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

I. Effects of Activation and Inhibition of the Fatty Acyl Coenzyme A Desaturation System

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SUMMARY

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The first of the 2 electrons required for the *N*-demethylation of ethylmorphine by the microsomal mixed-function oxidase system of liver is thought to be donated by TPNH, whereas the second has been postulated to be derivable from either TPNH or DPNH and transported to the cytochrome P-450 system via cytochrome b_5. DPNH is believed to support a larger pool of second electrons at cytochrome b_5 than can be supplied by TPNH, and this would explain the synergistic effect of DPNH on TPNH-dependent mixed-function oxidase reactions. This concept was strengthened by the current studies, which altered the pool of second electrons at cytochrome b_5 by manipulating the shunt of electrons from cytochrome b_5 to the microsomal fatty acyl-CoA desaturation system. When the activity of this shunt was inhibited with cyanide, DPNH synergism of ethylmorphine *N*-demethylation was increased from 30% (no cyanide) to 90% (0.1 or 0.5 mM cyanide). Cyanide had little or no effect on *N*-demethylation when TPNH was the sole source of electrons. When the activity of the desaturation system was stimulated by the addition of stearoyl-CoA, a natural substrate for the system, rates of *N*-demethylation declined when TPNH was the sole donor of electrons and when both TPNH and DPNH were present. Cyanide reversed the inhibitory effect of stearoyl-CoA. These results are interpreted to mean that second electrons, which are normally transported from DPNH through cytochrome b_5 to the cytochrome P-450 system, are dissipated in large part via cyanide-sensitive systems engaged in the oxidation of endogenous substrates; cyanide largely prevents this dissipation of second electrons, more of the electron pool at cytochrome b_5 is made available to the cytochrome P-450 system, and DPNH synergism is enhanced. Cyanide is without effect on the cytochrome P-450 system when TPNH is the sole source of electrons, because the steady-state concentration of reduced cytochrome b_5 that can be maintained by TPNH is below the level where a serious loss of electrons to endogenous systems via cytochrome b_5 can occur. Stearoyl-CoA increases the dissipation of electrons by serving as a substrate for the cyanide-sensitive desaturation system. It is postulated that stearoyl-CoA inhibits ethylmorphine

N-demethylation because the desaturation system and the mixed-function oxidase system compete for electrons supplied by an unidentified electron transfer component, which accepts electrons from either DPNH or TPNH via cytochrome b_5. These concepts were reinforced by studies in which rates of DPNH utilization were correlated with rates of ethylmorphine *N*-demethylation.

INTRODUCTION

The role of TPNH as the donor of reducing equivalents for the microsomal oxidations of drugs, other foreign compounds, and steroids in the liver is well established (1–5). DPNH will not substitute for TPNH in the cytochrome P-450-mediated oxidation of drugs, but reaction rates are increased when both nucleotides are present (6–8). Although this synergistic role of DPNH has been known since 1957 (6), DPNH has not been employed routinely in reaction mixtures, and the mechanism whereby DPNH contributes electrons to the system remained virtually unexplored until the recent investigations of Estabrook and associates (9–13).

The microsomal drug-metabolizing system involving cytochrome P-450 is classified as a mixed-function oxidase system; that is, 2 electrons are required for the coupled reduction of oxygen and the oxygenation of substrate. However, the reduction of cytochrome P-450 requires only 1 electron (14–16). The contribution of the first electron from TPNH in the reduction of cytochrome P-450 is well documented (14, 17, 18). In their search for the origin of the second electron, Estabrook and associates considered the possibility of its being contributed by DPNH and transferred via cytochrome b_5 to the oxygenated cytochrome P-450–substrate complex. They provided evidence for the following sequence: (a) substrate binds to oxidized cytochrome P-450; (b) an electron is transferred from TPNH via TPNH–cytochrome *c* reductase (TPNH–cytochrome P-450 reductase) to the oxidized cytochrome P-450–substrate complex; (c) the reduced cytochrome P-450–substrate complex combines with oxygen; and (d) this complex is reduced by an electron from cytochrome b_5, which may originate from either TPNH or DPNH. The increased over-all reaction rate seen when both DPNH and TPNH were present over that observed when TPNH was the only source of electrons was thought to occur because DPNH is a more efficient donor of second electrons than TPNH.

Recent studies by Oshino, Sato, and coworkers (19–23), which implicate cytochrome b_5 and a cyanide-sensitive factor in the microsomal desaturation of fatty acids, suggested means of investigating the mechanism of DPNH synergism of drug oxidation as well as the possible role of cytochrome b_5 in microsomal mixed-function oxidase reactions. In the desaturation system, DPNH reacts with a reductase to reduce cytochrome b_5, which in turn provides reducing equivalents via cyanide-sensitive factor for the desaturation of fatty acyl-CoA substrates. If DPNH and cytochrome b_5 are involved in the transfer of electrons to cytochrome P-450, it follows that any diversion of these electrons to the desaturation system would result in a decreased rate of drug oxidation. In the studies to be reported, stearoyl-CoA was employed to divert electrons in this manner, and the diversion was reversed with cyanide. Evidence is presented in support of the view that DPNH synergism of TPNH-dependent oxidation of drugs is mediated through cytochrome b_5.

MATERIALS AND METHODS

TPN^+, DPNH, glucose 6-phosphate, stearoyl-CoA, sodium oleate, sodium stearate, monosodium α-ketoglutarate, yeast alcohol dehydrogenase (salt-free), bovine liver L-glutamate dehydrogenase (type II), *p*-iodonitrotetrazolium violet, and triethanolamine HCl were obtained from Sigma Chemical Company; ethylmorphine HCl,

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from Mallinckrodt Chemical Works; glucose 6-phosphate dehydrogenase, from Boehringer/Mannheim; and acetaldehyde (analytical reagent grade), from Matheson, Coleman, and Bell.

Adult male Holtzman rats (150–280 g) were fed and watered ad libitum until death. Phenobarbital-treated rats received one daily intraperitoneal injection of 50 mg/kg of sodium phenobarbital (10% in NaCl) for 1 or 3 days. They were killed 24 hr after the last injection. Microsomes were prepared from livers perfused *in situ* with cold isotonic KCl solution as described elsewhere (24), and were used within 3 hr of preparation at protein concentrations of 0.6–1.0 mg/ml of incubation mixture. Protein was determined by the method of Lowry *et al.* (25). The incubation mixture was the same as that described previously (24), except that nicotinamide was omitted unless indicated. Ethylmorphine was used as the substrate at a concentration of 2 mM; reaction rates were determined by measuring formaldehyde formed (26). Reaction mixtures contained DPNH (1 mM), a TPNH-generating system, or both. The TPNH-generating system consisted of TPN^+ (0.4 mM), glucose-6-P (4 mM), and glucose-6-P dehydrogenase (2 enzyme units). Reaction mixtures were incubated under air at 37° for 15 min. Rates of ethylmorphine *N*-demethylation were demonstrated to be linear throughout the 15-min incubation period in the presence or absence of cyanide (0.1 and 0.5 mM) when TPNH was the sole electron donor and when TPNH and DPNH were present. The possibility was considered that cyanide might interfere with the determination of formaldehyde by the Nash method (26) because of formation either of cyanohydrin or of a metabolite of cyanide during incubation. The effects of cyanide (0.1, 0.5, 1.0, and 2.5 mM) on color development were studied when 0.1 and 0.2 mM concentrations of formaldehyde were incubated with the microsomes, as described previously, with the reaction mixture containing no ethylmorphine. Cyanide in concentrations lower than 2.5 mM had no effect on color development at either concentration of formaldehyde; color development was decreased by only 5.1 and 5.9% with 0.1 and 0.2 mM formaldehyde, respectively, when the concentration of cyanide was 2.5 mM

