MOLECULAR PHARMACOLOGY, 9, 470–485

Reduced Diphosphopyridine Nucleotide Synergism of the Reduced Triphosphopyridine Nucleotide-Dependent Mixed-Function Oxidase System of Hepatic Microsomes

II. Role of the Type I Drug-Binding Site of Cytochrome P-450

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(Received December 14, 1972)

SUMMARY

CORREIA, MARIA ALMIRA, AND MANNERING, G. J.: Reduced diphosphopyridine nucleotide synergism of the reduced triphosphopyridine nucleotide-dependent mixed-function oxidase system of hepatic microsomes. II. Role of the type I drug-binding site of cytochrome P-450. *Mol. Pharmacol.* **9**, 470–485 (1973).

Studies of the DPNH synergism of the TPNH-dependent mixed-function oxidase system of hepatic microsomes using a variety of substrates revealed that synergism occurred when the substrates were type I binding compounds (aminopyrine, benzphetamine, codeine, ethylmorphine, norcodeine), but not when the substrates were type II binding compounds (aniline, *p*-chloro-*N*-methylaniline). The role of type I binding in DPNH synergism of drug metabolism was investigated by employing microsomes which varied in their abilities to produce a type I binding spectrum with ethylmorphine. This was accomplished by selecting microsomes from different animal sources (male rats of different ages, female rats, 3-methyl cholanthrene-treated rats) or by subjecting microsomes from a given source to treatments known to diminish type I binding (treatment with SKF 525-A or with phospholipase C, storage). Using ethylmorphine as the substrate, type I binding was shown to be directly correlated with DPNH synergism and with DPNH utilization. The mean ratio of DPNH utilized to ethylmorphine metabolized by the various microsomes used in the study was 1.15. No correlation was seen between DPNH utilization and rate of hydroxylation of the type II compound, aniline. No increase in DPNH utilization above that seen in the absence of substrate was observed during aniline hydroxylation. The mechanism whereby type I substrates elicit DPNH synergism is postulated to occur as follows. The first of the 2 electrons required for drug oxidation is derived from TPNH and is utilized in the reduction of the oxidized cytochrome P-450–substrate complex. The second electron is derived from either TPNH or DPNH and is transported through cytochrome b_5 to the oxygenated, reduced cytochrome P-450–substrate complex, although the possibility remains that second electrons from TPNH may be contributed by a route that circumvents cytochrome b_5. When type I substrates are introduced into the system, the rate of entry of first electrons is accelerated and second electrons from the electron pool provided by DPNH are drawn via cytochrome b_5 into the system to balance the elevated input of first electrons. When type II substrates are introduced into the system, the rate of entry of first electrons is not accelerated and there is

neither the need nor the means for the system to utilize excess second electrons provided by DPNH. When only TPNH is present, the electron pool created at cytochrome b_5 is not large enough to match the pool of first electrons created by the addition of the type I substrate, and the over-all reaction is therefore slower than the DPNH-"synergized" reaction.

INTRODUCTION

In the preceding communication (1) the mechanism by which DPNH synergizes TPNH-dependent ethylmorphine *N*-demethylase was investigated by manipulating the activity of the microsomal cytochrome b_5-dependent fatty acyl coenzyme A desaturation system, which shunts reducing equivalents from DPNH away from the site where synergism of drug metabolism is mediated. When the shunt was activated with stearoyl-CoA, a substrate for the desaturation system, ethylmorphine metabolism was decreased; when the activity of the shunt was depressed by inhibiting a cyanide-sensitive factor required in the desaturation system, ethylmorphine metabolism was increased. These studies supported the concept that Estabrook and associates (2–6) have developed to explain DPNH synergism of TPNH-dependent drug metabolism, briefly, that the first of the 2 electrons required by the cytochrome P-450 system responsible for drug oxidations must come from TPNH, but that the second electron, which can be provided by either TPNH or DPNH through cytochrome b_5, is more available to the P-450 system when it is contributed by DPNH. The substrate in our studies was ethylmorphine, a compound which produces a type I spectrum (7) when complexed with cytochrome P-450. When these studies were expanded to determine the role of DPNH synergism in the oxidation of other type I compounds, as well as those which produce a type II spectrum, it became apparent that DPNH synergism occurred only when type I compounds were employed as substrates. The role of type I binding in DPNH synergism of drug oxidation was further investigated by employing microsomes which varied in their abilities to produce a type I binding spectrum with ethylmorphine. This was accomplished by selecting microsomes from different animal sources or by subjecting microsomes from a given source to treatments known to diminish type I binding. The P-450 hemoprotein in microsomes from female rats and young male rats shows less type I binding than that from adult male rats (8). The P-450 hemoprotein that results after the administration of 3-methylcholanthrene is highly deficient in a type I binding site (9, 10). Microsomes lose their ability to produce a type I binding spectrum with ethylmorphine after they have been treated with SKF 525-A (11)[1] or with phospholipase C (13). Microsomes lose type I binding during storage (9). In the current study both the degree of DPNH synergism of TPNH-dependent ethylmorphine *N*-demethylation and the amount of DPNH utilization were found to be correlated with the ability of microsomes to produce a type I binding spectrum.

MATERIALS AND METHODS

Aniline HCl was obtained from Eastman Organic Chemicals; *p*-chloro-*N*-methylaniline, from Calbiochem; aminopyrine (4-dimethylaminoantipyrine), from Aldrich Chemical Company; and codeine phosphate and norcodeine HCl, from Merck Sharp & Dohme Research Laboratory. Benzphetamine HCl and SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl) were gifts from the Upjohn Company, Kalamazoo, Mich., and Smith Kline & French Research Laboratories, Philadelphia, respectively. 3-Methylcholanthrene was obtained from Eastman Kodak. *dl*-Isocitrate (trisodium salt) and pig heart isocitrate dehydrogenase (type IV) were obtained from Sigma Chem-

This research was supported by United States Public Health Service Grant GM 15477. Part of this material appeared in abstract form [*5th Int. Cong. Pharmacol.* (*San Francisco*) p. 46 (1972)].

[1] K. M. Bidleman and G. J. Mannering, unpublished observations cited by Parli and Mannering (12).

ical Company. Sources of other materials used in the study are given in the preceding communication (1).

Male and female Holtzman rats, fed and watered ad libitum until dealth, were the source of hepatic microsomes, which were prepared as described previously (14). Microsomes were used within 3 hr of their preparation except when stored to reduce their capacity for type I binding, in which case they were suspended in isotonic KCl solution at −10° under air for as long as 168 hr. Stored microsomes were thawed and rehomogenized before use. Microsomes were treated with phospholipase C (EC 3.1.4.3, Sigma Chemical Company) as described previously (13); the reaction was stopped by chilling rather than with EDTA. Phospholipase C is thought to remove most of the type I binding sites (13). Microsomes were treated with SKF 525-A as follows. Washed, pelleted microsomes were suspended in 1.15 M Tris-KCl buffer, pH 7.4, to a concentration of approximately 6–8 mg of protein per milliliter. The microsomal suspension was divided into two portions. SKF 525-A was added to the first (final concentration, 0.75 mM), and the other was diluted to the same volume as the first portion. The two portions were sedimented at 100,000 × *g* for 30 min. The pellets were resuspended in 1.15% KCl solution, resedimented at 100,000 × *g* for 30 min, and resuspended in KCl solution to a protein concentration of 5 mg/ml. SKF 525-A is thought to bind irreversibly to the type I binding site (12, 15).

