ON THE MECHANISM OF DRUG HYDROXYLATION IN RAT LIVER MICROSOMES

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ABSTRACT

The TPNH- and O_2-dependent drug hydroxylation system of liver microsomes has been studied using normal rats and rats in which the drug-hydroxylating activity has been enhanced by repeated injections of phenobarbital. The oxidative demethylation of aminopyrine is employed as an assay. Optimal conditions for the assay with regard to the concentrations of TPNH and aminopyrine are established. TPN inhibits the reaction in a competitive manner, similarly to its effect on the microsomal TPNH–cytochrome *c* reductase. Drug hydroxylation, but not the "TPNH oxidase," TPNH–cytochrome *c*, –2,6-dichlorophenolindophenol, or –neotetrazolium reductase reaction, or the TPNH-dependent lipid peroxidation, is blocked by carbon monoxide. Microsomes from phenobarbital-treated rats exhibit increased activities of the various TPNH-linked reductase reactions, parallel to the increased drug hydroxylation activity, whereas the "TPNH oxidase" activity does not change appreciably. Measurements with microsomes from drug-treated animals reveal a 1:1:1 stoichiometry of aminopyrine-dependent oxygen uptake, TPNH oxidation, and formaldehyde formation. Attempts to solubilize the drug-hydroxylating enzyme system are also presented. It is concluded that the drug-hydroxylating enzyme system involves the microsomal TPNH–cytochrome *c* reductase and CO-binding pigment, and a hypothetic reaction scheme accounting for the data presented is proposed.

INTRODUCTION

In a previous paper (1) it has been reported that repeated administration of phenobarbital to rats results in an increased activity of the drug-hydroxylating enzyme system of liver microsomes. The activation was paralleled by selective increases in the activity of TPNH[1]–cytochrome *c* reductase (2–4) and the amount of the hemoprotein called "CO-binding pigment" (5) or "cytochrome P-450" (6, 7). It was concluded (1) that these two enzymes probably constitute components of the TPNH- and O_2-dependent drug-hydroxylating system.

In order to gain further insight into the nature and mechanism of this substrate-induced enzyme activation, it was thought desirable to investigate in some detail certain biochemical parameters of the drug-hydroxylating enzyme system. Con-

[1] Abbreviations used are: DPN and TPN, di- and triphosphopyridine nucleotide, oxidized forms; DPNH and TPNH, di- and triphosphopyridine nucleotide, reduced form; NT, neotetrazolium chloride; DCPIP, 2,6-dichlorophenolindophenol; PCMB, *p*-chloromercuribenzoate; SKF 525A, β-diethylaminoethyl-diphenylpropylacetate; DPPD, diphenylphenylenediamine; DIC, dicoumarol; FA, formaldehyde; MA, malonaldehyde; AP, aminopyrine; PP, inorganic pyrophosphate.

versely, induction *in vivo* provided a simple means of enriching the enzyme system in the microsomes, thus facilitating the study of its biochemical properties. The present work was undertaken with both these goals in mind. The data reported here provide information relating to the kinetics of the reaction, its stoichiometry and dependence on substrate concentration, and the effects of various electron donors and acceptors and of inhibitors, as well as attempts to solubilize the drug-hydroxylating enzyme system, using liver microsomes from rats before or after treatment with phenobarbital. The results have been reported in part in a preliminary form (8–11).

MATERIALS AND METHODS

Male Sprague-Dawley rats (200 to 300 gm) were starved overnight and killed by decapitation. Preparation of microsomes was performed as described by Ernster *et al.* (12). In the experiments with phenobarbital-treated rats the animals were given daily intraperitoneal injections of 100 mg of phenobarbital per kg body weight.

Oxidative demethylation activity of the microsomes was assayed with aminopyrine as substrate. The standard incubation system contained microsomes (*ca.* 2 mg of protein), 5 mM aminopyrine, 0.05 M tris buffer (pH 7.5), 50 mM nicotinamide, 5 mM $MgCl_2$, 0.5 mM TPN, and a TPNH-generating system consisting of 5 mM DL-isocitrate, 0.01 mM $MnCl_2$, and enough isocitric dehydrogenase to reduce 0.32 μmole TPN per minute, in a final volume of 2 ml. The time of incubation was 20 minutes and the temperature 37°C. The reaction was stopped by the addition of 0.25 ml 25 per cent $ZnSO_4$ and 0.25 ml saturated $Ba(OH)_2$. The amount of formaldehyde formed was measured by the Nash reaction (13).

Lipid peroxidation activity was measured by determining the amount of malonaldehyde formed with the thiobarbituric acid reaction (14). The incubation system (15) included microsomes, 0.025 M tris buffer (pH 7.5), 0.15 M KCl, 0.3 mM TPNH, and Fe^{2+}PP (12 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 0.2 mM NaPP), in a final volume of 2 ml. Incubation was for 6 minutes at 37°C. The reaction was stopped with 2 ml 30 per cent trichloroacetic acid.

TPNH–cytochrome *c* reductase, TPNH–DCPIP reductase, and TPNH–neotetrazolium (NT) reductase activities and the content of CO-binding pigment were determined as described by Dallner (16).

TPNH oxidase activity was assayed by measuring oxygen consumption with a Clark electrode in a system containing microsomes (*ca.* 5 mg protein), 0.05 M tris buffer (pH 7.5), and 0.3 mM TPNH. The reaction was started by the addition of TPNH. The temperature was 30°C.

