The UV, NMR and MS data obtained were found to be identical with data obtained for MC 1080, which also co-migrated with metabolite X on HPLC, identifying the metabolite as MC 1080.

The formation of MC 1080 was also observed in incubations of MC 903 with post-mitochondrial supernatant from liver from mini-pig and man. In the experiment with human liver an additional metabolite (Y) was prominent (Fig. 2b). The UV spectrum was different from that of MC 1080, but identical to that of MC 1046, with which compound metabolite Y also co-migrated exactly on HPLC. These results strongly indicate that metabolite Y is identical with MC 1046. Having observed MC 1046 in the experiments with human liver preparations further experiments were performed with rat and pig liver preparations (variation of incubation time and substrate concentrations). In these experiments the presence of minor amounts of MC 1046 was also observed, indicating the same qualitative pattern of metabolism in the three species.

The metabolism of MC 903 to MC 1080 and MC 1046 involves oxidation at the 24-position, similar to the C-24 oxidation pathway of metabolism of 1,25-dihydroxyvitamin D_3, the active form of vitamin D_3 [3, 4]. However, the 24-oxidation of 1,25-dihydroxy vitamin D_3 takes place mainly in the kidney and in the intestine, whereas the presently described metabolism is hepatic. Furthermore, the metabolism to MC 1080 involves a reduction of the 22,23 double bond in addition to the 24-oxidation step.

The formation of 24-oxidized metabolites appears to constitute a deactivation pathway for MC 903, since *in vitro* investigations of the effects of MC 1080 and MC 1046 on cell proliferation and differentiation show a considerably reduced activity compared to MC 903. These results and the results from *in vivo* investigations of metabolism of MC 903 in rat and pig will be published later.

In summary, a metabolite of MC 903 observed *in vivo* has been produced by incubation *in vitro* of MC 903 with liver post-mitochondrial supernatants from rat, pig and man. This metabolite was identified as MC 1080 by UV, NMR, MS and HPLC. An additional metabolite formed by incubation of MC 903 with human liver post-mitochondrial supernatant was identified as MC 1046. This metabolite was also detectable in rat and pig liver incubations.

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Effect of phenobarbital induction, charcoal treatment and storage on the spectral binding characteristics and NADPH–cytochrome P-450 reductase activity of hepatic microsomes

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The rate-limiting step in the hydroxylation of many foreign compounds and steroids by the hepatic microsomal cytochromes P-450 is the reduction of the cytochrome heme iron to Fe^{2+} by NADPH–cytochrome P-450 reductase [1–3]. Gigon *et al.* [4, 5] and Schenkman [6] found that, for a large number of water-soluble drugs, the substrate increases the rate of this reduction. Further, Gigon *et al.* [4] found that this stimulation showed a 1:1 stoichiometry with the hydroxylase activity. A number of studies from our laboratory have confirmed this observation [1, 7–10]. We and others have observed that there is an association between this substrate-induced increase in reductase activity and the presence of a type I difference binding spectrum [10–17], suggesting that the increased activity is associated with an increased fraction of the heme in the high spin state [10–17].

We have examined this hypothesis during studies that were stimulated by an apparent difference between the observations cited above and those of Peterson *et al.* [18]. This group reported that ethylmorphine does not stimulate NADPH–cytochrome P-450 reductase activity (cf. Table 3 of Ref. 18). A major difference between their studies and ours was that they utilized microsomes from phenobarbital-induced rats. Their failure to observe a stimulation with ethylmorphine could be due to residual phenobarbital, which we have detected previously in microsomes from treated animals [19]. This bound drug could be blocking the stimulatory action of ethylmorphine.

