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EFFECT OF PHENOBARBITAL PRETREATMENT ON THE METABOLISM AND BILIARY EXCRETION OF METHADONE*

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Abstract—The effect of phenobarbital (PB) pretreatment on the biliary excretion of methadone in rats was studied. Possible mechanisms by which PB pretreatment altered the biliary excretion of methadone were considered and studies *in vitro* on the metabolism of methadone were correlated with findings *in vivo*. For the biliary excretion studies, ^{14}C-methadone was administered intravenously and biliary excretion measured in anesthetized renal-ligated rats in which the common bile duct was cannulated. PB pretreatment increased the biliary excretion of ^{14}C after ^{14}C-methadone administration. The different metabolites of methadone formed *in vivo* and excreted into bile were separated by thin-layer chromatography and quantitated. The biliary excretion of the metabolite which results from *N*-demethylation and cyclization of methadone was not altered by PB pretreatment. However, the biliary excretion of metabolites which result from further *N*-demethylation, hydroxylation and glucuronidation was increased by PB pretreatment. Several determinants of biliary excretion (i.e. bile flow, hepatic blood flow and metabolism), which are enhanced by PB pretreatment, could cause the observed increase in the biliary excretion of methadone. Of these possibilities, we feel the data best support the suggestion that enhancement of methadone metabolism by PB pretreatment is responsible for the increased biliary excretion of methadone in PB-pretreated rats. Furthermore, metabolism studies *in vitro*, using microsomes from PB-treated rats, support the suggestion that PB pretreatment enhances the metabolism of methadone *in vivo*.

Methadone is metabolized in the liver [1–3], and the major portion of the metabolites is eliminated in the feces via biliary excretion [4–6]. One system of methadone metabolism is the liver microsomal mixed-function oxidase system, where methadone undergoes *N*-demethylation followed by cyclization to form 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [7]. Further *N*-demethylation of EDDP leads to the formation of 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) [2]. Both EDDP and EMDP have been shown in man and rat after methadone administration.

Baselt and Casarett [8] reported a highly "water-soluble metabolite" of methadone which they tentatively identified as a glucuronide conjugate of a derivative of EDDP. More recently, however, Baselt and Bickel [9] showed that the water-soluble metabolite was actually the glucuronide conjugate of EMDP. Most interestingly, Baselt and Casarett [8] found that pretreatment of rats with phenobarbital (PB) decreased the biliary excretion of the metabolite EDDP. The decreased biliary excretion of EDDP in PB-pretreated rats was particularly interesting to us, since we had observed a decrease in the biliary excretion of morphine glucuronide in PB-pretreated rats [10]. The findings of Baselt and Casarett with methadone and our own with morphine were unexpected, since PB pretreatment is a general inducer of drug-metabolizing enzymes and has been shown to enhance the metabolism of both methadone and morphine *in vitro* [10–13]. Baselt and Casarett [8] suggested that the decreased biliary excretion of EDDP in PB-pretreated rats was due to induction of pathways for the further metabolism of EDDP. They did not, however, present evidence to support this suggestion.

The purpose of the present experiment was to study the effect of PB pretreatment on the biliary excretion and metabolism of methadone. Experiments *in vivo* were concerned with the effect of PB pretreatment on the biliary excretion of methadone, as reflected both by total excretion and by the relative proportion of the different methadone metabolites excreted into bile. Studies *in vitro* were performed to determine the effect of PB pretreatment on the metabolism of methadone by hepatic microsomes.

MATERIALS AND METHODS

Chemicals. Methadone (heptanone-2-^{14}C) hydrochloride (5 μCi/μmole) was purchased from Mallinckrodt Chemical Co. The radiochemical purity of the ^{14}C-methadone was 98 per cent, as judged by thin-layer chromatography in two different solvent systems. Phenobarbital sodium and pentobarbital sodium were purchased from Winthrop Laboratories and Abbott

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Laboratories respectively. The methadone metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), were obtained from the Lilly Research Laboratories. Uridine diphosphoglucuronic acid (UDPGA), nicotinamide adenine dinucleotide phosphate (NADP), isocitric dehydrogenase and DL-isocitric acid were obtained from Sigma Chemical Co. The thin-layer plates used were Gelman instant thin-layer chromatography media (i.t.l.c.), type SA.

The liquid scintillation mixture used for counting ^{14}C consisted of 50 mg 1,4 bis-[2-(5-phenyl-oxazole)]-benzene (POPOP) and 4 g 2,5-diphenyloxazole (PPO) dissolved in 1·0 l. toluene and 0·5 l. Triton X-100.