The incubation mixtures containing a TPNH (TPN^+, 0.4 mM)-generating system, DPNH (1.0 mM), or both, have been described previously (1). Formaldehyde formed by demethylation of ethylmorphine, PCNMA,[2] aminopyrine, benzphetamine, codeine, and norcodeine was determined by the method of Nash (16). Aniline hydroxylase activity was determined as described by Kato and Gillette (17), with modifications described previously (13) or, where indicated, by the more rapid method of Imai *et al.* (18). Final concentrations of substrates were: aminopyrine, 5 mM; aniline, 1 mM; benzphetamine, 2 mM; codeine, 3 mM; ethylmorphine, 2 mM; norcodeine, 2 mM; and PCNMA, 1 mM. Reaction mixtures were incubated under air at 37° for 5 min when aminopyrine was the substrate, and for 15 min when other substrates were employed. A 10-min incubation time was employed when ethylmorphine was incubated with phospholipase C-treated microsomes. All reaction rates were linear during these incubation periods.

DPNH was measured as described by Estabrook and Maitra (19). Type I binding was determined by the procedure of Remmer *et al.* (20), as described previously (9, 13), using a saturating concentration of ethylmorphine (2.6 mM). Cytochromes P-450 and b_5 were determined as described by Omura and Sato (21). Protein was determined by the method of Lowry *et al.* (22).

3-Methylcholanthrene-treated rats received daily intraperitoneal injections of 3-methylcholanthrene (20 mg/kg in corn oil) for 4 days. They were killed 24 hr after the last injection. Control rats received corn oil for 4 days.

RESULTS

Absence of DPNH synergism of TPNH-dependent oxidation of type II drugs (aniline and PCNMA) and failure of cyanide to elicit DPNH synergism. In Figs. 1 and 2 it can be seen that DPNH does not synergize the TPNH-dependent hydroxylation of aniline or *N*-demethylation of PCNMA (CN^- concentration = 0), nor does cyanide stimulate the metabolism of these compounds appreciably at any of the concentrations employed. This is in contrast with the 30% DPNH synergism of ethylmorphine *N*-demethylation seen in the absence of cyanide and the 90% synergistic effect observed in the presence of optimal concentrations of cyanide (1). The highest concentrations of cyanide inhibited aniline and PCNMA metabolism. The inhibitory effect of high concentrations of cyanide on ethylmorphine *N*-demethylation has also been observed (1) and may be explained by the complexing of cyanide to cytochrome P-450 (23).

DPNH synergism of TPNH-dependent

[2] The abbreviation used is: PCNMA, *p*-chloro-*N*-methylaniline.

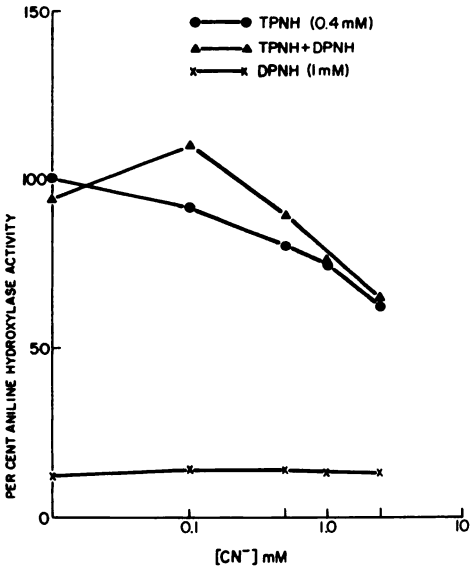

FIG. 1. *Effects of cyanide on aniline hydroxylase activity of rat hepatic microsomes in the presence of TPNH, DPNH, or both*

Aniline (1 mM) was incubated with microsomes in a mixture (final volume, 5 ml) containing a TPNH-generating system (TPN^+, 0.4 mM; glucose 6-phosphate, 2 mM; and glucose 6-phosphate dehydrogenase, 2 enzyme units); $MgCl_2$, 2 mM; 0.04 M phosphate buffer, pH 7.4; 1.15% KCl; and various concentrations of CN^-. The mixture minus microsomes was incubated for 3 min under air at 37°, the reaction was started by adding 1 ml of microsomal preparation (5 mg of protein per milliliter), and the incubation was continued for another 20 min. In systems containing both DPNH and TPNH, DPNH (final concentration, 1 mM) was added as a freshly made solution in 1.15% KCl just prior to incubation. In reactions containing DPNH only, the TPNH-generating system was omitted. Reaction rates were determined by measuring the *p*-aminophenol formed by the method of Kato and Gillette (17). All values (means of three experiments) are recorded as percentages of aniline hydroxylase activity observed when TPNH was the only source of electrons and no cyanide was present (100% = 58 nmoles of *p*-aminophenol formed per milligram of protein per hour). In each experiment livers were pooled from at least two male rats (180–220 g).

oxidation of type I drugs (aminopyrine, benzphetamine, codeine, ethylmorphine, norcodeine) and its enhancement with cyanide. DPNH synergism of TPNH-dependent drug metabolism has been observed when amino-

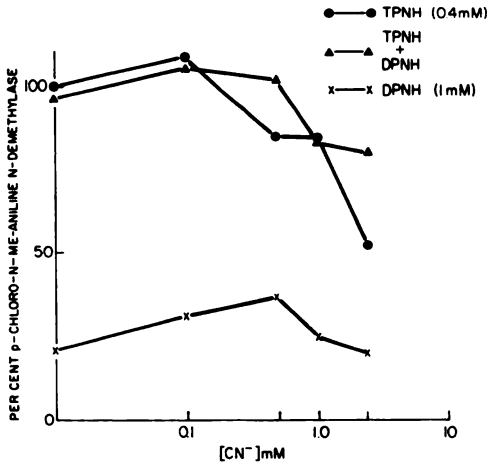

FIG. 2. *Effects of cyanide on p-chloro-N-methylaniline N-demethylase activity of rat hepatic microsomes in the presence of TPNH, DPNH, or both*

PCNMA (1 mM) was incubated with microsomes as described in Fig. 1, except that semicarbazide HCl (7.5 mM) was included in the medium, 3.0–5.0 mg of microsomal protein were employed, and the incubation period was 15 rather than 20 min. The formaldehyde formed was determined by the Nash method (16). All values (means of two experiments) are recorded as percentages of the PCNMA *N*-demethylase activity observed when TPNH was the only source of electrons and no cyanide was present (100% = 293 nmoles of formaldehyde formed per milligram of protein per hour). In each experiment livers were pooled from at least two male rats (180–220 g).