In the current study we examined this question by removing the residual phenobarbital from microsomes with charcoal. We found that the addition of ethylmorphine to this preparation gave a reverse type I difference binding spectrum and also markedly reduced the NADPH–cytochrome P-450 reductase activity. Charcoal treatment had no effect on either the spectral binding properties or the stimulation of cytochrome P-450 reduction by benzphetamine for microsomes from either control or phenobarbital-induced animals or on the effect of ethylmorphine

on control microsomes. Finally, after storage of the microsomes, both substrates showed the same binding spectra, but neither had any effect on the reduction of the cytochrome P-450. These data would suggest that the stimulation of cytochrome P-450 reduction is associated with more than just changes in the spin state of the cytochrome and, in confirmation of previous studies [9], may require a separate stimulatory site which is lost on storage.

Materials and methods

Microsomes were prepared as previously described from fed, male CD 180–220 g rats obtained from Charles River (Wilmington, MA) [1]. The treated animals had received sodium phenobarbital (0.1% in the drinking water) for 8 days [19]. The microsomal pellets were resuspended (50–60 mg protein/mL) in Tris (0.05 M, pH 7.4), 50% glycerol and 0.01% butylated hydroxytoluene and stored at −20° [20]. Just prior to each study, an aliquot of microsomes was thawed, washed and resuspended (60 mg protein/mL) in sodium pyrophosphate–EDTA (0.1 M–0.001 M; pH 7.5). All mixed-function oxidase activities are stable in this preparation.

Protein was determined by the method of Lowry *et al.* [21] and cytochrome P-450 by the method of Omura and Sato [22]. This type I difference binding spectra [9] and *N*-demethylase activities [2] were determined as previously described.

Some microsomes were treated with charcoal by the method of Schuster *et al.* [23]. The charcoal had been activated by heating to 90° for 60 min in 0.25 M sodium-EDTA, washing in deionized water and resuspending in buffer (100 mg charcoal/mL). Charcoal (2.5 mg/mg protein) and microsomes were gently stirred in buffer for 20 min at 4° under N_2, diluted 20-fold, centrifuged at 10,000 *g* for 20 min and then at 105,000 *g* for 60 min.

The NADPH–cytochrome P-450 reductase activity was determined in a modified Aminco–Morrow stopped-flow accessory mounted in an Aminco DW-2a spectrophotometer. The stopped-flow was fitted with nylon tubing, which is more rigid and less permeable to oxygen than the original teflon [24]. The NADPH-generating system [$NADP^+$ (1 mM), glucose-6-phosphate (14 mM), glucose-6-phosphate dehydrogenase (0.5 units/mL), glucose (10 mM) and catalase (65 μg/mL)] and the microsomal suspension [6 mg protein/mL, glucose (10 mM) and catalase (65 μg/mL)] were equilibrated with O_2 free CO for 15–20 min in a rotating tonometer [24]. Glucose oxidase (0.1 mL) (1385 units/mL) were added just prior to gassing the samples [16]. The stopped-flow data were collected and analyzed in an 8080a microprocessor-based computer [25]. Kinetic constants for each phase of the reduction were determined by graphical stripping of the log of the ferricytochrome P-450 remaining against time.

Results and discussion

As has been shown in previous studies with microsomes from control animals, ethylmorphine induced a type I difference binding spectrum and stimulated cytochrome P-450 reduction (Table 1). Charcoal treatment slightly increased the basal reductase activity, but not the stimulation (Table 1).

In contrast, ethylmorphine did not induce a binding spectrum with microsomes for phenobarbital-treated animals and had no effect on the reduction of cytochrome P-450 (Table 1). With charcoal-treated microsomes, we observed a reverse type I difference binding spectrum with ethylmorphine (peak = 423 nm) (Table 1). The rate of cytochrome P-450 reduction was 160% of that seen with charcoal-treated, control microsomes (Fig. 1, Table 1). Addition of ethylmorphine profoundly decreased the rate of reduction (Fig. 1, Table 1). Further, the semilogarithmic plot of the reduction in the presence of ethylmorphine was monophasic rather than multiphasic (Fig. 1), suggesting that there is marked alteration in cytochrome P-450 reduction. We believe that this is the first demonstration of the expected association between the presence of a reverse type I difference binding spectrum and a marked inhibition of cytochrome P-450 reduction [10].