Treatment of animals. Sprague–Dawley male rats (300–400 g) were obtained from Spartan Research, Haslett, Mich. The rats were divided into two groups. The control group was injected with saline (2·0 ml/kg, i.p.) once daily for 4 days, and the PB-pretreated group was injected with phenobarbital sodium (75 mg/kg, i.p.) for 4 days. All animals were used 1 day after their last injection and were fasted during this 24-hr period with free access to water.

The rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.) and the femoral vein, femoral artery and common bile duct were cannulated, as described by Peterson and Fujimoto [14]. The renal pedicles were ligated and body temperature was maintained at 37°. After the body temperature had stabilized, 1·0 μCi ^{14}Cmethadone (0·24 μmole/kg) was injected into the femoral vein. Bile was collected in 15-min samples for 90 min and 0·3-ml blood samples were collected in heparinized tubes at 2, 5, 10, 15, 20 and 30 min after methadone administration. It should be noted that, due to induction of drug metabolism by PB pretreatment, it was necessary to give additional pentobarbital to the PB-pretreated rats in order to maintain anesthesia. Routinely, an additional 45 mg/kg of pentobarbital was given in three intraveneous injections during the 90 min of bile collection.

Analytical procedures. The amount of radioactivity in each bile or blood sample was determined by liquid scintillation counting, as previously reported [10]. The per cent of ^{14}C present as EDDP, EMDP and other metabolites in each bile sample was measured by first separating the metabolites with thin-layer chromatography and then counting specific areas of the developed chromatogram. A portion of each bile sample (0·02 to 0·05 ml) was spotted on Gelman i.t.l.c. sheets (type SA) and developed to a solvent height of 14 cm in two different solvent systems: (A) methanol–ammonium hydroxide (100:1·5), and (B) ethyl acetate–butanol–ethanol–ammonium hydroxide (50:30:15:0·5). The chromatogram was allowed to air-dry thoroughly and strips 2 cm wide and 1 cm high were cut from 1 cm below the origin to 1 cm above the solvent front. The ^{14}C content of each strip was measured by liquid scintillation with quench correction using 10 ml of the scintillation mixture. The amount of each metabolite on the chromatogram was expressed as a percentage of the total dis/min on the chromatogram. These percentages were used as the relative percentages of each metabolite in bile. The per cent of the administered dose for each metabolite was calculated using the relative percentage of the metabolite in a given bile sample, and the per cent of the administered dose of ^{14}C recovered in that same bile sample. All counting was performed on a Packard Tri-Carb liquid scintillation counter, model 3380 (Packard Instrument Co.). The R_f values of EDDP and EMDP were determined by using nonradioactive compounds and spraying the developed chromatogram with iodoplatinate reagent. The R_f values of all ^{14}C-labeled metabolites in bile were measured by placing the developed chromatogram on Kodak RP x-ray film for 1 week. Comparison between treatment conditions was tested for statistical significance using the Student *t*-test.

Gas–liquid chromatography (g.l.c.) was used to confirm the identity of the EMDP spot isolated on thin-layer chromatograms developed in solvent system B. A Hewlett–Packard, model 5700, gas chromatograph equipped with a $\frac{1}{4}$ inch diameter by 3 ft glass column and hydrogen flame detector was used. The column was packed with 3% OV-17 on 100–120 mesh, high performance Chrom-Sorb W. Chromatography was performed with a column temperature of 240°, injector temperature of 250° and detector temperature of 300°. Flow rates for helium, hydrogen and air were 60, 60 and 240 ml/min respectively. Samples eluted from the thin-layer chromatograms with methanol were concentrated under nitrogen and injected directly.

Conversion of methadone in vitro to EDDP and EMDP. The conversion of methadone *in vitro* to EDDP and EMDP was studied by incubating ^{14}C-methadone with rat liver microsomes. Liver microsomes were obtained from control and PB-pretreated rats, as previously reported [10]. The incubation mixture contained: $MgCl_2$, 6·8 μmoles; isocitric acid, 2·6 μmoles; NADP, 0·5 μmole; UDPGA, 0·4 μmole; methadone HCl, 0·4 μmole; ^{14}C-methadone HCl, 0·15 μmole (0·25 μCi); 2·5 mg of microsomal protein and 0·05 M Tris–HCl buffer, pH 7·5, to a final volume of 1 ml. The reaction was initiated by the addition of 5 μl of isocitric dehydrogenase (5 units) and incubated at 37° for 15 min. The reaction was stopped by the addition of 50 μl of 1·0 N NaOH; 20 μl of the reaction mixture was spotted on type SA Gelman ITLC sheets and assayed for methadone, EDDP and EMDP in a manner identical to that used on the bile samples. UDPGA was included in the incubation mixture as a necessary factor for glucuronyl transferase, since Baselt and Casarett [8] and Baselt and Bickel [9] showed that the water-soluble metabolite of methadone was a glucuronide.