DPNH utilization during the oxidation of ethylmorphine was determined using the acetaldehyde–alcohol dehydrogenase system (27). TPNH levels in incubation mixtures were monitored using the NH_4^+–α-ketoglutarate–L-glutamate dehydrogenase system (27). The disappearance of DPNH or TPNH was recorded at 340 nm, using an Aminco DW-2 spectrophotometer. Determinations were made on aliquots of the incubation mixture taken at 0 and 15 min. DPNH and formaldehyde analyses were made on duplicate incubation mixtures in all individual experiments.

Cytochromes P-450 and b_5 were determined by the method of Omura and Sato (28), using an Aminco DW-2 spectrophotometer.

RESULTS

DPNH synergism of TPNH-dependent oxidation of drugs in the presence and absence of cyanide. Because the initial experimental design called for the activation of the cyanide-sensitive fatty acid desaturation system with stearoyl-CoA and subsequent inhibition of the system with cyanide, and because high concentrations of cyanide are known to combine with cytochrome P-450 (29–31), it was necessary to determine the effects of various concentrations of cyanide on drug oxidation in the absence of stearoyl-CoA. Figure 1 shows the synergistic effect of DPNH on TPNH-dependent ethylmorphine *N*-demethylation, namely, about 30% (CN^- concentration = 0), and the enhancement of the synergistic effect produced by cyanide. Enhencement, first observed at a cyanide concentration of 0.05 mM, reached a maximum of 90% at 0.1–0.5 mM, which coincides with the range of cyanide concentrations required to inhibit the desaturation system by 50% (19, 32). Cyanide enhancement of DPNH synergism declined at higher concentrations of cyanide and disappeared at 2.5 mM, which coincides with the K_s concentration of cyanide for cytochrome P-450 (19, 31). These results can be interpreted to mean that electrons from DPNH, normally destined for

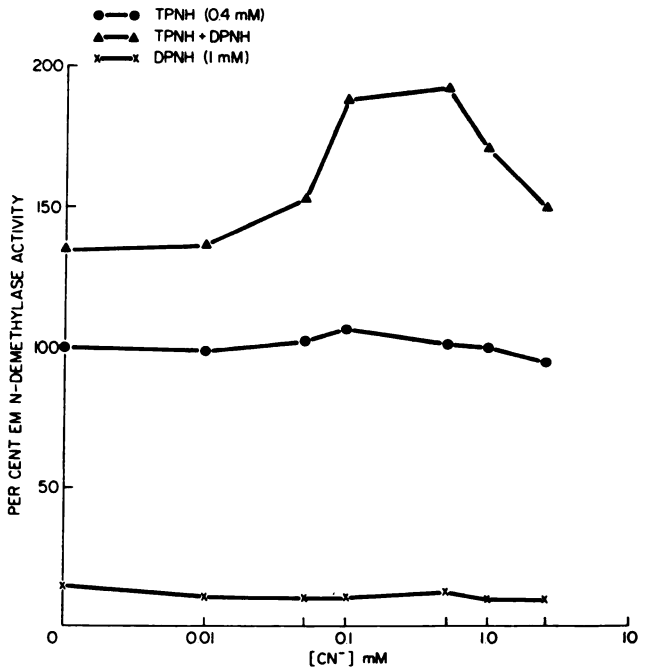


FIG. 1. *Effect of cyanide on ethylmorphine (EM) N-demethylase activity of hepatic microsomes from male rats in the presence of TPNH, DPNH, or both*

Ethylmorphine (2 mM) was incubated with microsomes in a mixture (final volume, 5 ml) containing a TPNH-generating system (MATERIALS AND METHODS), semicarbazide HCl (7.5 mM), $MgCl_2$ (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 (3.5–5 mg of protein per milliliter), and the incubation was continued for another 15 min. In systems containing both DPNH and TPNH, DPNH (final concentration, 1 mM) was added as a freshly prepared solution in 1.15% KCl just before the preliminary incubation. In mixtures containing DPNH only, the TPNH-generating system was omitted. Reaction rates were determined by measuring the formaldehyde formed. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity observed when TPNH was the only source of electrons and cyanide was absent from the medium (100% = 0.32 μmole of formaldehyde per milligram of protein per hour). In each experiment livers were pooled from at least two male rats (180–200 g).

cytochrome P-450, are diverted via cyanide-sensitive factor to endogenous substrates, presumably fatty acids, and that this diversion is blocked with cyanide. The effect becomes biphasic when cyanide reaches concentrations high enough to combine with a significant amount of cytochrome P-450. This inhibitory effect of cyanide in high concentrations is also seen in the absence of DPNH (Fig. 1, middle curve). The loss of cyanide enhancement of DPNH synergism at high concentrations of cyanide is greater than the inhibitory effect of cyanide on ethylmorphine *N*-demethylation when DPNH is absent, which suggests that cyanide in these high concentrations may affect components of the system other than cyanide-sensitive factor and cytochrome P-450. For reasons which will be given later, it is noteworthy that cyanide had little or no stimulatory effect on ethylmorphine *N*-demethylation when TPNH was the only source of electrons. Figure 1 also shows that DPNH supports *N*-demethylation to only about 15% of that achieved with TPNH, and that cyanide does not modify this reaction measurably when DPNH is the only source of electrons.

Inhibition of oxidation of drugs with stearoyl-CoA and its reversal with cyanide. The

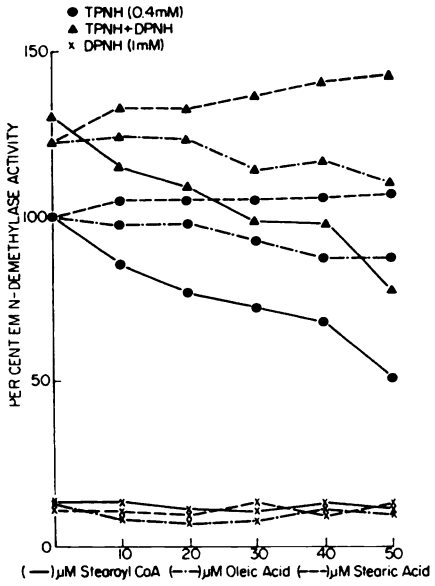


FIG. 2. *Effects of stearoyl-CoA, stearic acid, and oleic acid on ethylmorphine (EM) N-demethylation by hepatic microsomes from male rats*

Stearoyl-CoA (0–50 μM), stearic acid (0–50 μM), or oleic acid (0–50 μM) was added to the incubation mixture, and the reactions were carried out as described in Fig. 1, except that cyanide was excluded. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity observed when TPNH was the only source of electrons and stearoyl-CoA, oleic acid, and stearic acid were absent from the medium (100% = 0.43 μmole of formaldehyde/mg of protein per hour). In each experiment livers were pooled from at least two male rats (160–200 g).

