechol-*o*-methyltransferase inhibitor, pyrogallol [12], prior to the injection of salsolinol increased the half-life of this compound to 23.1 min. This supports the evidence already available that salsolinol serves as a good substrate for *O*-methylation [10].

In chronic infusion experiments, an infusion rate of 40 ng every half-hour, the lowest dose of salsolinol tested, caused an increase in alcohol consumption [8]. With a half-life of 12.5 min, it can be calculated from the equation

$$X^{oo} = \frac{X_0}{1 - e^{-k_0 td}}$$

(where X^{oo} is the amount of compound at $t = oo$, X_0 = the dose, k_0 = the disappearance rate constant and td is the dosing interval) that the peak amount steady state would be 49.0 ng per whole brain. Assays easily capable of detecting this amount of salsolinol or *O*-methyl salsolinol are available [13].

The amount of THP present in the brain at various intervals as a percentage of that given after injection is shown in Fig. 2. From the amount of THP recovered at different time points, a half-life of 17.3 min was calculated. The administration of 250 mg/kg of pyrogallol extended the half-life of THP to 69.3 min. This is a greater increase in $t_{1/2}$ than that observed for salsolinol and argues for the fact that *O*-methylation is a route of metabolism for this compound *in vivo* [10].

The lowest dose of THP tested in the chronic infusion experiments was 0.4 ng every half-hour [7]. With a half-life of 17.3 min, it is calculated that the maximum amount present at steady state would be 0.6 ng in the whole brain (0.3 ng/g). This is below the level of detection (2 ng/g) for the most sensitive assay which has yet been used to determine whether or not TIQs form after the ingestion of alcohol [11]. It is likely that even lower doses than those used in the original infusion experiments would be capable of altering behavior.

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Department of Pharmacology, University of Colorado Medical Center, Denver, CO 80262, U.S.A.

CHRISTINE L. MELCHOR
ALLAN MUELLER
RICHARD A. DEITRICH

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Morphine-induced depression of the hepatic microsomal drug-metabolizing enzyme—effect on the lipid component

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The oxidation of drugs in the liver is mediated primarily by an enzyme complex consisting of the proteins, cytochrome P-450 and NADPH-cytochrome P-450 reductase, and the phospholipid, phosphatidylcholine [1–5]. Resolution and reconstitution of the enzyme system have revealed the role of each component in the overall catalysis which requires molecular oxygen and NADPH [1]. The hemeprotein, cytochrome P-450, is the site of substrate binding, oxygen activation and oxidation. NADPH-cytochrome P-450 reductase functions as an electron carrier, transferring reducing equivalents from NADPH to cytochrome P-450. The role of the phospholipid is to provide a suitable

environment for the formation of a functional complex of the reductase and the hemeprotein and, hence, to facilitate the electron transfer and overall catalysis.

The enzyme activity is controlled by several factors including the administration of drugs, hormonal imbalance, age and sex [6–8]. In contrast to a large number of drugs which induce drug metabolism [6,7], Cochin and Axelrod [9] have shown that the administration of morphine and levorphanol to male rats depressed hepatic drug oxidation. Other investigators [10–12] have made similar observations, and Sladek *et al.* [12] reported a decline in cytochrome P-450 content in the microsomal fraction of morphine-treated male rats. Because the enzymatic activity is dependent not only on the protein but also on the lipid component of the enzyme complex, we have examined the effect of morphine treatment on the hepatic microsomal phospholipid content in this study. Some of the data have been reported previously [13].

All experiments were carried out with adult male Sprague–Dawley rats, weighing 200–300 g, purchased from Flow Laboratories (Dublin, VA). The animals were housed in groups of three or four in plastic cages with bedding and received Purina laboratory chow and water *ad lib.* They were maintained in a room at 25° with a light and dark cycle of 12 hr. Rats were rendered morphine dependent by the pellet implantation method of Way *et al.* [14]. The experimental group received (subcutaneously below the dorsal neck region) one pellet containing 100 mg of morphine base, 100 mg microcrystalline cellulose and 2 mg silica as fillers [15] on day 1 and day 4 and was killed on day 6. The control animals received placebo pellets in the same schedule as the experimental. We have ascertained that that animals treated with morphine-containing pellets were morphine dependent, since they exhibited typical withdrawal symptoms, such as diarrhea, wet dog shakes and teeth chattering, following the administration of naloxone.