N-demethylation studies. The rates of *N*-demethylation of methadone and EDDP were studied using a reaction mixture similar to the one above. The reaction volume was 5 ml, UDPGA was omitted and no radioactive methadone was included. For the studies on the *N*-demethylation of methadone and EDDP,

2·75 μmoles methadone and 2·75 μmoles EDDP, respectively, were used as substrates. The reaction was initiated by adding 5 μl (5 units) isocitric dehydrogenase and incubated at 37°. One-ml samples were withdrawn at 0, 5, 10 and 15 min. The rate of *N*-demethylation was monitored by the liberation of formaldehyde, which was determined by the method of Nash [15]. Protein was determined by the method of Lowry *et al.* [16].

RESULTS

Effect of PB pretreatment on plasma disappearance and biliary excretion of methadone. Figure 1 shows the disappearance of ^{14}C from plasma (top panel) and biliary excretion of ^{14}C (bottom panel) after intravenous administration of ^{14}C-methadone to rats pretreated with PB or saline (control). It should be noted that the abscissa for the two panels is different, since blood was collected during the first 30 min, whereas bile was collected for 90 min. While ^{14}C concentrations in plasma were similar for the two pretreatment conditions, the amount of ^{14}C excreted into bile during the 30-min period was significantly higher with PB-pretreated than with control rats. PB pretreatment significantly increased the biliary excretion of ^{14}C in the 15-, 30- and 45-min bile samples compared to controls. The amount of ^{14}C in the bile of PB-pretreated rats was significantly lower than that in controls for the 75- and 90-min bile samples. The total ^{14}C recovered in bile collected for 90 min after ^{14}C methadone injection was significantly greater for PB-treated rats (50·5 ± 0·7 per cent of the administered dose) as compared to controls (39·5 ± 1·1).

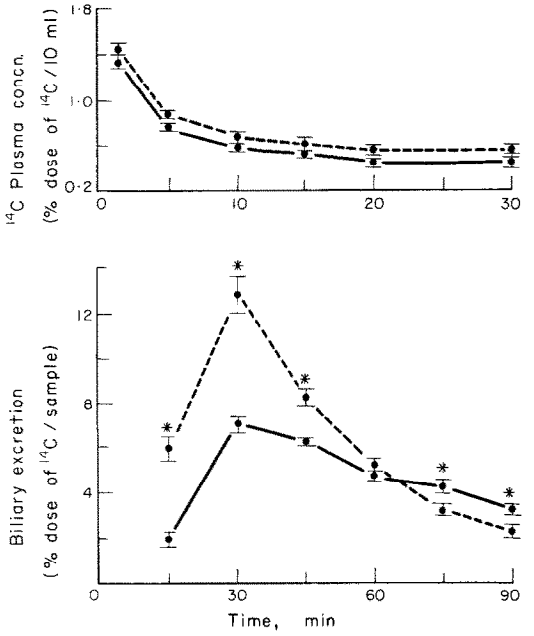

Fig. 1. Effect of saline and PB pretreatment on the ^{14}C concentration in plasma (top panel) and the biliary excretion of ^{14}C (bottom panel) after ^{14}C-methadone administration. The ordinate in the top panel gives the plasma concentration of radioactivity in 10·0 ml plasma, represented as a percentage of the dose of ^{14}C administered. The data are expressed per 10·0 ml plasma because this is the approximate plasma volume of a 300–400 g rat. In the bottom panel, the ordinate represents the biliary excretion of ^{14}C as per cent of the dose administered per 15-min bile sample. The pretreatments in all figures are designated as: —— saline (control) and - - - - PB. Each value is the mean ± S.E. of five or more animals. The asterisk (*) indicates values significantly different from controls ($P < 0·05$).

Effect of PB pretreatment on the methadone metabolite excretion pattern. In order to gain some insight into the causes of the increased biliary excretion of ^{14}C in PB-pretreated rats after methadone administration, each bile sample was analyzed by t.l.c. to determine the relative amounts of methadone and its metabolites. Solvent systems A and B each revealed three radioactive spots.