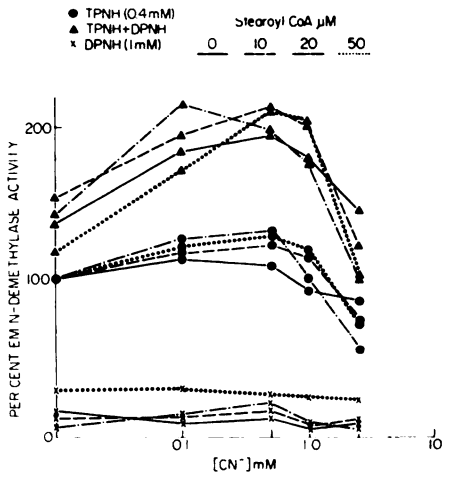


FIG. 3. *Reversal of inhibitory effect of stearoyl-CoA on hepatic microsomal ethylmorphine (EM) N-demethylation by cyanide*

Stearoyl-CoA (0–50 μM) and various concentrations of cyanide were included in the incubation mixture, and the reaction was carried out as described in Fig. 1. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activities observed at each of the concentrations of stearoyl-CoA when TPNH was the only source of electrons and cyanide was absent from the medium (100% at 0, 10, 20, and 50 μM stearoyl-CoA = 0.45, 0.41, 0.27, and 0.21 μmole of formaldehyde per milligram of protein per hour, respectively). In each experiment livers were pooled from at least four male rats (190–220 g).

dose-dependent, inhibitory effect of stearoyl-CoA, a natural substrate for the fatty acid desaturation system, on the *N*-demethylation of ethylmorphine in the presence of TPNH or TPNH plus DPNH is illustrated in Fig. 2. The inhibitory effect of stearoyl-CoA was as pronounced when TPNH was the only electron donor as when both TPNH and DPNH were present, which suggests that some of the electrons from TPNH that would normally have been used for *N*-demethylation when DPNH was absent were now being channeled to the desaturation system. In accordance with expectations, cyanide prevented this shunting of electrons and thereby restored the DPNH synergism of ethylmorphine *N*-demethylation (Fig. 3).

The rate of stearoyl-CoA desaturation was not measured, although Oshino *et al.* (20) reported that microsomes from rats fed ad libitum desaturated stearoyl-CoA at the rate of 2–4 nmoles/min/mg of protein. In the absence of stearoyl-CoA rates of ethylmorphine *N*-demethylation were 7.2 and 9.4 nmoles/min/mg of protein, respectively, when TPNH and TPNH plus DPNH were the sources of electrons. Thus, with the desaturation and cytochrome P-450 systems performing at similar rates, the observed inhibition of ethylmorphine *N*-demethylation by stearoyl-CoA is in keeping with the assumption that the two systems compete for a single source of electrons.

It is not possible to determine, in the study in which both nucleotides were present, how much of the decline in ethylmorphine *N*-de-

methylation with increasing concentrations of stearoyl-CoA was due to a loss of DPNH synergism and how much was due to a loss of TPNH-dependent *N*-demethylation; thus the parallel inhibition of *N*-demethylation observed with TPNH and with TPNH plus DPNH may be fortuitous and cannot be interpreted to mean that inhibition in both cases was due solely to a loss of electrons from TPNH to the desaturation system.

The involvement of TPNH in the microsomal hydroxylation of olefins and fatty acids has been demonstrated recently (33–38). Because microsomes are known to contain fatty acyl-CoA hydrolase (33, 38), it was important to determine the effects of stearic and oleic acids, which could have been present as metabolites of stearoyl-CoA. In Fig. 2 the effects of oleic and stearic acids on ethylmorphine *N*-demethylation are compared with the inhibition produced by stearoyl-CoA. It can be seen that oleic acid had little or no inhibitory effect on ethylmorphine *N*-demethylation and that, if anything, the effect of stearic acid on the reaction was stimulatory. Desaturated stearoyl-CoA (oleyl-CoA) was not available for testing. It was concluded from these studies that the inhibitory effect of stearoyl-CoA was not due to the formation of stearic or oleic acid.

DPNH utilization by microsomes as affected by TPNH, ethylmorphine, and cyanide. DPNH utilization during incubation of microsomes with TPNH, ethylmorphine, or both, in the presence of increasing concentrations of cyanide, is shown in Fig. 4. In the absence of cyanide the endogenous (ethylmorphine absent) utilization of DPNH is about as great as that seen during the oxidation of ethylmorphine in the presence of TPNH (compare curve *f* with curve *g*, where $[CN^-] = 0$), but in the presence of cyanide endogenous utilization of DPNH decreases with increasing cyanide concentration. Even at the highest concentration of cyanide, not all endogenous DPNH utilization was inhibited, which suggests that the fatty acyl-CoA desaturation system may not account for all of the endogenous utilization of DPNH. The presence of either TPNH (curve *d*) or ethylmorphine in the absence of

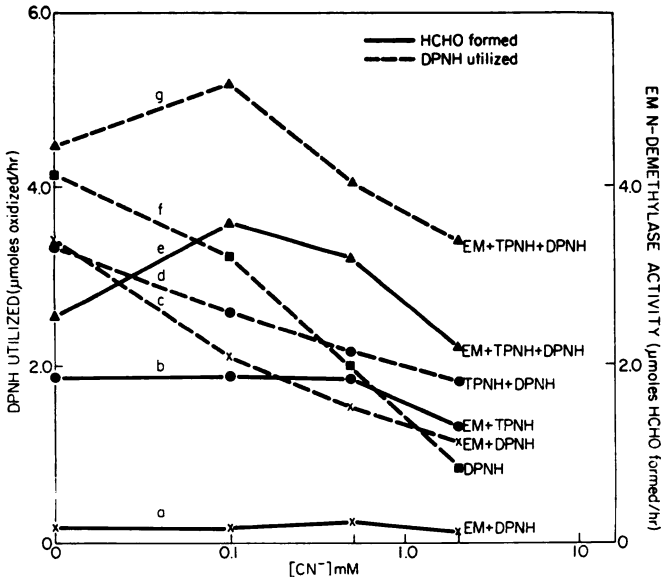


FIG. 4. *DPNH utilization during ethylmorphine (EM) N-demethylation*

Incubations were performed as described in Fig. 1, with each flask containing 3.5 mg of protein. At the end of the incubation period, 2-ml aliquots of the mixture were removed for DPNH and TPNH determinations (MATERIALS AND METHODS). The remaining 3 ml of incubation mixture were used for formaldehyde determinations. All values are the means of two experiments. In each experiment livers were pooled from at least two male rats (230–250 g).

TPNH (curve *c*) appears to decrease endogenous DPNH utilization, but not enough studies were conducted to determine whether the decreases were meaningful. The utilization of DPNH when both TPNH and ethylmorphine were present increases at a cyanide concentration of 0.1 mM and then decreases with the two higher concentrations of cyanide. This increase and subsequent decrease in DPNH utilization is paralleled by the increase and decrease in formaldehyde formation seen at the same concentrations of cyanide (compare curve *e* with curve *g*).