The hepatic microsomal fraction was isolated by differential centrifugation. The animals were decapitated and the livers were excised and placed in ice-cold 0.25 M sucrose–0.05 M Tris–HCl (pH 7.4) solution. The following manipulations were carried out at 4°. The organs were washed several times in the same medium to remove blood, minced, and homogenized in 3 vol. of sucrose–Tris solution in a Waring blendor for 40 sec. The homogenate was centrifuged at 600 *g* for 10 min and the pellet discarded. The supernatant fraction was centrifuged at 10,000 *g* for 10 min and the microsomal fraction was precipitated by submitting the 10,000 *g* supernatant fraction to centrifugation at 105,000 *g* for 1 hr. The microsomal pellets isolated from the placebo and morphine-treated animals were suspended in 0.05 M Tris–HCl (pH 7.4) containing 20% (v/v) glycerol and stored at −20° overnight. Enzyme assays and lipid extraction were performed the following day. No change in enzymatic activities was observed when the microsomal preparation was stored at −20° in the presence of glycerol for 1 week.

The procedure for the determination of the rate of *N*-demethylation of *p*-chloro-*N*-methylaniline (PCMA) was reported earlier [16]. The incubation mixture consisted of 50 mM Hepes–Na buffer (pH 7.4), 2.5 mM PCMA, 20 mM $MgCl_2$, and NADPH-generating system and the microsomal fraction (0.1 to 0.3 mg protein) in a final volume of 1.0 ml.* The reaction was initiated with the addition of 0.1 ml of the NADPH generating solution made up of 1 mM $NADP^+$, 5 mM glucose-6-phosphate and 2 I.U. of glucose-6-phosphate dehydrogenase. The mixture was incubated for 30 min at 37° in a metabolic shaker in air, and the reaction was terminated by the addition of 0.20 ml of a 30% (w/v) trichloroacetic acid (TCA) solution. After standing for 5 min, the reaction mixture was centrifuged at 10,000 *g* for 2 min in an Eppendorf centrifuge, and fluorescamine dissolved in acetone was added to an aliquot of the clear supernatant fraction. The fluorophore formed by coupling the *N*-demethylated product of PCMA, *p*-chloroaniline (PCA), with fluorescamine was extracted with ethyl acetate, and the fluorescence of the organic phase was measured in an Aminco–Bowman spectrofluorometer with excitation and emission wavelengths of 392 and 490 nm respectively. Standards and controls were run as the experimental except that TCA was added immediately after the NADPH solution. Under the conditions described above, the reaction was found to be linear with time and microsomal protein content.

Glucose-6-phosphate activity was estimated according to Harper [17], with slight modification. The incubation mixture contained 50 mM Hepes–Na (pH 6.5), 70 mM glucose-6-phosphate and the microsomal fraction (0.05 to 0.07 mg protein) in a final volume of 0.30 ml. The reaction was started by adding the substrate and allowed to proceed for 15 min at 37° in a metabolic shaker. Following the addition of 1.0 ml of 10% (w/v) TCA solution and centrifugation at 10,000 *g* for 2 min, an aliquot of the supernatant fraction was removed for the estimation of inorganic phosphate, determined according to Ames and Dubin [18].

Phospholipids were extracted from microsomes according to Rouser and Fleisher [19]. The microsomal suspension (2.0 ml, 10 mg protein) was homogenized with 20 ml of a chloroform/methanol solution (2/1, v/v) in a Potter–Elvehjem homogenizer at 4°. After centrifugation at 2000 *g* for 2 min, the lower organic phase was removed carefully and the aqueous phase extracted two more times with 10 ml of the chloroform/methanol mixture. The organic extracts were combined. An aliquot of the extract was evaporated, digested with $Mg(NO_3)_2$ and the pyrophosphate hydrolyzed with HCl. Inorganic phosphate was estimated by the procedure of Ames and Dubin [18]. The cytochrome P-450 content of the microsomal fraction was measured by the method of Omura and Sato [20], after solubilization with Triton X-100 as described [21]. NADPH-cytochrome *c* reductase was determined according to Masters *et al.* [22], except that the concentration of the phosphate buffer (pH 7.7) was raised to 0.3 M. The protein content was estimated by the method of Lowry *et al.* [23], with crystalline bovine serum albumin as standard.