With solvent system A, ^{14}C-labeled spots were found at the origin, at R_f 0·22 and R_f 0·85. The spots at R_f 0·22 and R_f 0·85 were chromatographically similar to EDDP and EMDP, which had R_f values of 0·20 and 0·84 in this solvent system. As a result, we will refer to these two ^{14}C-labeled metabolites as EDDP and EMDP. The metabolite found at the origin will be referred to as origin material (OM). Figure 2 shows the per cent of the administered dose of ^{14}C recovered from

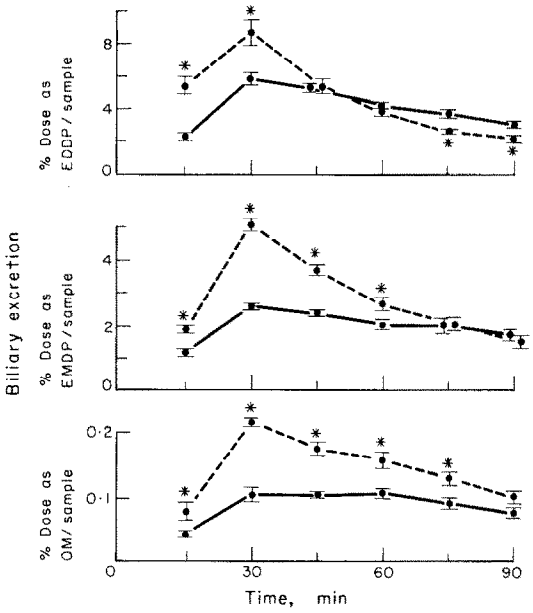

Fig. 2. Effect of saline and PB pretreatment on the biliary excretion of methadone metabolites separated by thin-layer chromatography using solvent system A. The ordinate represents the per cent of the administered dose of ^{14}C recovered in bile as EDDP (top panel), EMDP (middle panel) and OM (bottom panel). Abbreviations and methods of calculation are explained in Methods.

Table 1. Per cent recovery of ^{14}C-labeled compounds in bile 90 min after administration of ^{14}C-methadone*

^{14}C-recovered in bile as	Per cent administered dose of ^{14}C-recovered in 90 min in bile			
	Solvent system A		Solvent system B	
	Control	PB-pretreated	Control	PB-pretreated
Methadone	trace	trace	trace	trace
EDDP	24·8 ± 1·1	28·1 ± 2·04	24·5 ± 1·0	27·6 ± 1·7
EMDP	11·7 ± 0·3	16·9 ± 0·7†	2·0 ± 0·1	3·0 ± 0·1†
OM (origin material)	0·6 ± 0·03	0·9 ± 0·04†		
WSM (water-soluble metabolite)			10·8 ± 0·5	15·4 ± 0·8†

* The per cent of the administered dose of ^{14}C-methadone recovered in bile was calculated for the 90-min bile collection by summing up the per cent of the administered dose in the individual collection periods. Thin-layer chromatography was used to separate the different metabolites. Each value represents the mean ± the standard error from at least five rats. Experimental conditions and abbreviations are described in the text.

† Denotes values significantly different from respective controls ($P < 0·05$).

bile as EDDP, EMDP and OM with time from control and PB-pretreated rats. PB pretreatment significantly increased the biliary excretion of EDDP (top panel) in the 15- and 30-min collection periods. The amount of EDDP in bile was similar for the two pretreatment conditions in the 45- and 60-min collection periods. At the 75- and 90-min collection periods, however, the amount of EDDP found in the bile of PB-pretreated animals was significantly lower than that in controls. Although this curve is similar in shape to the curve for the recovery of total ^{14}C (Fig. 1), the amount of ^{14}C recovered in 90 min as EDDP was not significantly different between BP-pretreated and control rats (Table 1).

The amount of the metabolite tentatively identified as EMDP (middle panel, Fig. 2) was greater in bile of PB-pretreated rats in the 15-, 30-, 45- and 60-min collection periods and similar to that of controls in the 75- and 90-min collection periods. As a result, the per cent of the administered dose of ^{14}C found in bile in 90 min as EMDP increased by about 45 per cent in PB-pretreated rats as compared to controls (Table 1).

The third metabolite found with solvent system A was at the origin of the chromatogram and was present in much smaller quantities than EDDP or EMDP. The amount of the OM metabolite excreted into bile with time, after ^{14}C-methadone administration (lower panel, Fig. 3), was significantly greater for the first five collection periods with PB-pretreated rats than in controls. The increase in the biliary excretion of OM with time in PB-pretreated rats is reflected in the increase in the total 90-min recovery of this metabolite (Table 1).

A second solvent system (B, see Methods) was employed in order to check the results with solvent system A and to separate other possible metabolites. As previously mentioned, three radioactive spots were found when bile collected from rats given ^{14}C-methadone was applied to chromatograms and developed in solvent system B. Radioactive spots were found at R_f 0·31 and 0·95 as well as one spot at the origin. The ^{14}C-labeled spots at R_f 0·31 and 0·95 corresponded closely to EDDP and EMDP, which had R_f values of 0·32 and 0·94 respectively in this system.