These observations may be interpreted as follows. Electrons from DPNH may be used for the oxidation of endogenous substrates (e.g., through the fatty acyl-CoA desaturation system), and also to furnish the second electron for ethylmorphine *N*-demethylation. A measurable, but insignificant, part of the electron pool is used to reduce cytochrome P-450. The relative distribution of electrons through these pathways will depend upon the relative activities of the pathways, which in turn will depend in part upon the relative amounts of substrates available to each pathway. Thus, when ethylmorphine is present, endogenous DPNH utilization is suppressed. Endogenous DPNH utilization is suppressed further by the addition of cyanide. At a saturating level of ethylmorphine and an optimal concentration of cyanide, endogenous DPNH utilization is minimal. If endogenous DPNH utilization could be reduced to zero under these optimal conditions, which is unlikely, and if the system is a mixed-function oxidase system wherein the first electron is contributed solely by TPNH and the second electron solely by DPNH, then 2 moles of formaldehyde should be produced for each mole of DPNH utilized. However, in Fig. 4 it can be seen that more than twice the predicted amount of DPNH was utilized per mole of formaldehyde formed; at a cyanide concentration of 0.1 mM, 1.43 μmoles of DPNH were utilized for each micromole of formaldehyde formed (compare points on curves *e* and *g*); at a cyanide concentration of 0.5 mM the DPNH:HCHO ratio was 1.24. The DPNH:HCHO ratio varied considerably from experiment to experiment, and the ratios derived from Fig. 4 were among the highest observed. In six experiments in which optimal concentrations of cyanide were employed (0.1–0.5 mM), including those illustrated in Fig. 4, a mean ratio of 1.31 ± 0.10 was observed.

The TPNH level (not shown in Fig. 4) remained at a constant level of about 0.33 mM at all concentrations of cyanide and was not affected by the presence of DPNH.

Effect of cytochrome P-450:b_5 ratio on cyanide-enhanced DPNH synergism of ethylmorphine N-demethylation. If the DPNH synergism of TPNH-dependent drug metabolism is mediated through cytochrome b_5, changes in the relative amounts of cytochrome b_5 and P-450 might be expected to modify the synergistic effect. Cytochrome P-450:b_5 ratios can be altered in microsomes by phenobarbital administration, which causes a large increase in microsomal cytochrome P-450, but little or no change in the level of cytochrome b_5 (39–42). Rats which had received phenobarbital for 0, 1, and 3 days yielded microsomes with P-450:b_5 ratios of 2.25, 2.70, and 3.40, respectively. Figure 5 shows that in the absence of cyanide changes in the ratio had no effect on DPNH synergism, but that cyanide enhancement of DPNH synergism increased as the ratio decreased. Furthermore, changes in P-450:b_5 ratios had no effect on ethylmorphine *N*-demethylase activity, with or without cyanide, when TPNH was the only source of electrons. That changes in the ratio produce changes in the synergistic effect only in the presence of cyanide might be explained on the basis that in the absence of cyanide, when the synergistic effect is minimal, the concentration of b_5 at any of the three ratios is high enough to keep pace with the contribution of electrons from DPNH and the turnover of the P-450 cycle, but electrons from DPNH become more abundant when cyanide blocks the loss of electrons to other systems, and cytochrome b_5 then becomes relatively rate-limiting with respect to the activity of the P-450 system. Enthusiasm for this interpretation was dampened somewhat when it was observed that type I binding of ethylmorphine, unlike that of most type I compounds, is not increased after phenobarbital administration; the ratio of ethylmorphine binding to P-450

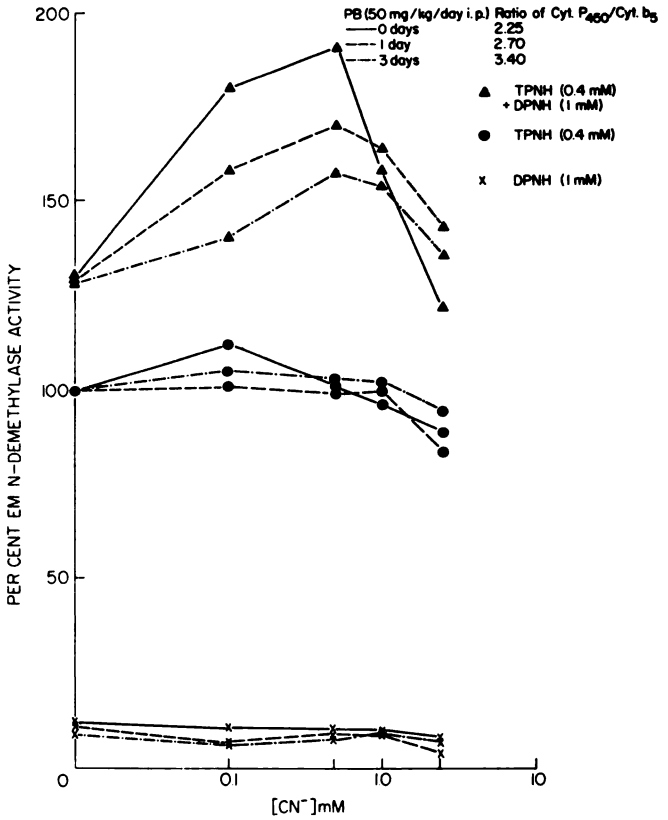


FIG. 5. *Effect of phenobarbital (PB) administration on relative concentrations of cytochromes P-450 and b_5 in microsomes and on DPNH synergism of TPNH-dependent ethylmorphine (EM) N-demethylation in the presence of cyanide*

Rats received intraperitoneal injections of sodium phenobarbital (50 mg/kg/day) for 1 or 3 days and were killed 24 hr after the last injection. Incubations were performed as described in Fig. 1. All values (means of two experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity observed when TPNH was the only source of electrons and cyanide was absent from the medium (100% = 0.34, 0.32, and 0.70 μmole of formaldehyde per milligram of protein per hour for microsomes from animals that had been treated with phenobarbital for 0, 1, and 3 days, respectively). In each experiment livers were pooled from at least two male rats (150–200 g).

decreases. As reported in the accompanying communication (43), the magnitude of the DPNH synergistic effect on ethylmorphine *N*-demethylation is directly related to the magnitude of type I binding of ethylmorphine to microsomes. Accordingly, the results in Fig. 5 might be explained by the loss of type I binding relative to cytochrome P-450 rather than by differences in the P-450:b_5 ratios.