In agreement with earlier studies [9–12], we observed a decline in hepatic microsomal drug oxidation in morphine-dependent male rats, as shown in Table 1. The *N*-demethylase activity, measured with PCMA as substrate, decreased 70 per cent, whereas the decline of cytochrome P-450 and NADPH-cytochrome *c* reductase activity was 25 and 15 per cent respectively. The large difference between the reductase of PCMA *N*-demethylation and that of cytochrome P-450 and NADPH-cytochrome *c* reductase suggests that the third component of the enzyme complex, the phospholipid, may have been affected by morphine treatment also. Assay of the phospholipid content of the hepatic microsomal fraction of morphine- and placebo-treated rats revealed a decrease of 22 per cent. Thus, chronic morphine treatment resulted in a decrease not only of the protein, but also of the lipid component of the hepatic microsomal mono-oxygenase.

The hepatic microsomal fraction contains several membrane-bound, lipid-dependent enzymes. It has been observed that the activity of glucose-6-phosphate, a phospholipid-dependent enzyme [24], was lowered to 60 per cent of the control level. The data do not reveal whether the decline in enzyme activity is due to a decrease in the enzyme level and/or phospholipid content of the microsmal fraction.

The alteration of the phospholipid content by morphine is significant, since the microsomal fraction contains several enzymes which require lipid for catalysis. The observed decline in glucose-6-phosphatase activity, a phospholipid-

* Hepes = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Table 1. Effects of morphine treatment on the activity and components of mono-oxygenase and on glucose-6-phosphatase activity in rat liver microsomes*

Component	Treatment	
	Placebo	Morphine
PCMA *N*-demethylation (nmoles PCA/min/mg protein)	18.6 ± 3.5 (4)	5.7 ± 0.8 (4)
Cytochrome P-450 (nmoles/mg protein)	0.37 ± 0.01 (4)	0.28 ± 0.03 (4)
NADPH-cytochrome *c* reductase (nmoles cytochrome *c* reduced/min/mg protein)	53.3 ± 4.8 (4)	45.3 ± 6.9 (4)
Glucose-6-phosphatase (μmoles inorganic phosphate/min/mg protein)	0.21 ± 0.02 (3)	0.12 ± 0.06 (3)
Phospholipid (μmoles inorganic phosphate/mg protein)	0.44 ± 0.06 (3)	0.33 ± 0.06 (3)

* All values are expressed as the mean ± S.E.M. The mean values of PCMA *N*-demethylation, cytochrome P-450, NADPH-cytochrome *c* reductase, glucose-6-phosphatase and phospholipid of the microsomal fraction from morphine-treated rats are different from placebo-treated animals at the $P > 0.05$ level, as calculated by student's *t*-test. The values in parentheses refer to the number of animals in each group.

dependent enzyme [24], was not unexpected, and the reported diminished glucuronyl transferase activity [25] could be due in part to the altered lipid composition. Other microsomal lipid-dependent enzymes may be affected similarly.

The mechanism of morphine-induced impairment of hepatic drug oxidation is not clear. It appears that the decrease in drug oxidation is not elicited by a direct action of the opiate on the liver cell. Amzel [26] failed to observe a change in *N*-demethylase activity in enzymatically isolated liver cells when they were incubated for 2 hr with the opiate. These findings suggest that the primary site of action of the opiate leading to a depression of hepatic drug oxidation must be sought elsewhere.

In summary, adult male rats were rendered morphine dependent by the pellet implantation method. Cytochrome P-450-dependent mono-oxygenase activity, cytochrome P-450 content, NADPH-cytochrome *c* reductase activity, phospholipid content and glucose-6-phosphatase activity in the hepatic microsomal fraction were found to be lowered. These results indicate that chronic morphine treatment affects not only the protein, but also the lipid component of the drug-metabolizing enzyme.

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Department of Medicinal Chemistry, School of Pharmacy, University of Maryland, Baltimore, MD 21201, *U.S.A.*

VIVIANA AMZEL*
THEO A. VAN DER HOEVEN†

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* Some of the work was performed in partial fulfillment of the requirements for the Ph.D. degree at the University of Maryland at Baltimore, MD. Holder of Dunning Fellowship. Present address: Department of Biophysics, The Johns Hopkins University, School of Medicine, 725 North Wolfe St., Baltimore, MD 21205.

† Present address: Department of Pharmacology and Experimental Therapeutics, Albany Medical College, Albany, NY 12208. Send reprint requests to this address.