Benzphetamine induced a type I binding spectrum rather than the reverse type I seen with ethylmorphine. Further, it stimulated the reduction of cytochrome P-450 with microsomes from both control (Table 1) and phenobarbital-

Table 1. Effect of phenobarbital pretreatment and charcoal on the NADPH–cytochrome P-450 reductase activity of hepatic microsomes from male rats

Microsomal preparation	Treatment of microsomes	Addition	Initial reduction rate constant sec^{-1}	Spectral binding type
Control			0.57	
		2 mM Ethylmorphine	1.57	I
		2 mM Benzphetamine	1.77	I
Control	Charcoal		0.89	
		2 mM Ethylmorphine	2.02	I
		2 mM Benzphetamine	2.02	I
Phenobarbital*			2.41	
		2 mM Ethylmorphine	2.41	None detected
Phenobarbital	Charcoal		1.43	
		2 mM Ethylmorphine	0.076	Reverse I
Phenobarbital†	Charcoal		2.31	
		2 mM Ethylmorphine	2.24	Reverse I
		2 mM Benzphetamine	2.24	I

* Phenobarbital was administered for 8 days as a 1 mg/mL solution in the drinking water.
† These microsomes were stored for 10 months at −20°.

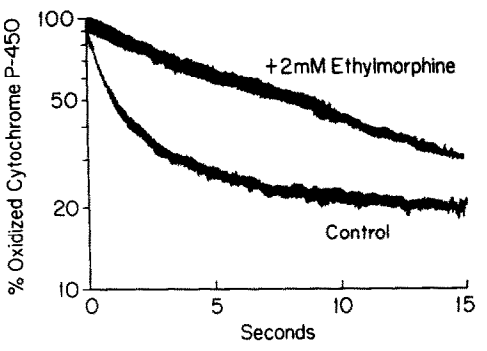

Fig. 1. Effect of 2 mM ethylmorphine on the NADPH reduction of hepatic, microsomal cytochrome P-450 in charcoal-treated microsomes from phenobarbital-induced rats.

treated animals (data not shown). Finally, in both preparations charcoal treatment did not affect either the binding spectrum of benzphetamine or the stimulation of cytochrome P-450 reduction which is seen with this agent (Table 1).

In all of the above studies the microsomal preparations were stored for no longer than 1 month at −20°. We have routinely used such microsomes and found that after washing they retain the activities observed in fresh preparations. On the other hand, after 10 months of storage, we found that microsomes from phenobarbital-treated animals retained the expected concentrations of cytochrome P-450, without any apparent cytochrome P-420 or loss of drug-metabolizing activity. Further, after charcoal treatment they showed a type I difference binding spectrum with benzphetamine and a reverse type I spectrum with ethylmorphine, but neither substrate affected the cytochrome P-450 reductase activity (Table 1).

The loss on storage of stimulation of cytochrome P-450 reduction without changes in the binding spectra suggests that cytochrome P-450 undergoes other changes besides changes in the spin state before there is a stimulation of reduction. A similar suggestion has been proposed by Schenkman's group [16, 17]. Further, we feel that our results support our earlier suggestion that those isoforms which show stimulation have a stimulatory site which is distinct from the type I binding site and which is lost upon extended storage [9, 10].

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Biothermodynamic characterization of erythrocyte hemolysis induced by phenothiazine derivatives and anti-inflammatory drugs

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Many ionic drugs which are distributed and/or bound to human blood cells frequently induce erythrocyte hemolysis with different rate and extent at high drug concentrations [1–4]. Drug-induced hemolysis has been recognized as being classified into two main categories: direct interaction of the drug with the erythrocyte membranes [4] and indirect disruption of the integrity in the cellular metabolism produced by the drug [5]. It is well known that phenothiazine derivatives and non-steroidal anti-inflammatory drugs induce hemolysis where the chief mechanism is the direct interactions with the membranes, whereby the hemolytic activity is dependent on both binding to, and membrane-perturbating ability of, the erythrocytes [6–9]. However, there has been, as yet, no satisfactory elucidation of the differences in hemolytic action between these drugs, nor has the precise mechanism by which drug-induced hemolysis is brought about at high concentrations been made known.