pyrine (2, 4, 5), codeine (4, 24), and ethylmorphine (1, 4, 6) have been used as substrates. All these compounds are known to give a type I binding spectrum with microsomes. The failure of the oxidation of the type II compounds, aniline and PCNMA, to be synergized by DPNH suggested that type I binding of substrates may be required for DPNH synergism. As a means of exploring this possibility, the effect of cyanide on the rates of oxidation of a number of type I substrates was studied with either TPNH or DPNH as the sole source of reducing equivalents, as well as in the presence of both TPNH and DPNH. Concentrations of cyanide previously shown to give optimal enhancement of DPNH synergism of ethylmorphine *N*-demethylation (0.1–0.5 mM) were used in all cases. The results are sum-

TABLE 1

DPNH synergism of TPNH-dependent rat liver microsomal oxidations of type I and type II substrates in the presence and absence of cyanide

Control values (micromoles of product formed per milligram of protein per hour) were obtained in the absence of CN^- and with TPNH as the only electron donor. Assay conditions are described under MATERIALS AND METHODS. Incubation mixtures contained 3.5–5 mg of microsomal protein in a volume of 5 ml. N = number of experiments, each of which employed microsomes pooled from at least two rats (150–250 g).

Substrate	N	Control	TPNH		DPNH		TPNH + DPNH	
			$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$	$-CN^-$	$+CN^-$
			% control		*% control*		*% control*	
Ethylmorphine	10	0.437 ± 0.024	100	111 ± 1.7	11 ± 1.8	10 ± 1.6	132 ± 1.6	202 ± 4.6
Codeine	3	0.281 ± 0.063	100	111 ± 4.4	11 ± 4.0	15 ± 7.5	113 ± 2.7	167 ± 17.5
Norcodeine	3	0.138 ± 0.028	100	108 ± 5.2	4 ± 0.9	4 ± 1.1	130 ± 6.3	146 ± 8.2
Benzphetamine	3	0.276 ± 0.017	100	99 ± 2.5	7 ± 0.6	9 ± 1.5	142 ± 1.6	132 ± 9.3
Aminopyrine	3	0.619 ± 0.005	100	95 ± 6.8	7 ± 2.0	5 ± 4.5	129 ± 0.9	117 ± 4.7
p-Chloro-*N*-methylaniline	3	0.195 ± 0.057	100	98 ± 6.7	35 ± 8.2	42 ± 8.4	106 ± 7.6	109 ± 6.6
Aniline	3	0.058 ± 0.002	100	95 ± 1.6	10 ± 1.7	8 ± 4.3	97 ± 3.6	108 ± 2.4

marized in Table 1. Results obtained with aniline and PCNMA are also given in the table. The metabolism of all the type I compounds was greater in the presence of both DPNH and TPNH than in the presence of TPNH only, but degrees of DPNH synergism varied greatly. Even greater variation was seen in the enhancing effect of cyanide on DPNH synergism, with comparable large effects seen with ethylmorphine and codeine, only a slight effect seen with norcodeine, and no effect, or perhaps even a negative effect, seen with aminopyrine and benzphetamine. Reasons for these differences are not immediately apparent; they are discussed later. As expected, the oxidation of aniline and PCNMA was not affeetcd by either DPNH or cyanide.

DPNH utilization during aniline hydroxylation. Figure 3 shows DPNH utilization and *p*-aminophenol formation during the oxidation of aniline in the presence of various concentrations of cyanide when both TPNH and DPNH or TPNH alone were added to the medium. Differences between DPNH utilization during aniline oxidation and during ethylmorphine N-demethylation under similar conditions are apparent when Fig. 3 is compared with Fig. 4 of the preceding communication (1). Cyanide did not stimulate utilization of DPNH during aniline hydroxylation, as was the case when ethylmorphine was the substrate, but DPNH utilization decreased continuously as cyanide concentrations were increased. The figure provides no evidence that DPNH is used during aniline hydroxylation, but neither does it exclude this possibility. At a concentration of 0.1 mM cyanide, where aniline hydroxylation was proceeding maximally, about 10 times more DPNH was utilized than could be accounted for by *p*-aminophenol formation. This is in contrast to the DPNH:HCHO ratio of 1.31 seen at an optimal cyanide concentration during ethylmorphine N-demethylation (1). TPNH was without effect on the utilization of DPNH in either the presence or absence of cyanide. The decline in DPNH utilization with increasing cyanide concentration is most likely attributable to the blocking of cyanide-sensitive, DPNH-dependent pathways, such as the fatty acyl-CoA desaturation system, and to unknown, cyanide-insensitive path-

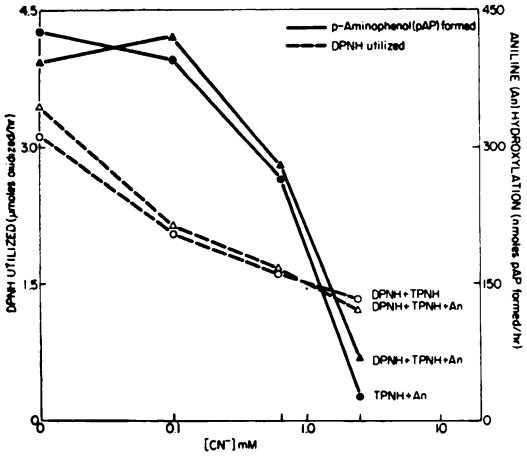

FIG. 3. *DPNH utilization during p-hydroxylation of aniline (An)*

Aniline (0.2 mM) was incubated with microsomes, a TPNH-generating system (TPN^+, 0.4 mM; dl-isocitrate, 4 mM; and isocitrate dehydrogenase, 1 enzyme unit); $MgCl_2$, 5 mM; phosphate buffer (0.04 M), pH 7.4; 1.15% KCl; and various concentrations of CN^-. The mixture minus microsomes was incubated for 3 min under air at 37°, the reaction was started by adding 1 ml of microsomal preparation (5 mg of protein per milliliter), and the incubation was continued for another 20 min. In reactions containing both DPNH and TPNH, DPNH (final concentration, 1 mM) was added as a freshly prepared solution in 1.15% KCl just prior to the incubation. At the end of 20 min, 2-ml aliquots of the mixture were removed and the DPNH and TPNH determinations were carried out as described previously (1). *p*-Aminophenol formed was determined in the remaining incubation mixture by the method of Imai *et al.* (18). All values are means of two experiments employing pooled livers from at least two rats (220–250 g).