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Stimulation and inhibition of cyclic AMP formation in isolated rat fat cell by prostacyclin (PGI_2)

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Since prostaglandins may be formed by adipose tissue and prostaglandins of the E-series are known to inhibit cyclic AMP formation and lipolysis in fat cells, a feed-back-regulatory role for these compounds has been proposed (for ref. see [1,2]). However, for several reasons it has been concluded that a significant physiological role for E-prostaglandins is unlikely [2–5]. After the isolation of prostaglandin endoperoxides, it was suggested that these compounds, rather than their metabolites (PGE's), played a regulatory role [6]. However, using intact fat cells we found that PGH_2 was considerably less potent than PGE_2 as an inhibitor of cyclic AMP formation and concluded that neither the endoperoxides nor the thromboxanes were likely to play a role as antilipolytic regulators [7]. Recently, still another biologically active metabolite of the prostaglandin endoperoxides, (5*Z*)-9-deoxy-6,9a-epoxy-Δ^5-$PGF_{1\alpha}$ (prostacyclin or PGI_2), was isolated and characterized by Vane and co-workers [8,9]. Since PGI_2 has similar effects as the PGE's in several systems, but is more potent [10,11], the effect of the PGI_2 on cyclic AMP formation in rat fat cells was tested.

Fat cells were isolated from male Sprague–Dawley rats (180–220 g) and incubated at a concentration of 50–150,000 cells/ml as described earlier [12]. Cyclic AMP accumulation was stimulated by noradrenaline (as the hydrochloride, Sigma, NA) in the presence or absence of theophylline (as the ethylenediamine salt, Oxyphylline, Astra). The incubation was terminated by trichloroacetic acid (final concentration 10%). After removing the TCA by extracting four times with ether, cyclic AMP content was determined directly on an aliquot of the deproteinized extract by the method of Brown *et al.* [13]. PGI_2 was dissolved in ethanol: 0.05 M Tris buffer, pH 9 (9:1), in which it is stable at −70°. Immediately prior to use it was diluted in 0.5 M Tris buffer, pH 9. Aliquots (10 μl) of this solvent with or without PGI_2 were added per ml of the incubate. The solvent *per se* had no effect on the fat cells. N^6-Phenylisopropyl adenosin (PIA), a kind gift of Dr. H. Storck of Boehringer, Mannheim, was dissolved in water.

NA (10^{-6}M) caused a rapid increase of fat cell cyclic AMP levels both in the absence and in the presence of theophylline (10^{-3} M). In both cases a maximal or close to maximal cyclic AMP level was found after 10 min incubation (Fig. 1). The accumulation was inhibited by a high (10^{-6} M) but not by a low (10^{-8} M) concentration of PGI_2. The inhibitory effect was also essentially maximal after 10 min incubation and decreased thereafter. Thus, for all subsequent studies this time of incubation was used.

In Fig. 2 the effect of preincubating the cells with PGI_2 before the addition of NA is shown. The inhibitory effect of PGI_2 (10^{-7} and 10^{-6} M) decreased as a consequence of preincubation. Thus the percentage inhibition caused by 10^{-7} M PGI_2 decreased from 34 ± 4 to 7 ± 4 per cent and that caused by 10^{-6} M from 68 ± 2 to 47 ± 5 per cent. This fall in inhibitory potency by incubation tallies with the known instability of PGI_2 in aqueous solution [9]. Two

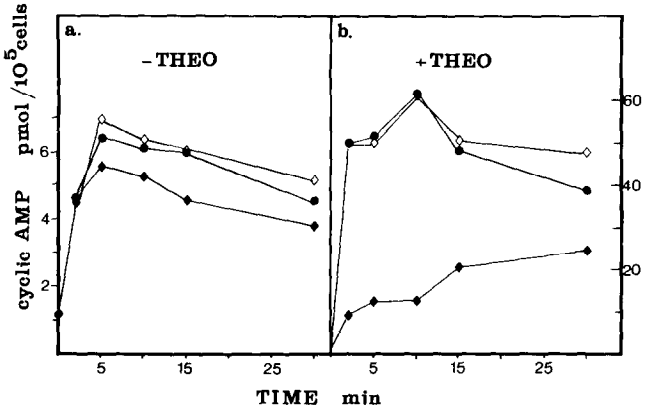

Fig. 1. Time-course of cyclic AMP accumulation in fat cells (85,000 cells/ml) following the administration of noradrenaline (1 μM) in the absence (●——●) and presence of PGI_2 (10^{-8} (◇——◇) and 10^{-6} M (◆——◆)). Panel a: no theophylline present. Panel b: theophylline (1 mM) added together with the other drugs. Mean of triplicate determinations.