Experiments aiming at solubilization of the oxidative demethylation system were performed by incubating microsomes (10 to 15 mg protein per ml) in a medium containing 0.05 M tris buffer (pH 9.0) and 0.1 per cent heat-treated (17) *Naja naja* venom aerobically for 30 minutes at 30°C. After the incubation, pH was adjusted to 7.0, a procedure which largely prevents the further action of the *Naja naja* phospholipase. The suspension was centrifuged at 105,000 *g* for 60 minutes.[2] The resulting supernatant, and the pellet resuspended in 0.25 M sucrose, were used for enzyme assays. In order to avoid disturbance of the spectrophotometric determination of CO-binding pigment by contaminating hemoglobin, the livers used in these experiments were perfused *in situ* with ice cold 0.9 per cent NaCl before homogenization.

Protein was determined according to the method of Lowry *et al.* (18).

All chemicals employed were standard commercial products.

RESULTS

Studies with Normal Rats

Figs. 1 and 2 illustrate the rate of oxidative demethylation of aminopyrine, measured by determining the amount of formaldehyde formed, as a function of the concentrations of aminopyrine and TPNH. The Lineweaver-Burk plots (Figs. 1*b* and 2*b*) reveal K_m (Michaelis constant) values of 8.0×10^{-4} M for aminopyrine and 2.5×10^{-5} M for TPNH. When saturating concentrations of aminopyrine and TPNH were used, the rate of the reaction was linear with time up to at least 50 minutes (Fig. 3 *b*), and with the amount of microsomal protein (Fig. 3 *a*). The maximal rate of oxidative demethylation of aminopyrine was 2.5 to 3.5 mμmoles formaldehyde formed per minute per milligram protein.

Under standard conditions, TPNH was generated by the isocitric dehydrogenase system. This was preferred to the glucose-6-phosphate dehydrogenase system, in view of the high glucose-6-phosphatase activity of the microsomes. The concentration of Mn^{++}, which is an activator of isocitric dehydrogenase, was kept lower than needed for maximal activation of the enzyme, since it was found that higher concentrations of Mn^{++}

[2] In order to make certain that the supernatant did not contain any poorly sedimentable particulate material, experiments were performed in which the time of centrifugation after solubilization was prolonged up to 4 hours at 105,000 *g*. This treatment, however, did not change the results.

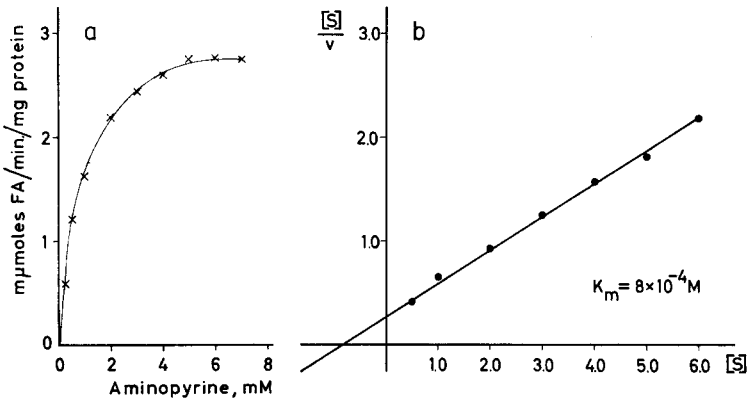

FIGURE 1 Fig. 1 *a*, rate of oxidative demethylation of aminopyrine with varying concentrations of aminopyrine. FA, formaldehyde.

Fig. 1 *b*, Lineweaver-Burk plot of Fig. 1 *a*. [*S*] aminopyrine, mM; *v*, relative reaction velocity.

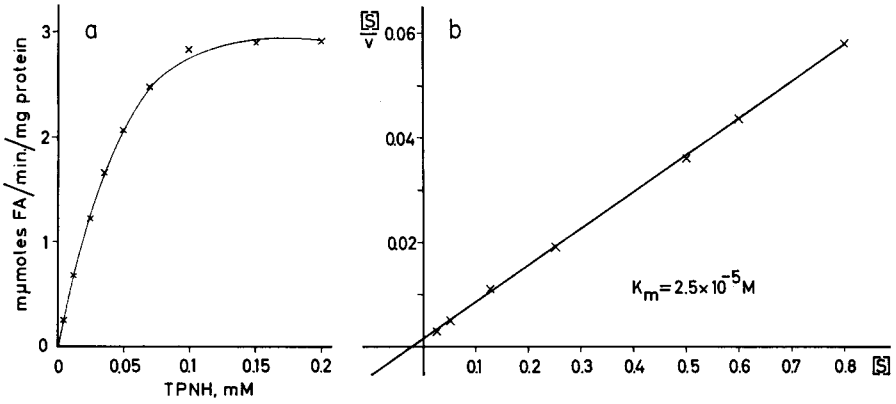

FIGURE 2 Fig. 2 *a*, rate of oxidative demethylation of aminopyrine with varying concentrations of TPNH. TPNH was generated by the isocitric dehydrogenase reaction (see Materials and Methods).