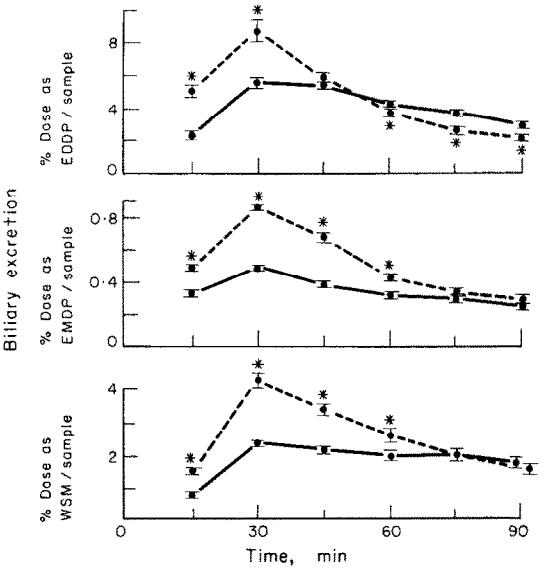

Fig. 3. Effect of saline and PB pretreatment on the biliary excretion of methadone metabolites separated by thin-layer chromatography using solvent system B. The ordinate represents the per cent administered dose of ^{14}C-recovered in bile as EDDP (top panel), EMDP (middle panel) and WSM (bottom panel). Abbreviations and methods of calculations are explained in Methods.

The recovery of EDDP from bile was very similar to that found with solvent system A. By comparing the curves in the upper panels of Figs. 2 and 3, it can be seen that the amount of EDDP found in bile with both solvent systems is greater with PB-pretreated animals in the 15- and 30-min collection periods, and becomes significantly less than that of controls in the last two or three collection periods. Also, the amount of ^{14}C re-

covered as EDDP in bile in 90 min (Table 1), using both solvent systems, is similar for PB-pretreated rats and controls.

The amount of radioactivity recovered in the spot corresponding to EMDP using solvent system B is different from the result obtained with solvent system A. The middle panel of Fig. 3 shows the time course recovery of ^{14}C as EMDP. The amount of EMDP in bile was significantly greater for the 15-, 30-, 45- and 60-min collection periods with PB-pretreated animals. While the general shape of these curves is similar to that found with solvent system A (middle panel, Fig. 2), it can readily be seen that only about one-fifth as much ^{14}C was separated as EMDP in solvent system B as compared to solvent system A.

The radioactive spot found at the origin in solvent system B differed in both amount and nature from the OM described in solvent system A. As will be shown later, the radioactive metabolite found at the origin in solvent system B is highly water soluble and possibly a glucuronide conjugate of some methadone derivative. This metabolite has characteristics similar to those of the metabolite described by Baselt and Casarett [8]; we have adopted their terminology and shall refer to this metabolite as WSM (water-soluble metabolite). It can readily be seen that the amount of WSM excreted in bile with time (lower panel, Fig. 3) is significantly greater with PB-pretreated rats as compared to controls in the first four collection periods. The total WSM recovered in 90 min in the bile of PB-pretreated rats was $15{\cdot}3 \pm 0{\cdot}8$ per cent of the administered dose as compared to only $10{\cdot}8 \pm 0{\cdot}5$ per cent for controls.

The amount of WSM found at the origin in solvent system B approximately equals the decrease in the amount of radioactivity in the EMDP spot between solvent systems A and B. From this observation, it appears that the ^{14}C-labeled spot, chromatographically similar to authentic EMDP in solvent system A, is a mixture of two metabolites. These two metabolites separate using solvent system B, resulting in the WSM spot at the origin and a smaller amount of ^{14}C at the same R_f as authentic EMDP. Further proof of this was obtained by eluting from the chromatogram the ^{14}C-labeled spot with an R_f similar to that of authentic EMDP in solvent system A. The radioactive eluate was re-chromatographed in solvent system B. This resulted in two ^{14}C-labeled spots. One spot corresponded to authentic EMDP, while the major portion of the radioactivity remained at the origin of the chromatogram (WSM).

In addition to the above studies, the EMDP and WSM spots obtained with solvent system B were eluted with methanol, concentrated by evaporation and analyzed by gas chromatography. Injection of the material from the EMDP spot resulted in a peak that had the same retention time as authentic EMDP. Furthermore, no other peaks were present that could be attributed to other metabolites of methadone, as judged by comparison with an identical experiment in which bile from rats that had not received methadone was chromatographed, eluted and analyzed by g.l.c. The WSM spot was found not to contain any EMDP, as judged by g.l.c.

From these data, it is our conclusion that the amount of EMDP and WSM excreted in bile should be determined from the results obtained with solvent system B. Solvent system A, on the other hand, demonstrates the presence of a small amount of an unidentified metabolite (OM), and serves as confirmation of the biliary excretion data for EDDP obtained using solvent system B.