Contribution of mitochondrial electron transport to DPNH electron shunt. Because microsomal preparations are contaminated with mitochondria, it seemed possible that mitochondrial cytochrome oxidase might shunt electrons from DPNH away from the drug-oxidizing system. In this event the enhancing effect of cyanide on the DPNH synergism of drug oxidation would be attributed to its inhibitory effect on mitochondrial cytochrome oxidase rather than to inhibition of microsomal cyanide-sensitive factor. Succinate *p*-iodonitrotetrazolium violet reductase activity, determined by the method of Shelton and Rice (44), was used as an enzyme marker to estimate the degrees of mitochondrial contamination of the microsomal preparations. Less than 1.5% of mitochondrial contamina-

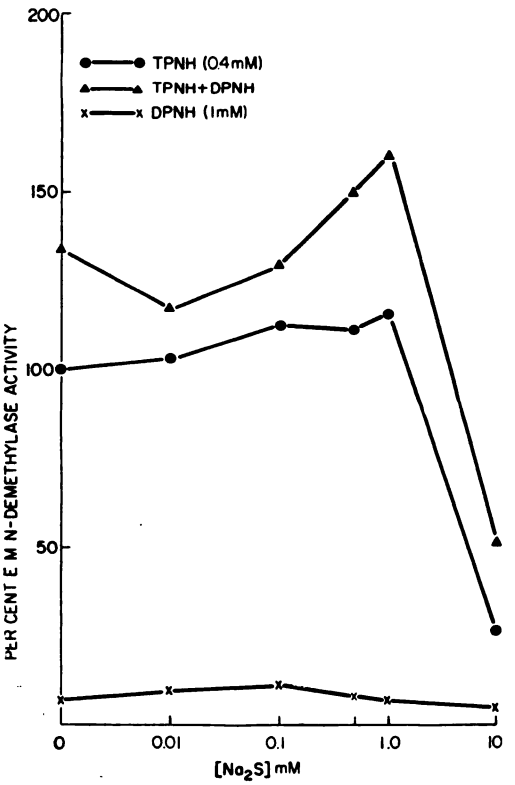


FIG. 6. *Effect of sodium sulfide on DPNH synergism of TPNH-dependent ethylmorphine (EM) N-demethylation*

Incubations were performed as described in Fig. 1. All values (means of three experiments) are recorded as percentages of the ethylmorphine *N*-demethylase activity observed when TPNH was the only source of electrons and Na_2S was absent from the medium (100% = 0.43 μmole of formaldehyde per milligram of protein per hour). In each experiment livers were pooled from at least two male rats (180–225 g).

tion was found in all but one of the five preparations examined, and it showed 2.9% contamination.

Although it seemed unlikely that mitochondrial contamination of this low magnitude could account for the DPNH electron shunt, inhibition of mitochondrial cytochrome oxidase with Na_2S was employed to test this possibility. Before studying the effect of sulfide on DPNH synergism it seemed pertinent to determine the effect of sulfide on mitochondrial cytochrome *c* oxidase. Accordingly, the inhibitory effects of various concentrations of sulfide on the rate of oxidation of reduced cytochrome *c* contained in mitochondrial and microsomal preparations from rat liver were determined. The method employed was that of Wharton and Tzagoloff (45), except that the disappearance of absorbance at 550 nm was determined by difference spectroscopy, using an Aminco DW-2 spectrophotometer, after first showing that the addition of sulfide caused no change in the absolute absorbance at 550 nm. The mitochondrial preparation was the 9000 × *g* pellet obtained during the preparation of microsomes, except that a preliminary centrifugation was performed at 600 × *g* to remove cell debris and nuclei. Using either mitochondrial or microsomal preparations, sulfide concentrations of 1, 10, 100, and 1000 μM inhibited cytochrome oxidase activity by about 45, 85, 100, and 100%, respectively. A similar study employed cyanide rather than sulfide. The use of difference rather than absolute spectral measurements at 550 nm eliminates the interfering peak at 548 nm, which occurs when cyanide is added to mitochondria (46). Cyanide concentrations of 5, 10, 50, 100, and 1000 μM inhibited cytochrome *c* oxidase activity in both microsomal and mitochondrial preparations by about 75, 90, 95, 98, and 99% respectively. In Fig. 6 it can be seen that a 0.1 mM concentration of sulfide, which inhibited mitochondrial cytochrome oxidase activity completely, did not enhance the DPNH synergistic effect, which it should have if it was blocking the DPNH electron shunt through cytochrome oxidase. This was not unexpected, because 0.01 mM cyanide, which inhibited mitochondrial cytochrome oxidase by 90%, had no effect on DPNH synergism (Fig. 1). At Na_2S concentrations of 0.5 and 1 mM, DPNH synergism of ethylmorphine *N*-demethylation was enhanced by 20 and 30%, respectively. The effect of Na_2S on cyanide-sensitive factor is not known, but it is conceivable that Na_2S at these high concentrations could have inhibited the cyanide-sensitive factor and thereby partially blocked the shunt in much the same way that cyanide produced the effect shown in Fig. 1.

A role of mitochondrial cytochrome oxidase was also ruled out by studies which uti-

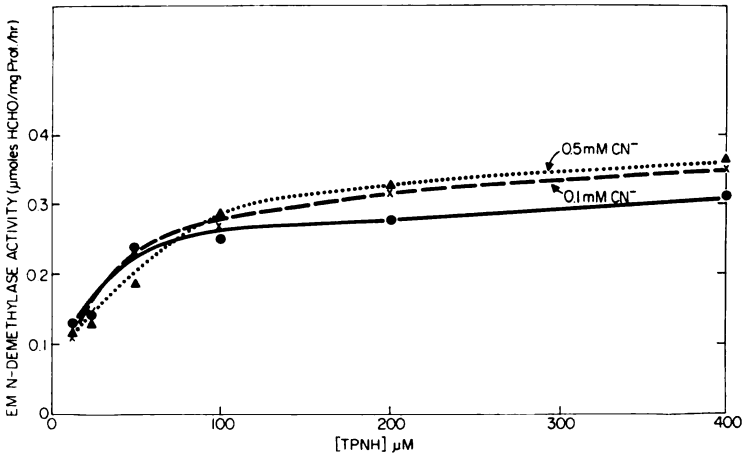


FIG. 7. *Effect of cyanide on ethylmorphine (EM) N-demethylation at various concentrations of TPNH*

Ethylmorphine was incubated as described in Fig. 1, except that the incubation time was 10 rather than 15 min, and various concentrations of TPNH were substituted for TPN^+. The amounts of glucose-6-P (4 mM) and glucose-6-P dehydrogenase (2 enzyme units) present in the medium were calculated to be sufficient to maintain essentially all the TPNH in its reduced form. All values are means of two experiments. In each experiment livers were pooled from at least two male rats (250–280 g).

lized suboptimal concentrations of TPNH. Cyanide enhanced DPNH synergism of TPNH-dependent ethylmorphine *N*-demethylation, but had no comparable stimulatory effect when TPNH was the sole source of electrons (Fig. 1). If the cyanide enhancement of DPNH synergism is attributable to contaminating mitochondria, cyanide should prevent the loss of electrons via cytochrome b_5, mitochondrial cytochrome *c*, and mitochondrial cytochrome oxidase when TPNH is the sole electron donor. This should become particularly observable when TPNH is present in suboptimal concentrations. Figure 7 shows that cyanide had no stimulatory effect on ethylmorphine *N*-demethylation when TPNH was supplied in suboptimal concentrations, namely, concentrations lower than 100 μM. The small stimulatory effect of cyanide seen at high concentrations of TPNH (Fig. 1) is probably due to the prevention of a small loss of electrons to the fatty acyl desaturation system.

In Fig. 4 of the accompanying publication (43) it can be seen that cyanide enhanced the DPNH synergism of ethylmorphine *N*-demethylation by only about 30% when microsomes from female rats were employed, a value much below the 90% enhancement observed with microsomes from male rats (Fig. 1). If mitochondrial contamination were responsible for DPNH synergism, this sex difference would not have been observed.