Fig. 2 *b*, Lineweaver-Burk plot of oxidative demethylation of aminopyrine with varying concentrations of generated TPNH. [*S*], TPNH, mM; *v*, relative reaction velocity.

may depress the rate of oxidative demethylation. It was also found essential to include 10^{-2} M nicotinamide in the reaction medium, in order to prevent breakdown of TPN by a TPNase present in the microsomes; as ascertained in complementary experiments, this enzyme appears to attack only the oxidized, and not the reduced, form of TPN.

TPN inhibited the oxidative demethylation of aminopyrine. In Fig. 4, the rates of oxidative demethylation are compared as measured with varying concentrations of *added* (not generated) TPNH in the absence and presence of nicotinamide and of varying concentrations of TPN. In the absence of nicotinamide and TPN, added TPNH supported the reaction with the same efficiency as did generated TPNH (*cf.* Figs. 2 *a* and *b* and 4 *a*). The presence of nicotinamide, allowing an accumulation of TPN from TPNH in the course of the oxidative demethylation, caused an apparent elevation in the saturating TPNH concentration, which could be increased further by added TPN (Fig. 4 *a*). The inhibition by TPN was competitive with respect to TPNH (Fig. 4 *b*), with a K_i (inhibitor constant) value of 2.8 $\times$ 10^{-4} M.

Replacement of TPNH by DPNH resulted in low rates of oxidative demethylation (Table I).

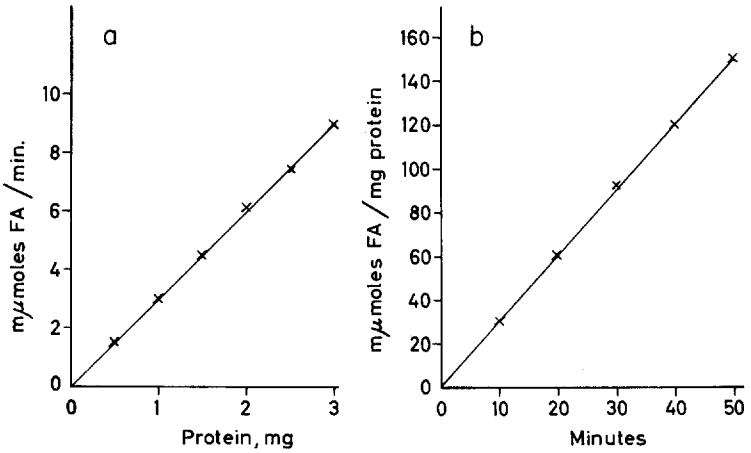

FIGURE 3 Fig. 3 *a*, rate of oxidative demethylation of aminopyrine with varying amounts of microsomes. Fig. 3 *b*, amount of formaldehyde formed from the oxidative demethylation of aminopyrine as a function of time of incubation.

Combination of TPNH and DPNH did not increase the maximal rate obtained with TPNH alone. Addition of 0.5 to 1.0 mM TPN to the DPNH system gave rise to an oxidative demethylation, the rate of which was approximately 40 per cent of that obtained with TPNH; decrease or increase of the TPN concentration beyond this range lowered the rate. These results confirm earlier observations (19) and suggest that microsomes may contain a transhydrogenase by which TPNH can be generated from DPNH. Also in accordance with previous reports (20), ascorbate was found inefficient as an electron donor for drug hydroxylation.

In addition to drug hydroxylation and reduction of cytochrome *c*, rat liver microsomes are known to catalyze TPNH oxidation linked to the reduction of various artificial electron acceptors such as DCPIP (12, 16, 21) and NT (3, 16, 22). Furthermore, as recently shown by Hochstein *et al.* (15, 23, 24), TPNH is able to induce a peroxidation of endogenous microsomal lipids provided that a catalytic amount of FePP is added; the reaction can be followed conveniently by measuring the amount of malonaldehyde, which is a split product of lipid peroxides, and which has been found (23, 24) to be produced during microsomal lipid peroxidation to an extent of approximately 5 per cent of the total oxygen consumption. Table II and Fig. 5 compare these various microsomal TPNH-oxidizing activities with regard to their response to known inhibitors of microsomal drug hydroxylation. Carbon monoxide has been shown to block drug hydroxylation in liver microsomes (8), analogously to its effect on steroid hydroxylation in microsomes from adrenal cortex (25), by interacting with the hemoprotein called "CO-binding pigment" (5) or "cytochrome P-450" (6, 7). As shown in Table II, the effect of CO on drug hydroxylation appears to be unique among microsomal TPNH-oxidizing reactions, in that a concentration of CO that blocks oxidative demethylation of aminopyrine completely is entirely without inhibitory effect on the TPNH-cytochrome *c*, –DCPIP, and –NT reductase activities The rate of lipid peroxidation was in fact even slightly stimulated by CO. As has been reported elsewhere (8), drugs undergoing oxidative demethylation inhibit lipid peroxidation, which is restored by CO. Hence, the stimulating effect of CO observed here might be due to the suppression of a slow rate of hydroxylation of some endogenous substance(s) possibly present in the microsomes.