Preliminary characterization of the water-soluble metabolite. The metabolite of methadone which remained at the origin of the chromatogram in solvent system B was characterized. Bile collected for 90 min from PB-pretreated rats which had been injected with ^{14}C-methadone was pooled, and 0·5 ml of this bile was made basic with 0·01 ml of 2·5 N NaOH and extracted with three 1·5-ml portions of chloroform. Thirty-five per cent of the radioactivity remained in the aqueous phase and 64 per cent was found in the pooled chloroform extracts. Both phases were applied to thin-layer chromatograms and developed in solvent system B. With the aqueous layer, 90 per cent of the radioactivity remained at the origin and only trace amounts of EDDP and EMDP were observed. With the chloroform layer, better than 85 per cent of the radioactivity was present as EDDP and EMDP with less than 5 per cent of the radioactivity remaining at the origin.

Further characterization of this metabolite was performed by incubation with β-glucuronidase to determine if the WSM is a glucuronide conjugate. The WSM was separated from bile of PB-pretreated rats which had been injected with ^{14}C-methadone. Bile (0·25 ml) was streaked across the origin of a chromatogram and developed in solvent system B. A strip extending 1 cm to each side of the origin was then cut out and the WSM eluted with methanol, evaporated to dryness and redissolved in 2·0 ml of pH 6·0 phosphate buffer. Hydrolysis with β-glucuronidase and subsequent extraction were carried out according to the method of Baselt and Casarett [8]. Prior to treatment with β-glucuronidase, chloroform extraction of the isolated WSM removed about 5 per cent of the ^{14}C from the aqueous phase. After 3 hr of incubation with β-glucuronidase, 63 per cent of the ^{14}C was extracted with chloroform. Thin-layer chromatography in solvent system B yielded a radioactive spot at R_f 0·96.

Studies in vitro. The effect of PB pretreatment on the metabolism of methadone *in vitro* was studied by incubating ^{14}C-methadone with isolated hepatic microsomes from control and PB-pretreated rats. Thin-layer chromatography was used to separate the metabolites formed. The results of this experiment are shown in Table 2. The amount of EDDP formed when methadone was incubated with microsomes from PB-pretreated rats was about twice as great when compared to controls. The amount of EMDP formed also was about double with PB-pretreated as compared to control microsomes. In addition, the amount of OM

Table 2. Conversion *in vitro* of ^{14}C-methadone to EDDP, EMDP and origin material by rat liver microsomes*

^{14}C-recovered as	Per cent of initial ^{14}C concentration	
	Control	PB-pretreated
Methadone	84·4 ± 0·5	71·9 ± 0·5†
EDDP	11·8 ± 0·5	22·0 ± 0·6†
EMDP	1·6 ± 0·1	2·7 ± 0·2†
OM (origin material)	0·8 ± 0·1	1·2 ± 0·03†

* Conversion *in vitro* of ^{14}C-methadone to metabolites was calculated as a per cent of the ^{14}C in the microsomal assay mixture which was spotted on the chromatogram. Each value represents the mean ± the standard error obtained with liver microsomes from at least five rats. Experimental conditions and abbreviations are described in the text.

† Denotes values significantly different from control ($P < 0.01$).

formed *in vitro* was significantly greater with PB microsomes, although the relative increase was not as great as that observed with the formation of EDDP or EMDP.

The amount of different ^{14}C-labeled metabolites formed *in vitro* from methadone was similar when determined with either solvent system A or B. The lack of any difference in the amount of ^{14}C found at the origin of the chromatograms indicates that the WSM was not formed *in vitro* in our incubation mixture containing methadone and hepatic microsomes. The small amount of ^{14}C which was present at the origin most probably represents the metabolite we have designated OM.

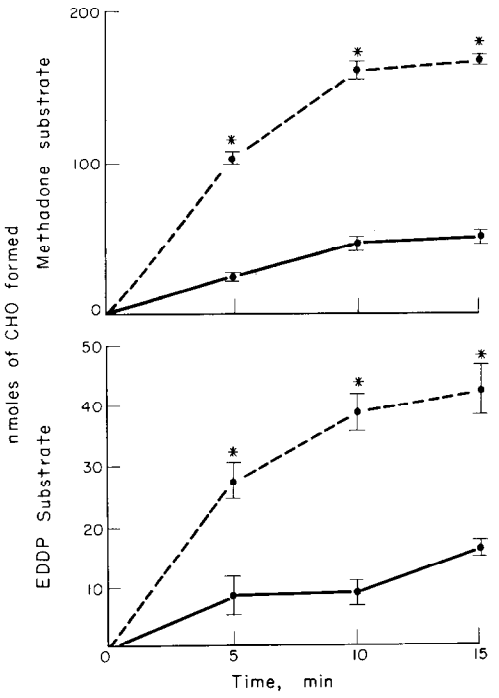

Fig. 4. Effect of saline and PB pretreatment on the *N*-demethylation *in vitro* of methadone (top panel) and EDDP (bottom panel) by hepatic microsomes. The ordinate represents nmoles formaldehyde formed/mg of microsomal protein. Assay conditions are stated in the text.