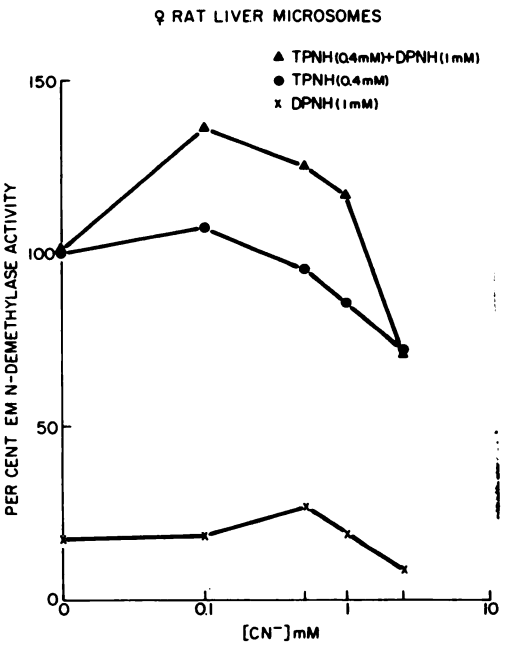

FIG. 4. *DPNH synergism of N-demethylation of ethylmorphine (EM), using microsomes from female rats*

Microsomal preparations from female rats were incubated with ethylmorphine (2 mM), using the mixture and incubation procedure described in Fig. 2. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity when TPNH was the sole electron donor and no cyanide was present (100% = 125 nmoles of formaldehyde formed per milligram of protein per hour). The mean value for ethylmorphine binding $\Delta A_{385-420}$ per milligram of microsomal protein was 0.009. In each experiment livers were pooled from at least three rats (160–180 g).

ways. Evidence for the latter is seen in the appreciable utilization of DPNH at the highest concentration of cyanide, where the hydroxylation of aniline was blocked almost completely.

Netter (25) has also observed that, in contrast to type I compounds, type II compounds do not increase the utilization of DPNH.

In passing, it is worth noting that cyanide inhibited aniline *p*-hydroxylation (Figs. 1 and 3) and PCNMA *N*-demethylation (Fig. 2) in concentrations that had little or no effect on ethylmorphine *N*-demethylation (1). This might have been predicted, because cyanide exhibits a type II binding spectrum with microsomes (26, 27).

DPNH synergism of N-demethylation of ethylmorphine, using microsomes from female rats. Female rats oxidize many drugs less rapidly than male rats, and this has been related to deficiency in type I binding sites in microsomes from females (8). If type I binding is a required feature of DPNH synergism of ethylmorphine *N*-demethylation, DPNH synergism should be less prevalent in microsomes from female rats than in microsomes from male rats. Com-

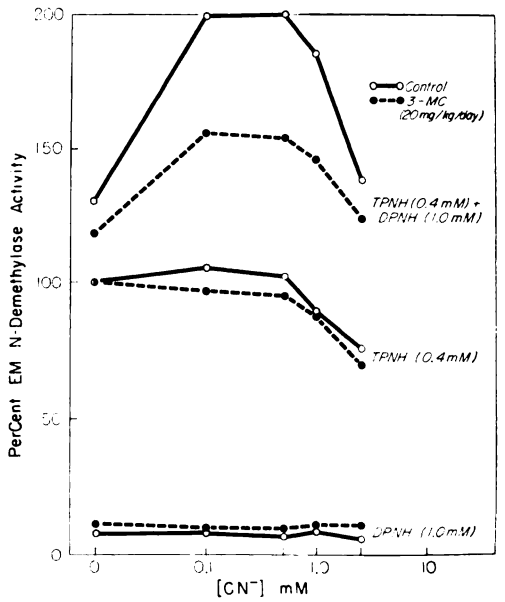

FIG. 5. *DPNH synergism of N-demethylation of ethylmorphine (EM), using microsomes from rats treated with 3-methylcholanthrene (3-MC)*

3-Methylcholanthrene (in corn oil) was administered intraperitoneally to male rats (180–200 g) at a dose of 20 mg/kg/day for 4 consecutive days. Control animals received an equivalent volume of the vehicle (corn oil) for the same time period. The rats were killed 24 hr after the last injection. Ethylmorphine *N*-demethylase activities were determined as described in Fig. 2, except that the microsomal suspension always contained 5 mg of protein per milliliter. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity when TPNH was the sole electron donor and no cyanide was present (100% = 534 and 529 nmoles of formaldehyde formed per milligram of protein per hour for microsomes from control and 3-methylcholanthrene treated rats, respectively). The mean values for ethylmorphine binding $\Delta A_{385-420}$ per milligram of protein were 0.022 and 0.005 for control and 3-methylcholanthrene-treated rat liver microsomes, respectively. In each experiment livers were pooled from at least two animals.

parison of Fig. 4 with Fig. 1 of the preceding communication (1) shows this to be the case. In the absence of cyanide, no DPNH synergism was seen when microsomes from female rats were employed, and cyanide caused a maximum synergistic effect of about 35% as compared to the 90% seen when microsomes from male rats were used.

DPNH synergism of N-demethylation of ethylmorphine, using microsomes from rats treated with 3-methylcholanthrene. Microsomes from rats treated with 3-methylcholanthrene are thought to be deficient in type I binding sites (9, 10). In accordance with the concept that type I binding is relevant to DPNH synergism, microsomes from animals treated in this manner should be less responsive to the synergistic effects of DPNH than microsomes from untreated animals. The results presented in Fig. 5 show this to be the case.

DPNH synergism of N-demethylation of ethylmorphine, using microsomes treated with SKF 525-A. SKF 525-A is thought to combine irreversibly with the type I site (12, 15). Microsomes deprived of type I binding sites in this manner could not take advantage of DPNH synergism while metabolizing ethylmorphine if DPNH synergism requires type I binding. Results presented in Fig. 6 show this to be the case.

DPNH synergism of N-demethylation of ethylmorphine, using microsomes treated with phospholipase C. Treatment of microsomes with phospholipase C is thought to destroy the type I binding site (13). Microsomes treated in this manner would not be expected to respond to synergistic effects of DPNH. As can be seen in Fig. 7, little or no DPNH synergism of ethylmorphine *N*-demethylation occurred when phospholipase C-treated microsomes were used in either the presence or absence of cyanide. "Control" microsomes exhibited about a 70% DPNH synergism in the absence of cyanide, in contrast to the 30% usually seen with "untreated" microsomes (1). The "control" microsomes in these studies differed from "untreated" microsomes in that they were treated exactly like those incubated with phospholipase C except that phospholipase C was omitted; this included the addition of Ca^{++}, which is essential for phospholipase C activity. Ca^{++} is known to stimulate DPNH synergism (28). Cyanide-sensitive factor is a relatively unstable enzyme (23), and its inactivation during incubation of the microsomes and the extra time required for preparation of the