Fig. 5 illustrates the effects of PCMB and SKF 525A on the various TPNH-oxidizing reactions. PCMB inhibited not only oxidative demethylation, but also lipid peroxidation and the TPNH-linked reduction of NT; but it had little effect on the reduction of DCPIP, and only slightly inhibited the reduction of cytochrome *c*. These effects were investigated without preincubation of the microsomes with PCMB prior to assay. As noticed by Phillips and Langdon (4), TPNH–cytochrome *c*

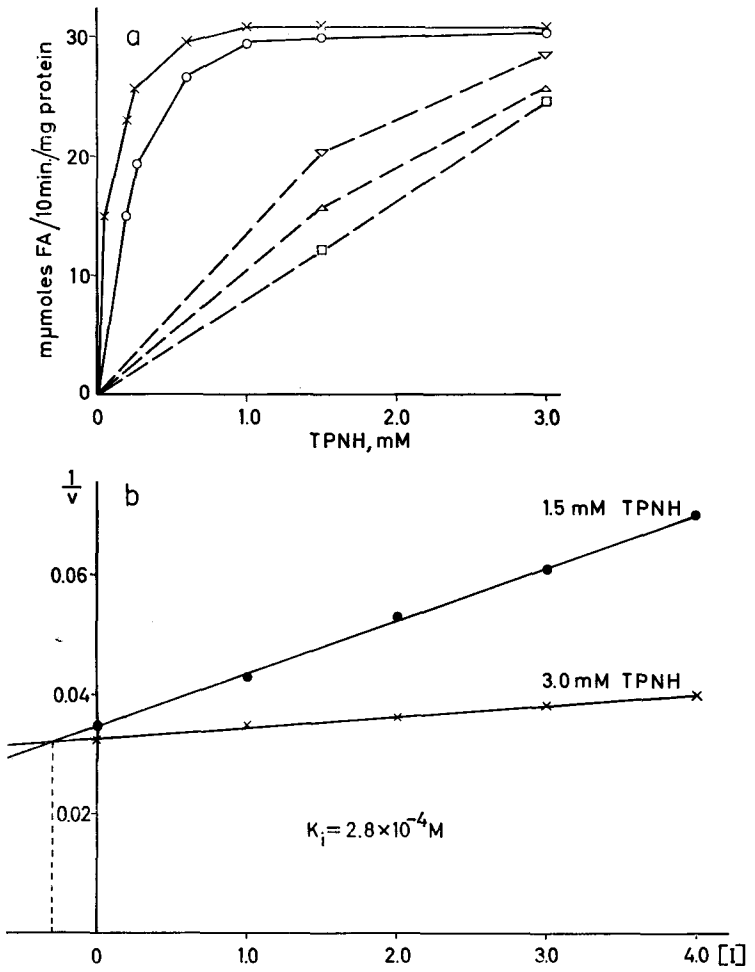

FIGURE 4 Fig. 4 *a*, rate of oxidative demethylation of aminopyrine with varying concentrations of added TPNH, in the absence and presence of nicotinamide, and in the presence of nicotinamide and varying concentrations of TPN. Crosses, TPNH; circles, TPNH + 50 mM nicotinamide; inverted deltas, TPNH + 50 mM nicotinamide + 1.5 mM TPN; deltas, TPNH + 50 mM nicotinamide + 3.0 mM TPN; squares, TPNH + 50 mM nicotinamide + 4.5 mM TPN.

Fig. 4 *b*, competitive inhibition of oxidative demethylation by TPN. [*I*], TPN, mM; *v*, relative reaction velocity.

reductase does acquire a PCMB sensitivity upon preincubation. SKF 525A, a well known inhibitor of microsomal hydroxylations (see reference 26), exerted a partial inhibition on both this reaction and the peroxidation of lipids, but had no effect on the TPNH–cytochrome *c*, –NT, and –DCPIP reductase reactions.

As first noticed by Gillette *et al.* (27), liver microsomes exhibit a slow rate of TPNH-dependent oxygen uptake, with the formation of H_2O_2, which requires no externally added hydrogen or hydroxyl acceptor. This "TPNH oxidase" reaction was later studied by Fouts (28) in connection with the submicrosomal localization of the drug-hydroxylating enzyme system. As shown in Table III, this reaction is entirely insensitive to CO, and is only partially inhibited by PCMB, properties which clearly distinguish it from drug hydroxylation. On the other hand, the reaction was found to be partially inhibited by DPPD, DIC, and KCN. Neither of

TABLE I

Oxidative Demethylation of Aminopyrine with Various Hydrogen Donors

The incubations were carried out as described in Materials and Methods, but TPN and the TPNH-generating system were omitted. Pyridine nucleotides and ascorbate were added in final concentrations as indicated.

Additions	Oxidative demethylation activity
	mμmoles FA/min./mg protein
TPNH, 3 mM	3.40
DPNH, 1 mM	0.18
2 mM	0.35
3 mM	0.49
4 mM	0.42
TPNH, 3 mM + DPNH, 3 mM	3.42
TPN, 2 mM	0
4 mM	0.15
DPNH, 3 mM + TPN, 0.25 mM	1.32
0.5 mM	1.46
1 mM	1.45
2 mM	1.00
4 mM	0.50
Ascorbate, 2 mM	0.10
4 mM	0.16
8 mM	0.14

these agents inhibited the oxidative demethylation of aminopyrine. The possible implications of these findings will be considered later (see Discussion).