Relationship of DPNH synergism of ethylmorphine N-demethylation to TPNH concentration. Using aminopyrine as a substrate, Cohen and Estabrook (11) showed that DPNH synergism was increased several fold as TPNH was decreased from concentrations in excess of 1 μM to the very low concentration (0.1 μM) required for the oxidation of aminopyrine by rabbit liver microsomes. They attributed this to the ability of DPNH to substitute for TPNH as the donor of the second electron in the absence of high concentrations of TPNH. It seemed pertinent to repeat their study using ethylmorphine as the substrate. The results (Fig. 8) confirm those of Cohen and Estabrook.

Part of the sharp decline in ethylmorphine *N*-demethylation seen at TPNH concentrations of 50 μM or less are probably attributable to the degradative effects of nucleotidases. Nicotinamide largely protects against this degradative action, but it also inhibits the mixed-function oxidase system, probably by complexing cytochrome P-450 (47). Nicotinamide largely prevented the sharp decline in ethylmorphine *N*-demethylation at the lowest TPNH concentrations, and DPNH

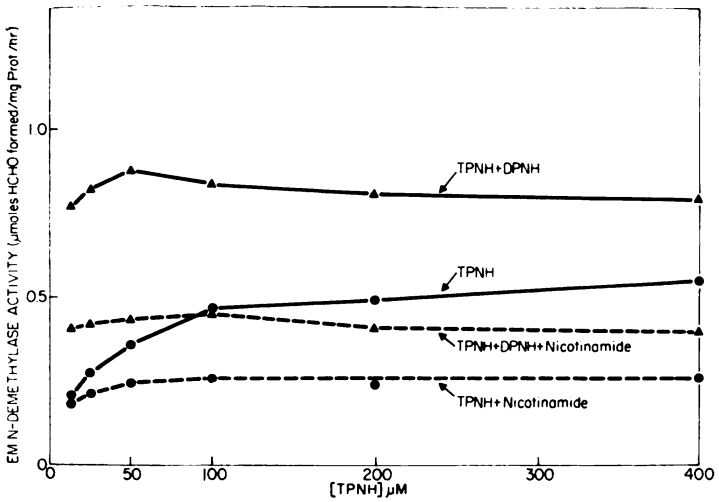


FIG. 8. *Effect of nicotinamide on DPNH synergism of ethylmorphine (EM) N-demethylation at various concentrations of TPNH*

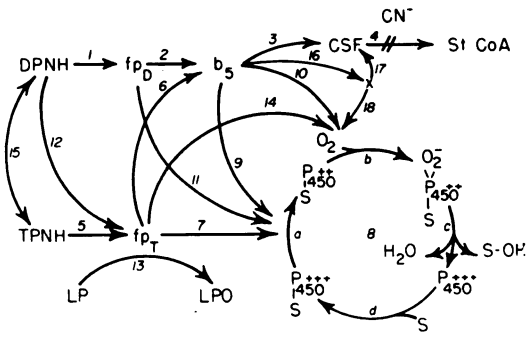
Incubations were performed as described in Fig. 7. DPNH was added as described in Fig. 1. Nicotinamide was employed in a concentration of 10 mM. All values are means of two experiments. In each experiment livers were pooled from at least two male rats (250–280 g).

synergism remained relatively constant throughout the entire range of TPNH concentrations (Fig. 8). Similar results have been reported by Cohen and Estabrook (10, 11), who used aminopyrine as the substrate. It thus appears that much of the exaggerated DPNH synergism observed at very low TPNH concentrations is due to a sparing effect of DPNH on TPNH degradation.

DISCUSSION

The proximal distribution of two electron transfer chains in hepatic microsomes, one involving cytochrome P-450 and the other cytochrome b_5, suggests that the two chains may interact in the performance of certain metabolic functions (9). Particular attention has been directed by Estabrook and associates (9–13) to a possible associative role of the cytochrome b_5 chain with the mixed-function oxidase system which employs cytochrome P-450 in the oxidation of drugs, other foreign compounds, and steroids (1–5). Scheme 1 is provided to facilitate discussion of a variety of ways in which the two electron chains could interact.

According to the concept proposed by Cohen, Estabrook, and Hildebrandt (10–13), the first of the 2 electrons required for the



SCHEME 1

CSF, cyanide-sensitive factor; St. CoA, stearoyl-CoA; fp_D, DPNH-dependent flavoprotein; fp_T, TPNH-dependent flavoprotein; LP, lipid; and LPO, lipid peroxide.

oxidation of drugs is used in the reduction of oxidized cytochrome P-450–substrate complex (step 8a) and is derived almost exclusively from TPNH (steps 5 → 7 → 8a). Steady-state conditions for this reaction are achieved with very low concentrations of TPNH. DPNH will neither substitute appreciably in this reaction nor act synergistically to TPNH. The second electron involves the reduction of the oxygenated, reduced cytochrome P-450–substrate com-

plex (step 8b). This electron is accepted from cytochrome b_5, which obtains reducing equivalents from TPNH, when it is the sole source of electrons (steps 5 and 6), or from either TPNH or DPNH (steps 1 and 2), when both nucleotides are present. DPNH exerts a synergistic effect because it maintains a higher steady-state level of reduced cytochrome b_5 during drug oxidation than does TPNH. Our studies do not prove this concept, but they support it.

DPNH *synergism* implies that DPNH produces an effect on the function of the cytochrome P-450 system above that which should be expected of the system. We prefer to think of the DPNH effect not as synergistic, as the word is usually defined, but rather as satisfying a requirement of the system. At any given steady-state turnover of the cytochrome P-450 cycle (steps 8a–d), first and second electrons must obviously be introduced at the same rate. Thus any rate limitation of the overall reaction imposed by the input of the first and second electrons must necessarily represent a coupled rate limitation. However, relative rate limitations within the coupled rate limitation can be imposed by the relative availability of electrons supplying each of the two sites of entry. When type I substrates, such as ethylmorphine, are added to microsomes, the potential for an increased rate of reduction of cytochrome P-450–substrate complex (first electron) is created (steps 5 → 7 → 8a), and this increases the demand for second electrons. If we assume a role of cytochrome b_5 in the reaction involving the second electron, as well as an abundance of first electrons, the turnover rate of the cytochrome P-450 cycle is controlled by the steady state of reduced cytochrome b_5, which, in turn, determines the rate at which electrons can be transferred from reduced cytochrome b_5 to the cytochrome P-450 system either directly or through an intermediate carrier. The potential for input of second electrons created by the addition of ethylmorphine, and presumably other type I substrates, cannot be satisfied by the second electrons that can be made available by TPNH, which is incapable of maintaining the high steady state of cytochrome b_5 reduction required for maximal turnover of the cytochrome P-450 cycle. However, advantage is taken of this increased potential for input of first electrons when DPNH is added, because DPNH raises the steady state of reduced cytochrome b_5 above that attainable with TPNH. It has been shown, for example, that DPNH maintains a higher steady-state level of reduced cytochrome b_5 than TPNH during stearoyl-CoA desaturation (20). Another way of expressing this concept is to say that the addition of ethylmorphine creates a deficiency of second electrons relative to the input of first electrons and that this deficiency can be met more adequately by second electrons from DPNH than by second electrons from TPNH. After addition of DPNH the rate of entry of second electrons still does not preceed maximally, because second electrons from DPNH are being fed to endogenous pathways. When optimal concentrations of cyanide were added, much of the loss of second electrons to endogenous systems was blocked, and the resulting enlarged pool of second electrons more nearly satisfied the demand for second electrons created by the addition of ethylmorphine. Cyanide did not enhance ethylmorphine *N*-demethylation when TPNH was the only source of reducing equivalents, because the drain of electrons to endogenous substrates through cytochrome b_5 and cyanide-sensitive factor (step 5 → 6 → 3 → 4) was not great enough to disturb the steady-state level of reduced cytochrome b_5 that can be achieved with TPNH. When stearoyl-CoA was added, the drain of electrons from both DPNH and TPNH through cytochrome b_5 to cyanide-sensitive factor (steps 5 → 6 → 3 → 4 and 1 → 2 → 3 → 4) was greatly accelerated, and the cytochrome P-450 system was deprived of second electrons not only when TPNH and DPNH were present, but also when TPNH was the only source of electrons. Under both conditions the rate of ethylmorphine *N*-demethylation was depressed, but, in accordance with the proposed concept, cyanide reversed the inhibitory effects of stearoyl-CoA. In fact, when cyanide was present, the DPNH synergism appeared to be somewhat more pronounced in the presence of stearoyl-CoA (compare Fig. 3 with Fig. 1). This is in keeping with the observation that maximum inhibition of the desatu-