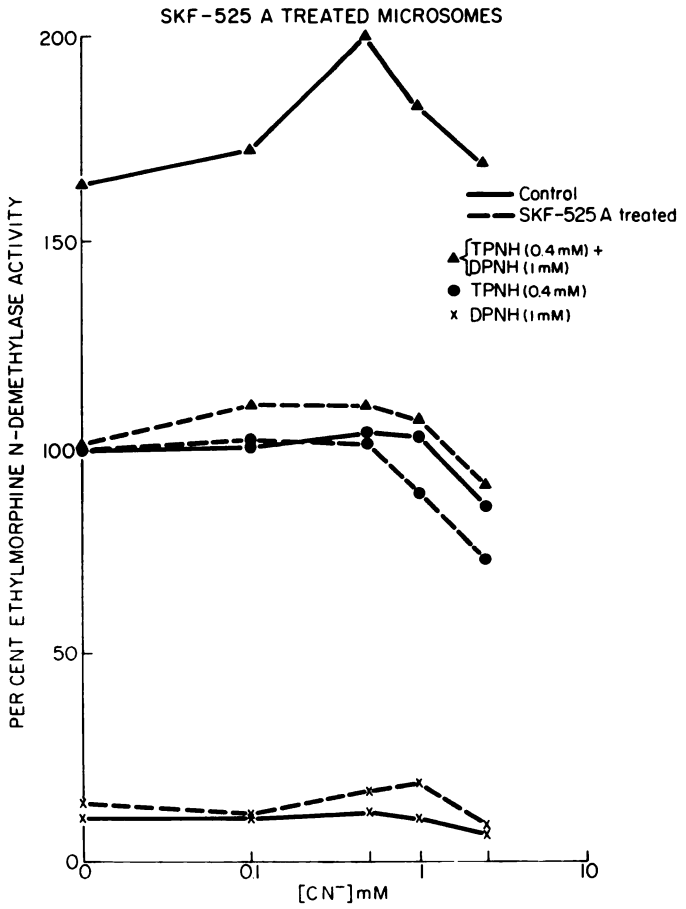

FIG. 6. *DPNH synergism of N-demethylation of ethylmorphine (EM), using microsomes treated with SKF 525-A*

Ethylmorphine *N*-demethylase activities were determined as described in Fig. 4, using SKF 525-A-treated microsomes (MATERIALS AND METHODS) from male rats (250–300 g). Values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity when TPNH was the sole electron donor and no cyanide was present (100% = 560 and 393 nmoles of formaldehyde formed per milligram of protein per hour for control and SKF 525-A-treated microsomes, respectively). The mean values for ethylmorphine binding $\Delta A_{385-420}$ per milligram of protein were 0.020 and 0.000 for control and SKF 525-A-treated rat liver microsomes, respectively. In each experiment livers were pooled from at least three animals.

microsomes might also explain the exaggerated DPNH effect seen in the absence of cyanide. Loss of cyanide-sensitive factor because of the additional manipulation required for the preparation of SKF 525-A-treated microsomes might also account for the large DPNH effect seen in the absence of cyanide in Fig. 6.

A possibility other than the loss of type I binding sites must be considered as an explanation for the decrease in DPNH synergism that results when microsomes are treated with phospholipase C, namely, that DPNH–cytochrome b_5 reductase activity may have been greatly diminished in these microsomes. Phospholipid is thought to be required for the activity of this reductase (29), and microsomes lose about 80% of their phospholipid when treated with phospholipase C (13). However, it seems unlikely that the reductase could have been reduced to a rate-limiting concentration, since Oshino

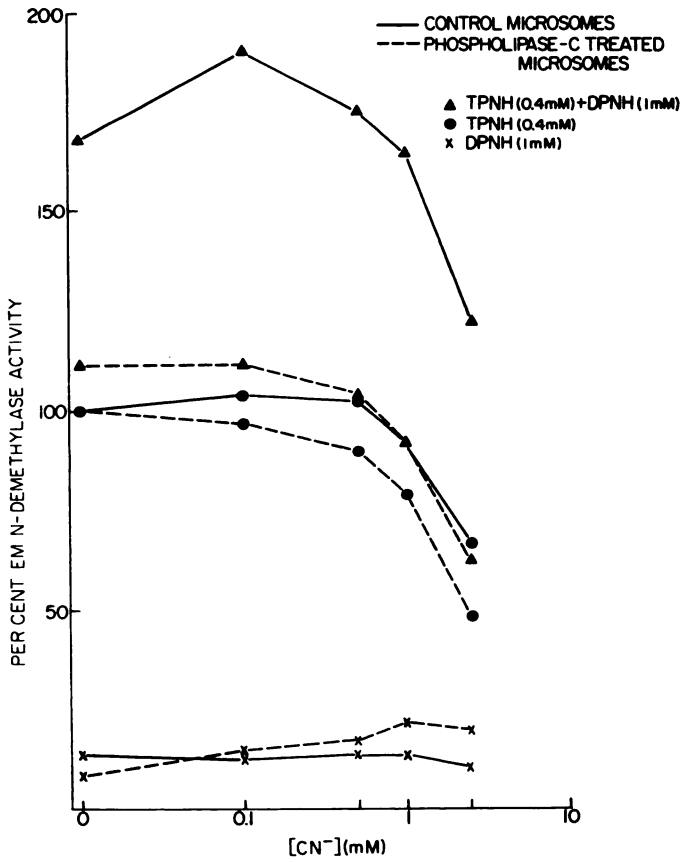

FIG. 7. *DPNH synergism of N-demethylation of ethylmorphine (EM), using microsomes treated with phospholipase C*

Phospholipase c-treated microsomes were prepared as described previously (13). Control microsomes underwent similar treatment, but phospholipase C was omitted. Ethylmorphine *N*-demethylase activities were determined as described in Fig. 5, except that the incubation time was 10 rather than 15 min. Values (means of three experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity when TPNH was the sole electron donor and no cyanide was present (100% = 620 and 418 nmoles of formaldehyde formed per milligram of protein per hour for control and phospholipase C-treated microsomes, respectively). The mean values for ethylmorphine binding $\Delta A_{385-420}$ per milligram of protein were 0.020 and 0.002 for control and phospholipase C-treated rat liver microsomes, respectively. In each experiment livers were pooled from at least four animals (230–280 g).

and co-workers (30) have shown that microsomal DPNH–cytochrome b_5 reductase activity could be inhibited by 99% with *p*-chloromercuribenzenesulfonate without impairing the cytochrome b_5-dependent fatty acyl-CoA desaturation system.

Effect of storage of microsomes on DPNH synergism of N-demethylation of ethylmorphine. Storage of microsomes is known to cause a loss of type I binding sites (9). DPNH synergism of ethylmorphine metabolism using microsomes stored for 0, 1, 3, and 7 days under air at −10° is shown in Fig. 8; it declined with aging of the microsomes. Maximum synergism seen for microsomes stored for 0, 1, 3, and 7 days was 103, 78, 66, and 40%, respectively. Ethylmorphine binding was also seen to fall during the 7-day period. As can be seen in Fig. 9, where data from Fig. 8 are plotted with binding as a function of synergism, the relationship between loss of binding during storage of