Studies with Phenobarbital-Treated Rats

As already reported (1), treatment of rats with a daily dose of 100 mg of phenobarbital per kg body weight during 5 days leads to a 4 to 6-fold increase in the oxidative demethylating activity of the liver microsomes. A parallel and selective increase occurred in the amount of CO-binding pigment and the activity of TPNH–cytochrome *c* reductase. In the present study it could be established that the affinities of the drug-hydroxylating system and TPNH–cytochrome *c* reductase for TPNH were unchanged as compared with those found in the untreated animals, as was also the affinity of the drug-hydroxylating system for aminopyrine. Likewise unchanged were the K_i values for the inhibition by TPN.

As shown in Table IV, the TPNH–DCPIP and –NT reductase activities of the liver microsomes increased upon treatment of rats with phenobarbital, in a fashion parallel to the TPNH–cytochrome *c* reductase. The lipid-peroxidating activity of the microsomes from phenobarbital-treated animals was not investigated, since it might be expected that the liver microsomes of these animals would contain bound drug which inhibits lipid peroxidation (8). A drug-induced increase was also found in the TPNH-linked oxidation of luminol, as reported elsewhere (29). On the other hand, there was no increase in the drug-hydroxylating activ-

TABLE II

Effect of Carbon Monoxide on Microsomal TPNH Oxidation

The incubations were carried out in Warburg vessels, which were filled with the gas mixture, except for the recordings of cytochrome *c* reductase and DCPIP reductase activities, in which the gas mixture was bubbled through the cuvette for 20 seconds before the activity was measured.

System	Gas phase	
	4% O_2; 96% N_2	4% O_2; 56% N_2; 40% CO
Cytochrome *c* reductase (mμmoles cyt. *c* red./min./mg protein)	60	58
NT reductase (mμmoles NT red./min./mg protein)	109	120
DCPIP reductase (mμmoles DCPIP red./min./mg protein)	60	56
Lipid peroxidation (mμmoles MA formed/min./mg protein)	4.12	5.40
Oxidative demethylation (mμmoles FA formed/min./mg protein)	3.11*	0.38

* When air was used as gas phase, the corresponding value was 3.09.

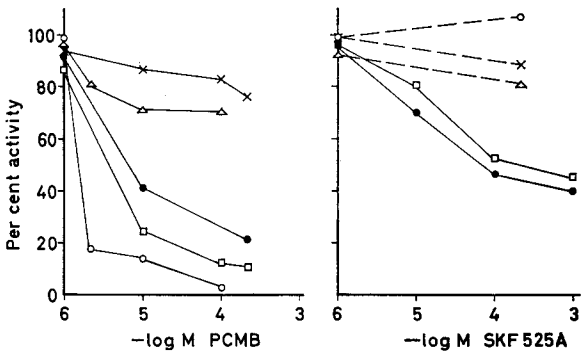

FIGURE 5 Effects of PCMB and SKF 525A on various microsomal TPNH-linked oxidoreductions. Crosses, cytochrome *c* reductase; open circles, NT reductase; deltas, DCPIP reductase; squares, lipid peroxidation; closed circles, oxidative demethylation.

TABLE III

Effects of Various Inhibitors on Microsomal TPNH-Dependent Oxygen Uptake

TPNH oxidase was recorded in the absence of drug as described in Materials and Methods.

Additions	Oxygen consumption
	mμmoles O_2 consumed/min./mg protein
TPNH, 10^{-4} M	3.28
TPNH, 3.0×10^{-4} M	4.02
TPNH, 6.0×10^{-4} M	3.92
TPNH, 3.0×10^{-4} M	3.90
+ 40% CO	4.02
TPNH, 3.0×10^{-4} M	4.00
+ PCMB, 10^{-4} M	1.95
+ PCMB, 10^{-3} M	1.90
TPNH, 3.0×10^{-4} M	4.15
+ DPPD, 1 μg	4.00
+ DPPD, 3 μg	3.40
+ DPPD, 5 μg	3.41
TPNH, 3.0×10^{-4} M	4.15
+ DIC, 5.0×10^{-7} M	3.02
+ DIC, 1.5×10^{-6} M	2.54
+ DIC, 3.0×10^{-6} M	2.52
TPNH, 3.0×10^{-4} M	4.10
+ KCN, 1.2×10^{-4} M	3.70
+ KCN, 2.5×10^{-4} M	3.60
+ KCN, 5.0×10^{-4} M	3.60
TPNH, 3.0×10^{-4} M	4.03
+ TPN, 2.0×10^{-4} M	3.02
+ TPN, 4.0×10^{-4} M	2.50

ity as measured with DPNH alone or in combination with TPN.

Liver microsomes from untreated animals revealed only a relatively modest difference in oxygen uptake when incubated with TPNH alone and with TPNH plus aminopyrine (1); the values usually observed were about 2 to 4 mμmoles O_2 consumed per minute per mg protein in the absence, and 5 to 7 in the presence, of aminopyrine. This difference was strikingly increased in the phenobarbital-treated animals, owing to a selective activation of the drug-induced oxygen uptake. This situation provided favorable conditions for determining with some accuracy the stoichiometry of the oxidative demethylation reaction with regard to oxygen consumption, TPNH disappearance, and formaldehyde formation. Results of a series of such determinations, summarized in Table V, show a molar relationship near to 1 between aminopyrine-induced increase in oxygen consumption and TPNH disappearance, and the amount of formaldehyde formed.