N-demethylation of methadone and EDDP. An additional measure of PB induction of methadone metabolism was made by studying the rate of *N*-demethylation of methadone and EDDP by rat liver microsomes. Figure 4 shows a comparison of the rate of formaldehyde formation from methadone and EDDP, using microsomes from control and PB-pretreated rats. It is evident that PB pretreatment increases the rate of *N*-demethylation of both methadone (upper panel) and EDDP (lower panel). With microsomes from PB-pretreated rats, the initial rate of *N*-demethylation of methadone to EDDP and EMDP increases to about 4-fold, whereas the initial rate of *N*-demethylation of EDDP to EMDP increases to about 3-fold the rates observed with controls.

DISCUSSION

The present study shows that PB pretreatment of rats increases the biliary excretion of ^{14}C after ^{14}Cmethadone administration. The explanation for the increased biliary excretion of methadone in PB-pretreated rats, however, is complex, since the net effect of PB pretreatment on biliary excretion could be due to the influence of PB pretreatment on a number of factors. For example, Roberts and Plaa [17] have shown that PB pretreatment increased bile flow in rats and suggested that increased biliary excretion of bilirubin in PB-pretreated rats was due to increased bile flow. In addition, Hart *et al.* [18] found that PB pretreatment increased the biliary excretion of phenol red and probenecid, and Goldstein and Tauroy [19] reported increased biliary excretion of thyroxine in PB-pretreated rats. Both groups of investigators concluded that the increased biliary excretion of these compounds was due primarily to increased bile flow in PB-pretreated animals. In the present study, increased bile flow in PB-pretreated rats was found to be the same as we reported previously [10] and was similar in magnitude to that originally reported by Roberts and Plaa [17].

Increased metabolism of methadone in PB-pretreated rats is another possible mechanism by which PB pretreatment could increase biliary excretion. PB pretreatment is well recognized as a potent inducer of the metabolism of many drugs and has been shown to enhance the metabolism of methadone *in vitro* [11]. Levine [20, 21] has shown that PB pretreatment increased the biliary excretion of 3-methylcholanthrene and benzpyrene by enhancing the metabolism of both drugs. Furthermore, Levine and Singer [22] have suggested that induction or inhibition of drug metabolism will correspondingly increase or decrease the biliary

excretion of most drugs which are metabolized prior to their excretion into bile. PB pretreatment has also been shown to increase hepatic blood flow [23] and Whitsett *et al.* [24] have shown that the hepatic removal rate and hepatic metabolism of oxyphenbutazone depended directly upon hepatic blood flow. Furthermore, Broduer and Marchand [25] have suggested that hepatic blood flow is important in maintaining proper levels of drug-metabolizing enzyme activity. It is possible therefore that hepatic blood flow is a determinant in the rate of biliary excretion of a compound.

The increased biliary excretion of ^{14}C after ^{14}C-methadone administration in PB-pretreated rats could be explained by any one of the possible effects of PB pretreatment just presented. However, it is not unreasonable to expect that increases in hepatic blood flow or bile flow would result in proportionate increases in the biliary excretion of total ^{14}C and any one of the metabolites. In a similar manner, one would expect that PB-induced increases in these flows would also result in proportionate increases in the amount of all metabolites excreted into bile. In contrast to these expectations, we found that while PB pretreatment increased the total ^{14}C and the amount of metabolites EMDP, OM and WSM excreted into bile, the amount of metabolite EDDP was not significantly changed by PB pretreatment (Table 1).

The above argument tends to discount major involvement of increased hepatic blood flow or bile with observed increases in biliary excretion of ^{14}C after ^{14}Cmethadone administration in PB-pretreated rats. Another possible explanation of the biliary excretion data presented here is that PB pretreatment could alter the mechanism of the excretion process, resulting in increased excretion of methadone metabolites from the liver into bile. Such an explanation is supported by the fact that PB pretreatment increased the initial biliary excretion with time of all methadone metabolites (see Figs. 2 and 3). However, an altered excretion mechanism caused by PB pretreatment does not account for the fact that the biliary excretion of EDDP (upper panel, Figs. 2 and 3) was increased in PB-pretreated rats in only the 15- and 30-min collection periods and became significantly less than that of controls in the 75- and 90-min collection periods. In comparison, PB pretreatment increased the biliary excretion of the metabolites OM, EMDP and WSM (lower panel, Fig. 2; middle and lower panel, Fig. 3) throughout most of the 90 min of bile collection. Further studies in this area are necessary. Our present effort is devoted to studying the effect of PB pretreatment on the biliary excretion of administered metabolites of methadone, a situation where the effect of metabolism in the excretion process will be more limited or not a competing process.