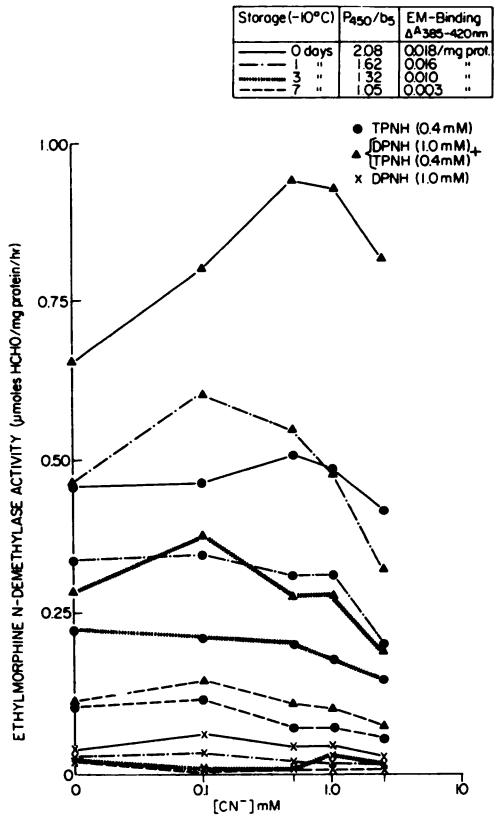

FIG. 8. *Effect of storage of microsomes on DPNH synergism of N-demethylation of ethylmorphine (EM)*

Microsomal suspensions were prepared as described previously (14), with a protein concentration of 10 mg/ml. Aliquots were frozen at −10° for 1, 3, and 7 days. At the end of the storage period, microsomal suspensions were thawed, resuspended by homogenization, and diluted with 1.15% KCl so as to contain 3.5 mg of protein per milliliter. On day 0, the microsomal suspension (3.5 mg of protein per milliliter) was used within 2 hr of preparation. Ethylmorphine *N*-demethylase activities were determined as described in Fig. 2, except that microsomal suspensions always contained 5 mg of protein per milliliter. Cytochromes P-450 and b_5 were determined as described by Omura and Sato (21). Ethylmorphine binding to hepatic microsomes was measured as described by Remmer *et al.* (20) at a final ethylmorphine concen tration of 2.6 mM. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity when TPNH was the sole electron donor and no cyanide was present. The 100% values for microsomes stored for 0, 1, 3, and 7 days were 460, 340, 230, and 100 nmoles of formaldehyde formed per milligram of protein per hour, respectively. Microsomes were prepared from livers pooled from at least six male rats (150–250 g).

microsomes and loss of DPNH synergism is linear. About 60% of the cytochrome P-450 disappeared during the 7-day storage period, but only about 10% of the cytochrome b_5 was lost.

Correlation of DPNH synergism and type I binding. In Fig. 9 the degrees of type I binding by microsomes from different sources (male and female rats, rats treated with 3-methylcholanthrene) or by microsomes from male rats treated with SKF 525-A or phospholipase C, or microsomes from male rats stored for various time periods are plotted against the degrees of DPNH synergism of ethylmorphine *N*-demethylation in the presence of optimal concentrations of cyanide obtained with corresponding microsomes. The correlation coefficient of 0.94 obtained from the statistically constructed plot is highly significant ($p < 0.001$). No correlation was observed between the cytochrome P-450 content of the same microsomes and DPNH synergism (Fig. 10).

Correlation of DPNH utilization and type I binding. In Fig. 11 the degrees of type I binding by microsomes from different sources are plotted against DPNH utilization during ethylmorphine *N*-demethylation in the presence of optimal concentrations of cyanide. The very significant correlation ($p < 0.001$) of $r = 0.88$ was calculated. The statistically drawn plot revealed an intercept at 0.2 μmole of DPNH utilized per milligram of protein per hour, which may be interpreted to mean that at least this amount of DPNH utilization is unrelated to type I binding. The exclusion from the figure of data obtained with SKF 525-A-treated microsomes was deliberate, because about as much DPNH was utilized by these microsomes in the absence as in the presence of ethylmorphine. We have observed the production of acetaldehyde during the incubation of SKF 525-A-treated microsomes in the absence of added substrate,[3] indicating the *N*-demethylation of SKF 525-A bound to microsomes. Because SKF 525-A is a type I

[3] K. M. Bidleman and G. J. Mannering, unpublished observations.

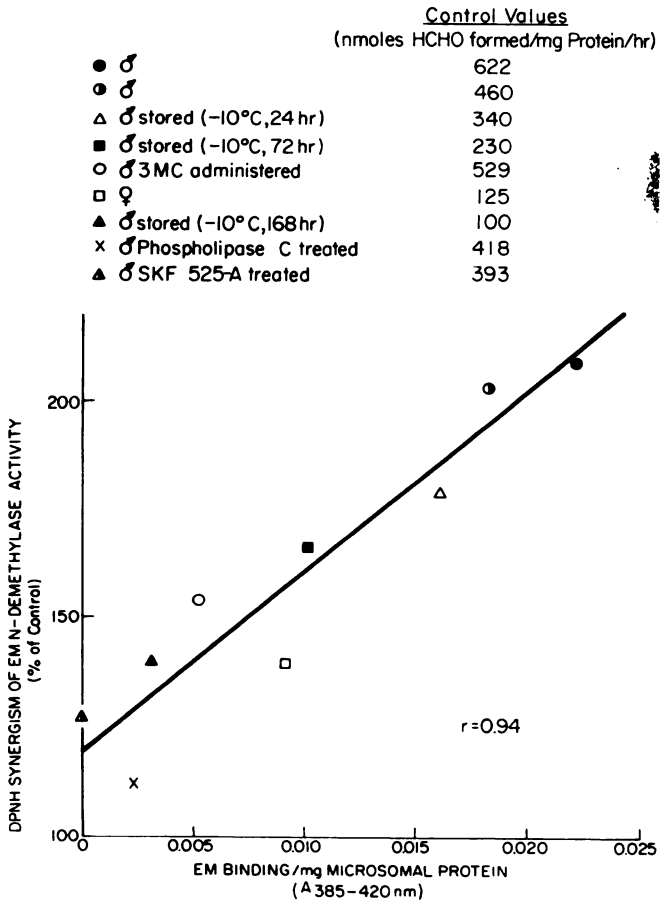

FIG. 9. *Relationship of maximal DPNH synergism of TPNH-dependent ethylmorphine (EM) N-demethylase activity in the presence of cyanide to ethylmorphine binding*

DPNH synergism was studied as described in the preceding figures; ethylmorphine binding was determined as described in Fig. 8, using the same microsomes employed to obtain the synergism data. The positive correlation ($r = 0.94$) of synergism with binding is highly significant ($p < 0.001$). All values (means of two experiments) were recorded as percentages of the ethylmorphine *N*-demethylase activity observed when TPNH was the only source of electrons and no cyanide was present. Values were taken from Figs. 4–8 and represent the maximum DPNH synergism observed at either 0.1 or 0.5 mM cyanide. Values designated ● were obtained in the same way, using microsomes from male rats (300–310 g). In each experiment livers were pooled from at least two rats. 3MC, 3-methylcholanthrene.

compound, it would be expected to solicit electrons from DPNH during its metabolism, and this would account for the accelerated utilization of DPNH by these microsomes in the absence of ethylmorphine.