TABLE IV

Effect of Phenobarbital Treatment on the Microsomal Oxidation of TPNH with Various Electron Acceptors

Measurements were made by following the reduction of the acceptors (16) in the presence of 10^{-6} M dicoumarol.

No. of phenobarbital treatments	Acceptor		
	Cytochrome *c*	DCPIP	NT
	μmoles TPNH ox./min./mg protein		
0	0.032	0.039	0.100
3	0.096	0.145	0.348
5	0.130	0.190	0.420

Many attempts have been made to solubilize the drug-hydroxylating enzyme system of liver microsomes (30). However, only a minor part of

TABLE V

Stimulation of Microsomal TPNH Oxidation by Aminopyrine

Exp. no.	Oxygen consumption			TPNH oxidation*			FA formation
	−AP	+AP	Δ	−AP	+AP	Δ	+AP
	mμmoles/min./mg protein			*mμmoles/min./mg protein*			*mμmoles/min./mg protein*
1	5.3	20.1	14.8	4.9	18.0	13.1	14.0
2	4.9	21.5	16.6	6.1	20.5	14.4	15.2
3	4.9	20.8	15.7	6.0	19.5	13.5	15.8
4	6.0	20.1	14.1	6.2	20.0	13.8	15.0
5	5.3	16.2	10.9	6.2	15.4	9.2	10.6

* TPNH oxidation was determined spectrophotometrically by following the change of absorbancy at 340 mμ.

TABLE VI

Solubilization of the Oxidative Demethylating System by Naja naja Venom Treatment of Microsomes Isolated from Phenobarbital-Treated Rats and Controls

No. of phenobarbital treatments	Fraction	Protein	Oxidative demethylation activity	TPNH–cytochrome *c* reductase activity	CO-binding pigment			
					P-450		P-420	
					Reduced by $Na_2S_2O_4$	Reduced by TPNH	Reduced by $Na_2S_2O_4$	Reduced by TPNH
		mg/gm liver	*mμmoles FA/min./mg protein*	*μmoles TPNH ox./min./mg protein*	$\Delta E_{450-500}$/*mg protein*		$\Delta E_{420-500}$/*mg protein*	
0	Microsomes	23.1	2.75	0.024	0.020	0.019	—	—
	Solubilized	11.0	0.68	0.024	—	—	0.019	0.003
	Pellet	11.3	0.00	0.010	—	—	0.012	0.000
4	Microsomes	26.2	11.50	0.090	0.077	0.077	—	—
	Solubilized	16.0	2.40	0.095	—	—	0.083	0.019
	Pellet	9.3	0.00	0.017	—	—	0.048	0.000

the drug-hydroxylating activity has been recovered upon solubilization, and further purification has failed to give a reasonable concentration of the activity on the protein basis. Omura and Sato (6, 7) have shown that solubilization of the CO-binding pigment of liver microsomes by treatment with heated snake venom or deoxycholate is accompanied by a shift of the absorption peak from 450 to 420 mμ. Whereas the P-450 form is readily reduced by TPNH, the P-420 form is only poorly reducible enzymatically. Table VI shows the results of attempts to solubilize the oxidative demethylating system by treatment with heat-treated snake venom, using liver microsomes isolated from both normal and phenobarbital-treated rats. It may be seen that, in both cases, the supernatant after solubilization exhibited about the same specific activity of TPNH–cytochrome *c* reductase as did the original microsomes. *Naja naja* venom treatment also solubilized the CO-binding pigment and converted it into the P-420 form, which was only slightly reducible by TPNH. However, a minor part of the P-420 could be reduced by TPNH, and this part was found to be increased by phenobarbital stimulation of the rats *in vivo*. Parallel to this, an increased activity of aminopyrine demethylation was recovered in the supernatant from phenobarbital-stimulated rats as compared with the controls. Apparently, the decrease of the drug-hydroxylating activity upon solubilization is related to the modification of the CO-binding pigment.

DISCUSSION

For studying the rates of hydroxylation of various drugs by liver microsomes and especially for stud-

ying the enhancement of these rates caused by *in vivo* treatment with inducing drugs, it is of great importance that the activities are assayed under optimal conditions. Kinetic studies presented in this paper establish the quantitative requirements for drug and TPNH in obtaining maximal reaction rates of oxidative demethylation which are linear with time and with the amount of microsomal protein. The fact that the K_m values for drug and TPNH are unchanged in the microsomes from the drug-treated animals eliminates the possibility that the enhancement of drug hydroxylation found in these microsomes might be due to an increased substrate affinity rather than to a net increase in enzyme content. Activation of microsomal glucose-6-phosphatase in alloxan-diabetic rats, reversed by insulin, has earlier been found by Segal and Washko (31) to be connected with shifts in the affinity of the enzyme for its substrate.

Of particular importance in establishing optimal conditions for determination of the microsomal drug-hydroxylating activity may be the present finding that TPN is a competitive inhibitor of the process. Earlier observations that DPNH stimulates TPNH-linked drug hydroxylation (32, 33) may be explained on the basis of a removal of TPN by way of transhydrogenase. In the present experiments, using conditions preventing TPN accumulation, no stimulation of the reaction rate by DPNH was found. Whether the slow reaction driven by DPNH alone proceeds by a separate pathway or by a trace of TPN bound to the microsomes cannot be decided on the basis of the data available. Also the nature of the microsomal transhydrogenase reaction and its relationship to those occurring in mitochondria (34, 35) will require further investigation. The fact that the DPNH + TPN-driven drug hydroxylation activity of the microsomes does not increase parallel to the TPNH-driven one upon drug treatment indicates that the drug-induced activation of the hydroxylating enzyme system does not include the transhydrogenase.