ration system is achieved only in the presence of substrate (20).

The reduction of cytochrome b_5 with DPNH is much more rapid than either the desaturation of stearoyl-CoA or the *N*-demethylation of ethylmorphine, which would seem to rule out the possibility that the desaturation system and the cytochrome P-450 system compete for electrons at cytochrome b_5. It is therefore necessary to postulate an unknown electron transfer component, x, which accepts electrons from cytochrome b_5 at a relatively slow rate and transfers them to the cyanide-sensitive factor via step 17 and to the oxygenated cytochrome P-450–substrate complex via step 18. Stearoyl-CoA desaturation and ethylmorphine *N*-demethylation proceed at comparable rates and would be expected to compete for electrons donated by a common carrier.

Recent studies by Sasame and coworkers (48), which employed antibody to cytochrome b_5, can be interpreted to support this concept. Antibody to cytochrome b_5 prevented DPNH synergism of microsomal ethylmorphine *N*-demethylation, but did not inhibit the reaction when TPNH was the only source of electrons. The authors interpreted this to mean that the transfer of the second electron from DPNH to the oxygenated cytochrome P-450–substrate complex is mediated by cytochrome b_5, but that transfer of the second electron from TPNH to the same complex is not. This may very well be the case, but an alternative explanation for this seeming selective effect of antibody to cytochrome b_5 does not require the invocation of another pathway for the introduction of second electrons. Antibody to cytochrome b_5 did not completely inactivate cytochrome b_5. The half-times of cytochrome b_5 reduction by DPNH and TPNH were increased from 156 to 368 msec and from 250 to 385 msec, respectively. Ethylmorphine *N*-demethylation was decreased by the addition of antibody to cytochrome b_5 from 187 to 161 nmoles/0.5 mg of protein per 10 min when TPNH was the only source of reducing equivalents, and from 236 to 166 nmoles/0.5 mg of protein per 10 min when both TPNH and DPNH were present. It would appear that the antibody altered the nature of the cytochrome b_5 (or selectively inactivated one of two forms of cytochrome b_5) so that it was reduced as rapidly by TPNH as by DPNH, thereby eliminating any previous advantage to the system offered by DPNH. Thus, in the absence of antibody and when both DPNH and TPNH were present, the steady state of reduced cytochrome b_5 was determined by DPNH, which produced a half-time reduction of cytochrome b_5 of 156 msec, and not by TPNH, which produced a half-time reduction of cytochrome b_5 of 250 msec.

When both nucleotides were present, the higher steady-state level of reduction of cytochrome b_5 resulted in a 30% elevation in ethylmorphine *N*-demethylation above that seen when TPNH was the sole source of reducing equivalents (i.e., a 30% DPNH synergism). After the addition of antibody to cytochrome b_5, the potential abilities of DPNH and TPNH to maintain a steady state of reduced cytochrome b_5 were almost identical, as represented by their respective half-time rates of reduction of cytochrome b_5, 368 msec and 385 msec. Accordingly, when antibody to cytochrome b_5 was present, the rate of ethylmorphine *N*-demethylation was essentially the same when TPNH was the only source of electrons (161 nmoles/0.5 mg of protein per 10 min) as when TPNH and DPNH were present (166 nmoles/0.5 mg of protein per 10 min). In view of these considerations, the question remains unanswered as to whether or not second electrons can be introduced to the oxygenated, reduced cytochrome P-450–substrate complex in microsomes by pathways that do not involve cytochrome b_5.

A number of alternative pathways which might explain DPNH synergism were considered, including such possibilities as the role of a transhydrogenase, DPNH pathways alternative to TPNH pathways, and sparing actions of DPNH that prevent the destruction of TPNH or the utilization of TPNH by systems not involved in drug metabolism. The possibility that DPNH might exert its synergistic effect through the action of a transhydrogenase (step 15) can be hastily discounted in view of the studies of Cohen, Hildebrandt, and Estabrook (11, 13), which showed that DPNH had no synergistic effect on the reduction

of cytochrome P-450 under anaerobic conditions or on the TPNH-dependent lipid peroxidation reaction, thus eliminating pathway 15 → 5 → 7 → 8a. We have been able to confirm both these observations. The same authors also pointed out that TPN^+ inhibited the DPNH-supported reaction under conditions in which this could not have occurred if a transhydrogenase system had been operational. It is also inconceivable that, in the presence of saturating concentrations of TPNH, a transhydrogenase could stimulate the over-all reaction by contributing to the formation of more TPNH. Alternative pathways such as those involving steps 12 and 7 or 1 and 11 would also not be expected to produce synergistic effects in the presence of saturating concentrations of TPNH, and are eliminated because of the failure of DPNH either to reduce cytochrome P-450 effectively or to synergize TPNH reduction of cytochrome P-450 (step 8a).

The possible sparing effect of DPNH on the destruction of TPNH by microsomal nucleotidases might conceivably account for DPNH synergism when concentrations of TPNH are very low, but, as was demonstrated in Fig. 8, this is not a factor when concentrations of TPNH are as high as those employed in the current studies. The possibility that DPNH might produce an apparent synergistic effect by sparing TPNH in pathways than can accept electrons from either DPNH or TPNH loses credibility with the finding that DPNH oxidation was greater in the presence of TPNH than in its absence, a result contrary to what would be expected if TPNH and DPNH were competing for a common electron-accepting pathway (12). Gillette *et al.* (49) suggested that the DPNH-dependent flavoprotein (fp_D) might compete with the TPNH-dependent flavoprotein (fp_T) for the reduction of cytochrome b_5, in which case electrons from TPNH diverted from cytochrome P-450 by way of step 6 are spared when DPNH is present to provide electrons for the reduction of oxidized P-450–substrate complex by channeling more electrons from TPNH through step 7; as emphasized previously, however, DPNH does not synergize the reduction of cytochrome P-450. Sodium sulfide, in a concentration known to inhibit mitochondrial cytochrome oxidase almost completely, failed to enhance DPNH synergism of ethylmorphine *N*-demethylation (Fig. 6). This tends to eliminate the possibility that cyanide spared the loss of electrons from DPNH or TPNH by inhibiting cytochrome oxidase contained in the small amount of contaminating mitochondria. If the shunting of electrons from TPNH to mitochondria is an important pathway, cyanide should have stimulated ethylmorphine *N*-demethylation when TPNH in low concentrations was the sole source of electrons; it did not (Fig. 7).