Correlation of DPNH utilization and ethylmorphine N-demethylation under optimal conditions for DPNH synergism. In Fig. 12 ethylmorphine *N*-demethylation by microsomes from different sources is plotted against DPNH utilization. Cyanide concentrations (0.1–0.5 mM) were determined to have provided optimal conditions for DPNH synergism for each microsomal preparation. The very significant correlation ($p < 0.001$) of $r = 0.94$ was calculated. If the stoichiometry of 1 TPNH:1 DPNH:1 HCHO prevails, the slope should be unity. The observed slope of 1.15 was not significantly different ($p < 0.05$) from unity. An estimate

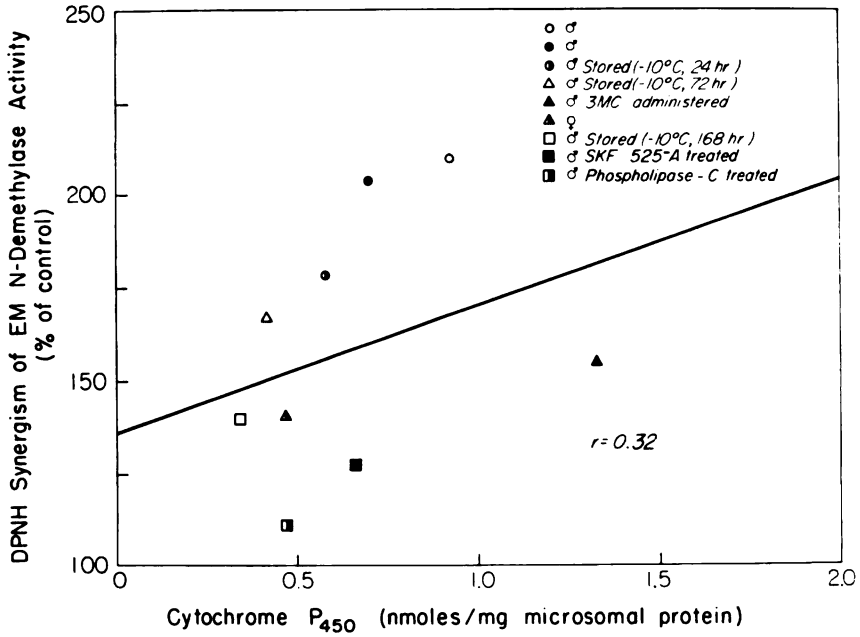

FIG. 10. *Relationship of maximal DPNH synergism of TPNH-dependent ethylmorphine (EM) N-demethylase activity in the presence of cyanide to cytochrome P-450 content of rat liver microsomes*

DPNH synergism was studied as described in the preceding figures; cytochrome P-450 was determined by the method of Omura and Sato (21), using the same microsomes employed to obtain the synergism data. No correlation (r = 0.32) of synergism with cytochrome P-450 content was observed. All values (means of two experiments) were recorded as percentages of the ethylmorphine N-demethylase activity observed when TPNH was the only source of electrons and no cyanide was present. Values were taken from Figs. 4–8 and represent the maximum DPNH synergism observed at either 0.1 or 0.5 mM cyanide. In each experiment livers were pooled from at least two rats. 3MC, 3-methylcholanthrene.

closer to unity is obtained if we take into account the O-de-ethylation of ethylmorphine that occurs simultaneously with N-demethylation. Takemori and Mannering (31) incubated codeine with a 9000 × g supernatant fraction of mouse liver and observed that the conversion of codeine to morphine (O-dealkylation) occurred at about 15% of the rate of N-demethylation of codeine. Preliminary results in our laboratory[4] indicate similar relative rates of N- and O-dealkylation of ethylmorphine in rat microsomes. Ratios of 1.09 and 1.28 obtained when untreated microsomes from adult male rats were used (last two points in Fig. 12) are to be compared with the mean value of 1.31 obtained in comparable studies (1).

[4] S. A. Oyegbile and G. J. Mannering, unpublished observations.

DISCUSSION

In the preceding communication (1) evidence was presented in support of the mechanism proposed by Estabrook and associates (2–6), whereby the first of the 2 electrons required for the oxidation of drugs by the cytochrome P-450 system is provided by TPNH and the second electron is provided by either TPNH or DPNH via cytochrome b_5, with DPNH being the more efficient of the two donors of the second electron. We visualized DPNH synergism as occurring only when the demand for second electrons, caused by the addition of substrate, exceeded the supply of second electrons that could be furnished by TPNH. If we accept this concept, it is not surprising that in the current study type I substrates were observed to elicit DPNH synergism whereas type II substrates did not; type I compounds are known

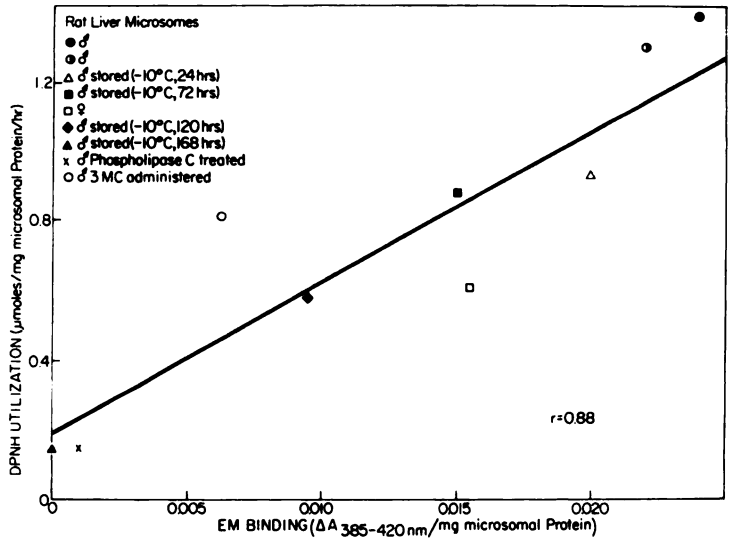

FIG. 11. *Relationship of DPNH utilization during maximal DPNH synergism of TPNH-dependent ethylmorphine (EM) N-demethylase activity, in the presence of cyanide, to ethylmorphine binding*

DPNH utilization during maximal DPNH synergism was determined as described under MATERIALS AND METHODS. Incubation mixtures and sources of microsomes were the same as those used in Fig. 9. All reactions were conducted in the presence of 0.1 or 0.5 mM CN^-. The maximal values for DPNH utilization obtained at one or the other concentration of CN^- were used in the figure. Ethylmorphine binding was determined (20) using the same microsomes. The positive correlation ($r = 0.88$) of DPNH utilization with binding is highly significant ($p < 0.001$). Values were obtained using livers pooled from at least two rats (180–250 g). 3MC, 3-methylcholanthrene.

to stimulate the transfer of first electrons whereas type II compounds do not, and may even impede their transfer (32–34). Accordingly, when type I substrates are introduced into the system, the rate of entry of first electrons is accelerated and second electrons from the electron pool provided by DPNH are drawn via cytochrome b_5 into the system to balance the elevated input of first electrons. When type II substrates are introduced into the system, the rate of entry of the first electron is not accelerated and there is neither the need nor the means for the system to utilize excess second electrons providable by DPNH. In other words, the rate of entry of the first electron is rate-limiting relative to the rate of entry of the second electron, but when type I substrates are introduced this rate limitation is lessened or, in effect, reversed. If, in the absence of DPNH, second electrons provided by TPNH had been sufficient to keep pace with the demand for first electrons created by the presence of the type I substrate, no synergism of ethylmorphine *N*-demethylation would have been seen when DPNH was added to the system, because the over-all reaction would already have been proceeding at its maximal rate.