A similar conclusion seems to apply to the microsomal "TPNH oxidase" reaction. This reaction is unaffected by CO, a fact which suggests that it is not a functional reflection of the entire hydroxylating enzyme system including the cytochrome P-450. The effect of DPPD suggests the involvement of lipid peroxidation (23, 24), a possibility also supported by the observation of Staudinger and Zubrzycki (36) that the molar ratio of oxygen consumed to TPNH oxidized in the TPNH oxidase reaction slightly exceeds 1. Inhibition by low concentration of DIC suggests the involvement in the TPNH oxidase reaction of the enzyme DT diaphorase (37), small amounts of which are consistently found in rat liver microsomes (38). By reacting with a quinone possibly present in the microsomes, and subsequent autooxidation of the reduced quinone, DT diaphorase might account for a part of the TPNH oxidase reaction. Finally, the partial sensitivity to KCN suggests the involvement of cytochrome oxidase, possibly present in the form of contaminating traces of mitochondrial fragments, although the pathway by which TPNH would be oxidized by this system is unclear. In any case, the present data warrant caution in using TPNH oxidase as a parameter in measuring microsomal drug hydroxylation.

By using microsomes isolated from phenobarbital-pretreated rats, it has been possible in this work to establish the stoichiometry of the oxidative demethylation reaction. This was shown to involve a 1:1:1 molar relationship between TPNH oxidized, O_2 consumed, and formaldehyde produced, which substantiates the generally proposed equation (see reference 26):

$$RCH_3 + TPNH + H^+ + O_2 \rightarrow RH + HCHO + TPN^+ + H_2O$$

Studies with various electron acceptors and inhibitors have provided important information concerning the components of the drug-hydroxylating system. This information is summarized schematically in Fig. 6. Involvement of the flavoprotein known as TPNH–cytochrome *c* reductase and the CO-binding pigment called cytochrome P-450 in the hydroxylating system has been strongly suggested by the selective increase in the levels of these two enzymes parallel to the drug-induced increase in the over-all hydroxylating activity (1). The involvement of TPNH–cytochrome *c* reductase is further supported by the similar K_m values for TPNH of the cytochrome *c* reductase and the drug-hydroxylating reaction, as well as by their similar inhibition by TPN, as revealed by the present data. The CO-binding pigment, which was first described by Klingenberg (5), has recently been solubilized by Omura and Sato (6, 7) and shown to be a cytochrome of the *b* type. The participation of this enzyme in the hydroxylation of steroids by adrenal microsomes was first demonstrated by Estabrook *et al.* (25), whereas its in-

volvement in the drug hydroxylation of liver microsomes was shown in this laboratory (8) and later confirmed by Staudinger *et al.* (39).

The interaction between TPNH–cytochrome *c* reductase and the CO-binding pigment may prove to be a complex process, as suggested by several observations. The fact that PCMB strongly and consistently inhibits oxidative demethylation while it exerts a variable influence—inhibition, no effect, or stimulation, depending on the assay conditions —on the TPNH–cytochrome *c* reductase (4) suggests that the flavoenzyme involves sulfhydryl groups, which are essential for the coupled reaction but not for the simple cytochrome *c* reductase function. The occurrence of a NT reductase activity, which is more labile than the cytochrome *c* reductase (3, 16), and which again is consistently PCMB sensitive, further suggests the existence of multiple sites or modes of action of the flavoprotein. The involvement of additional electron transfer catalysts in the coupled reactions, such as non-heme iron or a quinone, is also a conceivable possibility.

Finally, the question as to whether the drug-hydroxylating system involves specific enzymes for the activation of the individual drugs cannot be answered on the basis of the information presented in this paper. Data from experiments with various inducing drugs are presented in a succeeding paper. (40) and discussed in relation to this problem.

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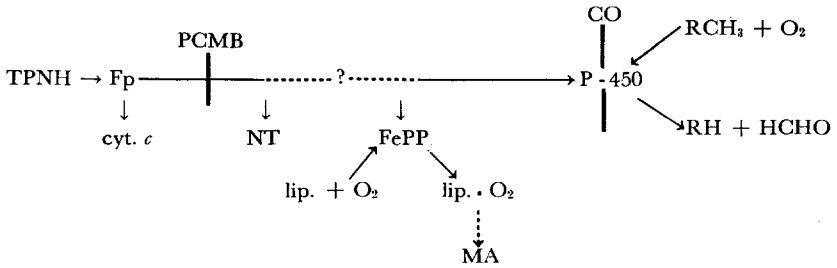

FIGURE 6 Hypothetic reaction pathways of microsomal drug hydroxylation and related reactions. *Fp* flavoprotein.