Recently we reported the usefulness of microcalorimetry for the study of drug interaction in the blood system [10–12]. The thermodynamic parameters in the drug reaction process may include structural contributions arising from different molecular events and changes in the aggregation state of either reactant. In the present study, the biothermodynamic characteristics of the erythrocyte hemolysis induced by ionic drugs, phenothiazine derivatives as cationics and non-steroidal anti-inflammatory drugs as anionics, were observed, in an attempt to define the mechanism of hemolysis.

Materials and methods

Fluphenazine dimaleate (FPZ), perphenazine dimaleate (PPZ), trifluoperazine dimaleate (FPRZ), prochlorperazine dimaleate (CPRZ) and perazine dimaleate (PRZ) were obtained from Yoshitomi Pharm. Ind. Ltd (Osaka, Japan). Flufenamic acid (FA), mefenamic acid (MA), ibuprofen (IP) and indomethacin (IM) were purchased from the Sigma Chemical Co. (St Louis, MO), and flurbiprofen (FP) was a gift from Kaken Pharm. Co. Ltd (Tokyo, Japan). Stock solutions (0.2 M) of IM, FA and MA were prepared in dimethyl sulfoxide and diluted with phosphate-buffered isotonic saline (10 mM sodium phosphate, 140.5 mM NaCl, pH 7.4) (PBS). Other drugs were directly dissolved in PBS. Human erythrocytes were supplied by the Red Cross Blood Centre (Fukuoka, Japan) and washed three times with PBS by centrifugation at 1000 *g* for 10 min and then resuspended in the same buffer as a stock solution. Before each use, the erythrocytes were washed with PBS until the supernatant fraction was clear and colorless, in order to obtain packed cells with a 100% erythrocyte concentration. The number of erythrocytes in each experimental suspension was measured by a Coulter Counter model TA-2 (Hialeah, FL).

In the experiments of drug-induced hemolysis, a drug solution was added to a 2% (v/v) erythrocyte suspension in PBS by microsyringe at a drug concentration between 10^{-5} and 10^{-2} M. The mixture was incubated for 90 min at 37° and then centrifuged at 1000 *g* for 10 min. The supernatant fraction was separated, and the absorbance E^{1cm}_{543nm} of hemoglobin released from the erythrocytes was determined. The percentage of hemolysis is expressed by the ratio of absorbance at 543 nm against the complete hemolysis of the erythrocytes in water.

Calorimetric measurements were carried out at 37.0 ± 0.05° using a differential flow-microcalorimeter [12] and a Rikadenki chart recorder (Tokyo, Japan). The reaction solutions were introduced into the calorimeter through Tigon tubin with a four-channeled peristaltic pump (Gilson minipuls 2, Villers-Le-Bel, France). The procedures used in the flow experiments have been described in detail elsewhere [12]. For the heat effect on drug-induced hemolysis, the dilution heat of erythrocytes was measured continuously. A calorimetric record of the heat produced by erythrocyte hemolysis during incubation of a 4% (v/v) erythrocyte suspension with FPRZ is shown in Fig. 1. The enthalpy change of the hemoglobin released from one red cell (ΔH_{hemol}) was calculated from a steady-state level of the signal representing the complete hemolysis by use of the following equation:

$$\Delta Q = \Delta H_{hemol} \cdot Fr \cdot [E],$$

where ΔQ, Fr and $[E]$ represent the heat effect, the flow rate, and the number of erythrocyte cells respectively.

Results and discussion

Figure 2 shows the calorimetric (upper) and percent hemolysis (bottom) profiles during incubation of 4% (v/v) erythrocyte suspensions at various concentrations of PPZ and FA. As shown in the case of PPZ (Fig. 2A) and FPRZ (Fig. 1), the phenothiazines brought about immediate hemolysis upon drug exposure, and the heat of hemolysis was increased endothermically with an increased degree of hemolysis. On the other hand, a different pattern was observ-