The mechanism whereby type I binding facilitates the rate of input of the first electron from TPNH is suggested by recent studies of Sies and Kandel (35), which showed that the oxidation-reduction potential of cytochrome P-450 is raised when it is complexed with the type I substrates hexobarbital and aminopyrine. Employing submicrosomal particles containing cytochrome P-450, Waterman and Mason (36) obtained an oxidation-reduction potential for cytochrome P-450 of −410 mV, which is not favorable for the acceptance of electrons from cytochrome b_5 ($E^\circ = +30$ mV). However, as they pointed out, the oxidation-reduction potential of cytochrome P-450 in the intact membrane may be different from that of cytochrome P-450 located in submicrosomal particles.

It is conceivable that type I compounds

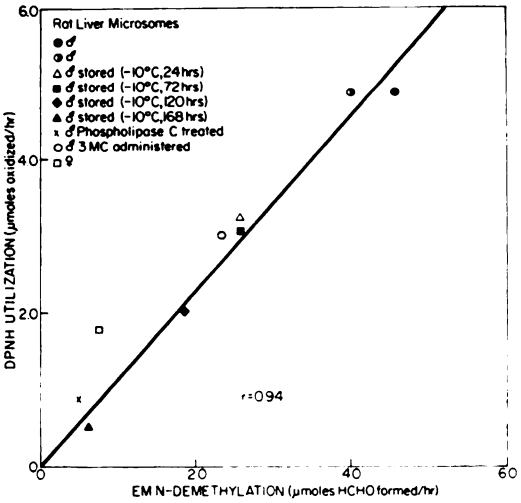

FIG. 12. *Relationship of DPNH utilization to formaldehyde formed during maximal DPNH synergism of TPNH-dependent ethylmorphine (EM) N-demethylase activity in the presence of cyanide*

Values for DPNH utilization and formaldehyde formation were obtained from the experiment described in Fig. 11. The positive correlation (r = 0.94) of DPNH utilized with formaldehyde formed is highly significant ($p < 0.001$). 3MC, 3-methylcholanthrene.

may also facilitate the input of second electrons. Evidence that this may be the case is provided by the cytochrome P-450$_{cam}$ model. Gunsalus and Lipscomb (37) showed that the complexing of camphor with cytochrome P-450$_{cam}$ elevates the oxidation-reduction potential of the hemoprotein from −270 to −170 mV. This adduct complex is followed by the first-electron reduction of the hemoprotein in the hydroxylation cycle. The reduced cytochrome P-450$_{cam}$–substrate complex then accepts molecular oxygen as the second adduct. The oxidation-reduction potential of this complex is about 0 mV. This is above the —60 mV potential of putidaredoxin, which contributes the second electron to the complex. By analogy, type I substrates would raise the oxidation-reduction potential of the oxygenated, reduced cytochrome P-450–substrate complex to a level where it could more readily accept electrons from cytochrome b_5 or an intermediate component. The degree of type I binding would determine the degree of elevation of the oxidation-reduction potential of the oxygenated complex, which in turn would regulate the turnover rate of the system when DPNH was present.

The cytochrome P-450$_{cam}$ system may also serve as a model to support the concept that the rate of the over-all hydroxylation reaction may be controlled by the rate of introduction of the second rather than the first electron. The following rate constants determined by Gunsalus and Lipscomb (37) for the cytochrome P-450$_{cam}$ system establish the rate-limiting step at the second-electron reduction of the oxygenated complex: binding of camphor to cytochrome P-450$_{cam}$, k = 7000 sec^{-1}; first-electron reduction, k = 35 sec^{-1}; formation of the reduced P-450$_{cam}$–camphor–O_2 complex, k = 470 sec^{-1}; second-electron reduction of the reduced P-450$_{cam}$–camphor–O_2 complex, k = 20 sec^{-1}.

Cyanide enhanced DPNH synergism of the metabolism of ethylmorphine, codeine, and norcodeine, but not that of benzphetamine or aminopyrine (Table 1). This discrepancy is not surprising in view of the alternative pathways of metabolism available to at least two of these substrates. *N*-Demethylation accounts for only 50% of aminopyrine metabolism, and hydroxylation accounts for the other 50% (38). The removal of one *N*-methyl group from aminopyrine yields a metabolite which gives a type II spectrum (39) and which in turn is *N*-demethylated. The second demethylation would not be synergized by DPNH, nor would its rate be enhanced appreciably by cyanide. The metabolism of benzphetamine offers similar possibilities. It can be hydroxylated as well as *N*-demethylated (40–43), and it can be cleaved to *l*-amphetamine, a type II compound (44). Benzphetamine forms an *N*-oxide derivative without the participation of cytochrome P-450 (40, 43, 44). In view of the variety of metabolic pathways utilized in the metabolism of these compounds, it is not unlikely that pathways other than *N*-demethylation may have been favored by a cyanide-mediated shunting of electrons, thereby reducing or eliminating the cyanide enhancement of DPNH synergism as determined by the measurement of rates of *N*-demethylation.

The evaluation of the stoichiometry of microsomal mixed-function oxidase reactions is complicated by pathways which use TPNH and DPNH in the oxidation of endogenous substrates. The addition of drug substrate should channel much of this endogenous drain of electrons to the cytochrome P-450 system, and cyanide should facilitate an inventory of electrons by blocking the loss of electrons to endogenous cyanide-sensitive systems.

Using an atmosphere of 9:1 $CO:O_2$ to differentiate between cytochrome P-450-related and -nonrelated TPNH utilization by microsomes, Stripp *et al.* (45) obtained a stoichiometric relationship between the oxidation of several type I drugs and TPNH utilization of about 1:1, which is in keeping with the stoichiometry required of mixed-function oxidase reactions. The ratios of product formed to TPNH utilized were reasonably close to unity when ethylmorphine and aminopyrine were employed as substrates, but deviated considerably from unity with other type I substrates. On the other hand, Cohen and Estabrook (4), employing aminopyrine as a substrate, observed a TPNH:HCHO ratio of about 2, and offered several explanations for this discrepancy in the stoichiometry of a mixed-function oxidase reaction. If it is assumed that all of the first electrons were donated by TPNH and that all of the second electrons were donated by DPNH, the stoichiometry of approximately 1 DPNH:1 HCHO observed in our studies with ethylmorphine is in agreement with that seen by Cohen and Estabrook with aminopyrine.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the able technical assistance of Miss Virginia Kickertz and Miss Viola Abbott.

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