REFERENCES

1. ORRENIUS, S., ERICSSON, J. L. E., and ERNSTER, L., *J. Cell Biol.*, 1965, **25,** 627.
2. HORECKER, B. L., *J. Biol. Chem.*, 1950, **183,** 593.
3. WILLIAMS, C. H., and KAMIN, H., *J. Biol. Chem.*, 1962, **237,** 587.
4. PHILLIPS, A. H., and LANGDON, R. G., *J. Biol. Chem.*, 1962, **237,** 2652.
5. KLINGENBERG, M., *Arch. Biochem. and Biophysics*, 1958, **75,** 376.
6. OMURA, T., and SATO, R., *J. Biol. Chem.*, 1964, **239,** 2370.
7. OMURA, T., and SATO, R., *J. Biol. Chem.*, 1964, **239,** 2379.
8. ORRENIUS, S., DALLNER, G., and ERNSTER, L., *Biochem. and Biophysic. Research Commun.*, 1964, **14,** 329.
9. ORRENIUS, S., DALLNER, G., and ERNSTER, L., Abstracts, First Meeting of the Federation of European Biochemical Societies, London, 1964, 13.
10. ORRENIUS, S., DALLNER, G., NORDENBRAND, K., and ERNSTER, L., Abstracts, 6th International Congress of Biochemistry, New York, 1964, 326.
11. ORRENIUS, S., and ERNSTER, L., Abstracts, 14th Scandinavian Congress on Pathology and Microbiology, Oslo, 1964, 145.
12. ERNSTER, L., SIEKEVITZ, P., and PALADE, G. E., *J. Cell Biol.*, 1962, **15,** 541.
13. NASH, T., *Biochem. J.*, 1932, **26,** 292.
14. BERNHEIM, F., BERNHEIM, M. L., and WILBUR, K. M., *J. Biol. Chem.*, 1948, **174,** 257.
15. HOCHSTEIN, P., NORDENBRAND, K., and ERNSTER, L., *Biochem. and Biophysic. Research Commun.*, 1964, **14,** 323.
16. DALLNER, G., *Acta Path. et Microbiol. Scand.*, 1963, suppl. 166.
17. IMAI, Y., and SATO, R., *Biochim. et Biophysica Acta*, 1960, **42,** 164.
18. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A.

L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193,** 265.
19. NILSSON, A., *Ark. Kemi*, 1963, **21,** 97.
20. MITOMA, C., POSNER, H. S., REITZ, H. C., and UDENFRIEND, S., *Arch. Biochem. and Biophysics*, 1956, **61,** 431.
21. ERNSTER, L., *Acta Chem. Scand.*, 1958, **12,** 600.
22. WILLIAMS, C. H., GIBBS, R. H., and KAMIN, H., *Biochim. et Biophysica Acta*, 1959, **32,** 568.
23. HOCHSTEIN, P., and ERNSTER, L., *Biochem. and Biophysic. Research Commun.*, 1963, **12,** 388.
24. HOCHSTEIN, P., and ERNSTER, L., *in* Ciba Foundation Symposium on Cellular Injury, (A. V. S. de Reuck and J. Knight, editors), London, Churchill, 1963, 123.
25. ESTABROOK, R. W., COOPER, D. Y., and ROSENTHAL, O., *Biochem. Z.*, 1963, **338,** 741.
26. BRODIE, B. B., *in* Ciba Foundation Symposium on Enzymes and Drug Action, (J. L. Mongar and A. V. S. de Reuck, editors), London, Churchill, 1962, 317.
27. GILLETTE, J. R., BRODIE, B. B., and LA DU, B. N., *J. Pharmacol. and Exp. Therap.*, 1957, **119,** 532.
28. FOUTS, J. R., *Biochem. and Biophysic. Research Commun.*, 1961, **6,** 373.
29. NILSSON, R., ORRENIUS, S., and ERNSTER, L., *Biochem. and Biophysic. Research Commun.*, 1964, **17,** 303.
30. SCHUSTER, L., *Ann. Rev. Biochem.*, 1964, **33,** 571.
31. SEGAL, H. L., and WASHKO, M. E., *J. Biol. Chem.*, 1959, **234,** 1937.
32. CONNEY, A. H., BROWN, R. R., MILLER, J. A., and MILLER, E. C., *Cancer Research*, 1957, **17,** 628.
33. NILSSON, A., and JOHNSON, B. C., *Arch. Biochem. and Biophysics*, 1963, **101,** 494.
34. KAPLAN, N. O., COLOWICK, S. F., and NEUFELD, E. F., *J. Biol. Chem.*, 1953, **205,** 1.
35. DANIELSSON, L., and ERNSTER, L., *Biochem. Z.*, 1963, **338,** 188.
36. STAUDINGER, HJ., and ZUBRZYCKI, Z., *Z. Physiol. Chem.*, 1963, **332,** 109.
37. ERNSTER, L., DANIELSSON, L., and LJUNGGREN, M., *Biochim. et Biophysica Acta*, 1962, **58,** 171.
38. DANIELSSON, L., ERNSTER, L., and LJUNGGREN, *Acta Chem. Scand.*, 1960, **14,** 1837.
39. STAUDINGER, HJ., KERÉKJÁRTÓ, B. V., ULLRICH, V., and ZUBRZYCKI, Z., *in* An International Symposium on Oxidases and Related Oxidation Reduction Systems, (T. E. King, H. S. Mason, and M. Morrison, editors) Amherst, Massachusetts, 1964, in press.
40. ORRENIUS, S., *J. Cell Biol.*, 1965, **26,** 725.