Perhaps the best correlation exists between increased metabolism of methadone and its increased biliary excretion in PB-pretreated rats. Increased metabolism of methadone in PB-pretreated rats is consistent with the observed increases in the biliary excretion of total ^{14}C, EMDP, OM and WSM. Furthermore, the lack of increase in the total amount of EDDP excreted into the bile of PB-pretreated animals could be due to PB-induced enhancement of further metabolism of EDDP.

As previously stated, methadone is *N*-demethylated followed by cyclization to form EDDP. Further *N*-demethylation of EDDP results in EMDP. Recently Baselt and Bickel [9] reported that EMDP undergoes hydroxylation and glucuronidation to form *para*-hydroxy EMDP glucuronide. This glucuronide, as described by Baselt *et al.* [8, 9], is identical in properties to the WSM (water-soluble metabolite) observed in the present study. Therefore, the increased biliary excretion of EMDP and WSM in PB-pretreated rats could reflect increased metabolism of EDDP to EMDP. The fact that the total amount of EDDP excreted into bile was similar for the two pretreatment conditions and the fact that the total amounts of EMDP and WSM excreted were greater from PB-pretreated rats suggest that the metabolism of methadone to EDDP is also enhanced by PB pretreatment. Even though the metabolism of methadone to EDDP is increased by PB pretreatment, increased conversion of EDDP to EMDP could result in the observed lack of increase in the total amount of EDDP excreted into bile.

It could be argued that the effect of PB pretreatment on the biliary excretion of EDDP may be due, in part, to the low dose of methadone used in these experiments. However, Baselt and Casarett [8], using higher doses of methadone (16 mg/kg) and a longer bile collection time (24 hr), found that the total amount of EDDP excreted into bile in PB-pretreated rats was actually less than that in control rats.

Support for the suggestion that PB pretreatment enhances the conversion of methadone to EDDP and of EDDP to EMDP was found with studies *in vitro*. In agreement with the results of Alvares and Kappas [11], we found that microsomes from PB-pretreated rats converted methadone to EDDP and EMDP to a greater extent that did microsomes from control rats (Table 2). In addition, we found that PB pretreatment enhanced the formation *in vitro* of a metabolite that corresponded to the metabolite OM found in the studies *in vivo* (Table 2). The formation of the metabolite WSM was not observed *in vitro*. Further studies are in progress to optimize assay conditions in order to study the effect of PB pretreatment on the formation of WSM *in vitro*.

In addition to the above studies *in vitro*, we studied the effect of PB pretreament on the microsomal *N*-demethylation of methadone and EDDP (Fig. 4). Microsomes from PB-pretreated rats were found to *N*-demethylate methadone at a significantly greater rate than did control microsomes. Using EDDP as a substrate, it is possible to study just the conversion of EDDP to EMDP. In agreement with the results *in vivo*, it appeared that PB pretreatment also enhanced the second *N*-demethylation in the metabolism of methadone.

While the metabolites EDDP, EMDP and WSM have been characterized by others and their structures determined, the metabolite OM has not been studied. Further characterization of this metabolite is now in progress.

It is interesting to note that the ^{14}C concentrations in plasma were similar for PB-pretreated and control rats (upper panel, Fig. 1), while, during this same time period, the ^{14}C concentrations in bile were much greater in PB-pretreated rats. Since the concentration of ^{14}C in plasma was too low to permit identification of the ^{14}C-labeled compound, we cannot offer an explanation for the apparent lack of correlation between ^{14}C plasma concentrations and the increased metabolism and biliary excretion of ^{14}C-methadone in PB-pretreated rats.

In conclusion, PB pretreatment could be increasing the biliary excretion of methadone by altering several different determinants of biliary excretion. However, we feel the data best support the idea that PB pretreatment is enhancing the metabolism of methadone and its metabolites *in vivo*. The enhanced metabolism of methadone results in metabolites which are more readily excreted into bile. In agreement with the suggestion of Baselt and Casarett [8], we feel that the reason for the lack of increase in the biliary excretion of EDDP in PB-pretreated rats is induction of the further metabolism of EDDP by PB pretreatment. For more definitive proof of these suggestions, it will be necessary to study the effects of PB pretreatment on the biliary excretion of the administered metabolites of methadone. Work is in progress to purify the ^{14}C-labeled metabolites of methadone for this purpose.

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