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REFERENCES

1. J. R. Gillette, *Advan. Pharmacol.* **4**, 219–261 (1966).
2. A. H. Conney, *Pharmacol. Rev.* **19**, 317–366 (1967).
3. G. J. Mannering, *in* "Selected Pharmacological Testing Methods," (A. Burger, ed). pp. 51–119. Marcel Dekker, New York, 1968.
4. R. W. Estabrook, A. Shigematsu, and J. B. Schenkman, *Advan. Enzyme Regul.* **8**, 121–130 (1970).
5. G. J. Mannering, *in* "Fundamentals of Drug Metabolism and Drug Disposition" (B. N. La Du, H. G. Mandel, and E. L. Way, eds.), pp. 206–252. Williams & Wilkins, Baltimore, 1971.
6. A. H. Conney, R. R. Brown, J. A. Miller, and E. C. Miller, *Cancer Res.* **17**, 628–633 (1957).
7. A. Nilsson and B. C. Johnson, *Arch. Biochem. Biophys.* **101**, 494–498 (1963).
8. V. Ullrich, *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 357–365 (1969).
9. R. W. Estabrook and B. S. Cohen, *in* "Microsomes and Drug Oxidations" (J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, eds.), pp. 95–109. Academic Press, New York, 1969.
10. B. S. Cohen and R. W. Estabrook, *Arch. Biochem. Biophys.* **143**, 37–45 (1971).
11. B. S. Cohen and R. W. Estabrook, *Arch. Biochem. Biophys.* **143**, 46–53 (1971).
12. B. S. Cohen and R. W. Estabrook, *Arch. Biochem. Biophys.* **143**, 54–65 (1971).

13. A. Hildebrandt and R. W. Estabrook, *Arch. Biochem. Biophys.* **143**, 66–79 (1971).
14. V. Ullrich, B. Cohen, C. Y. Cooper, and R. W. Estabrook, *in* "Structure and Function of Cytochromes" (K. Okunuki, M. D. Kamen, and I. Sekuzu, eds.), pp. 649–655. University of Tokyo Press, Tokyo, 1969.
15. R. W. Estabrook, A. Hildebrandt, H. Remmer, J. B. Schenkman, O. Rosenthal, and D. Y. Cooper, *in* "19th Colloquium der Gesellschaft für biologische Chemie" (B. Hess and H. Staudinger, eds.), pp. 142–177. Springer, Berlin, 1969.
16. M. Waterman and H. S. Mason, *Biochem. Biophys. Res. Commun.* **39**, 450–454 (1970).
17. P. L. Gigon, T. E. Gram, and J. R. Gillette, *Biochem. Biophys. Res. Commun.* **31**, 558–562 (1968).
18. P. L. Gigon, T. E. Gram, and J. R. Gillette, *Mol. Pharmacol.* **5**, 109–122 (1969).
19. N. Oshino, Y. Imai, and R. Sato, *Biochim. Biophys. Acta* **128**, 13–28 (1966).
20. N. Oshino, Y. Imai, and R. Sato, *J. Biochem. (Tokyo)* **69**, 155–167 (1971).
21. N. Oshino and R. Sato, *J. Biochem. (Tokyo)* **69**, 169–180 (1971).
22. N. Oshino and R. Sato, *Arch. Biochem. Biophys.* **149**, 369–377 (1972).
23. N. Oshino, *Arch. Biochem. Biophys.* **149**, 378–387 (1972).
24. N. E. Sladek and G. J. Mannering, *Mol. Pharmacol.* **5**, 174–185 (1969).
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265–275 (1951).
26. T. Nash, *Biochem. J.* **55**, 416–421 (1953).
27. R. W. Estabrook and P. K. Maitra, *Anal. Biochem.* **3**, 369–382 (1962).
28. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2370–2378 (1964).
29. J. B. Schenkman, H. Remmer, and R. W. Estabrook, *Mol. Pharmacol.* **3**, 113–123 (1967).
30. C. R. E. Jefcoate, J. L. Gaylor, and R. L. Calabrese, *Biochemistry* **8**, 3455–3463 (1969).
31. J. L. Gaylor, N. J. Moir, H. E. Seifried, and C. R. E. Jefcoate, *J. Biol. Chem.* **245**, 5511–5513 (1970).
32. J. L. Gaylor and H. S. Mason, *J. Biol. Chem.* **243**, 4966–4972 (1968).
33. J. B. Marsh and A. T. James, *Biochim. Biophys. Acta* **60**, 320–328 (1962).
34. P. Hochstein and L. Ernster, *Ciba Found. Symp. Cellular Injury* 123–135 (1964).
35. F. Wada, K. Hirata, H. Shibata, K. Higashi, and Y. Sakamoto, *J. Biochem. (Tokyo)* **62**, 134–136 (1967).
36. A. Y. H. Lu and M. J. Coon, *J. Biol. Chem.* **243**, 1331–1332 (1968).
37. M. L. Das, S. Orrenius, and L. Ernster, *Eur. J. Biochem.* **4**, 519–523 (1968).
38. T. Watabe, Y. Ueno, and J. Imazumi, *Biochem. Pharmacol.* **20**, 912–913 (1971).
39. H. Remmer, *Naturwissenschaften* **45**, 189 (1958).
40. H. Remmer and B. Asleben, *Klin. Woehenschr.* **36**, 332–333 (1958).
41. H. Remmer, *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiedebergs)* **235**, 279–290 (1959).
42. H. Remmer, *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiedebergs)* **237**, 296–307 (1959).
43. M. A. Correia and G. J. Mannering, *Mol. Pharmacol.* **9**, 470–485 (1973).
44. E. Shelton and M. E. Rice, *J. Nat. Cancer Inst.* **18**, 117–125 (1957).
45. D. C. Wharton and A. Tzagoloff, *Methods Enzymol.* **10**, 245–250 (1967).
46. W. W. Wainio and J. Greenlees, *Arch. Biochem. Biophys.* **90**, 18–21 (1960).
47. J. B. Schenkman, J. A. Ball, and R. W. Estabrook, *Biochem. Pharmacol.* **16**, 1071–1081 (1967).
48. H. A. Sasame, J. R. Mitchell, S. Thorgeirsson, and J. R. Gillette, *J. Drug Metab. Dispos.* **1**, 150–155 (1973).
49. J. R. Gillette, D. C. Davies, and H. A. Sasame, *Annu. Rev. Pharmacol.* **12**, 57–84 (1972).