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Mechanism of the Nucleotide Pyrophosphatase Induced Distortion of Stoichiometry of TPNH Utilization and Product Formation by Hepatic Cytochrome P-450 Linked N-Demethylase Systems

ELIZABETH H. JEFFERY AND GILBERT J. MANNERING

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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SUMMARY

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By definition, an hepatic cytochrome P-450- and TPNH-linked monooxygenase reaction requires that a mole of TPNH be utilized for each mole of product formed. When rat hepatic microsomes have been used to study the stoichiometry of N-demethylase reactions, considerably more TPNH utilization has usually been observed than can be accounted for by product (HCHO) formation. The presence of nucleotide pyrophosphatase in microsomes has been shown to cause the apparent substrate-induced enhancement of endogenous TPNH oxidation. Demethylation of the substrate (aminopyrine, benzphetamine, codeine or ethylmorphine) increases the rate of TPNH oxidation and this enhances the rate limitation of TPNH for the pyrophosphatase reaction (a first order reaction) without inflicting a TPNH rate limitation on the endogenous oxidation of TPNH (a zero order reaction). As a consequence, less 2′5′ ADP—an inhibitor of TPNH oxidation—is formed from TPNH by the pyrophosphatase when the substrate is present than when it is absent. Because less 2′5′ ADP is formed in the presence of substrate, less inhibition of endogenous TPNH oxidation occurs; substrate thus appears to stimulate endogenous TPNH oxidation when in fact it is only permitting the reaction to proceed at a less inhibited rate. When EDTA (0.2 mM) or 5′AMP (1 mM) is used to inhibit pyrophosphatase activity, a stoichiometry of TPNH oxidized:HCHO formed of unity was observed when microsomes from several mammalian species were used. Unity was also observed when the influence of pyrophosphatase was diminished by increasing the ratio of demethylase activity to pyrophosphatase activity in microsomes by administering phenobarbital to rats; by modulating the ratios of the two enzymes in this manner, it was shown that disparity in the 1:1 ratio of TPNH oxidation: HCHO formation is directly related to the nucleotide pyrophosphatase content of the microsomes. When EDTA or 5′AMP is added to the medium, the rate of monooxygenase activity of hepatic microsomes can be measured by monitoring the disappearance of the absorbance of TPNH at 340 nm.

INTRODUCTION

The hepatic cytochrome P-450- and NADPH-dependent systems involved in the oxidation of many drugs and other xenobiotics are classified as monooxygenase (1) or mixed function oxidase systems (2), i.e., the systems catalyze the transfer of one of the oxygen atoms of molecular oxygen to the substrate and the other oxygen atom undergoes two equivalent reductions to

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form water. The stoichiometry of hepatic microsomal monooxygenase reactions therefore demands equivalent utilization of TPNH, O_2 and substrate. However, widely different ratios of TPNH utilization to product formation have been observed. For example, when aminopyrine was the substrate, Cohen and Estabrook (3) observed twice as much TPNH oxidized as product formed, whereas Stripp *et al.* (4) observed about four times more product formed than TPNH utilized. Hepatic microsomes oxidize TPNH in the absence of added substrate. This endogenous enzyme activity (TPNH oxidase activity) may consume as much or more TPNH than that required for the oxidation of exogenous substrate. The apparent increase in TPNH oxidation above that required for product formation, which occurs when drug substrates are incubated with microsomes, might be explained in several ways. a) The measured product of the reaction may be metabolized further to a product which is not measured; this is not a likely possibility when formaldehyde is the product (HCHO) because it is not metabolized by microsomes, at least not when semicarbazide is present (5). b) Uncoupling may cause the formation of more water than product (6). c) Cytochrome P-450 generates H_2O_2 (7–9), thus raising the possibility that H_2O_2 may be formed at the expense of product formation. We do not consider b) or c) a strong possibility when ethylmorphine is the substrate because a 1:1 stoichiometry of TPNH oxidation and HCHO formation was achieved in the presence of EDTA (10), which is not known to prevent uncoupling; however, this possibility cannot be ignored. d) Endogenous TPNH oxidation is stimulated by exogenous substrate; this is a possibility to be considered in view of our observation that the rate of TPNH oxidation when ethylmorphine was present was about the same with or without the addition of EDTA (10); since EDTA increased endogenous TPNH oxidation to the degree that TPNH oxidation and HCHO formation were equivalent, it could be implied that ethylmorphine stimulated endogenous TPNH oxidation as effectively as EDTA. e) The formation of an inhibitor of endogenous TPNH oxidation during incubation is decreased when exogenous substrate is present; with respect to the action of EDTA, the result would be indistinguishable from that produced by mechanism d). Of the five possibilities, e) appeared most likely. TPNH is hydrolyzed by microsomal nucleotide pyrophosphatase to nicotinamide mononucleotide (NMNH) and 2′5′ ADP (11) which, as will be seen, is an inhibitor of hepatic monooxygenase reactions. Indirect evidence will be presented which shows that less 2′5′ ADP is formed when exogenous substrate is present than when absent, and that this explains why more TPNH is oxidized when substrates are N-demethylated by microsomes than can be accounted for by product formation.

This report is an extension of an earlier communication (10) which showed that a TPNH:HCHO ratio of unity was achieved when ethylmorphine was N-demethylated by microsomes from male rats when EDTA was added to the incubation medium in a concentration that inhibits pyrophosphatase activity. The current communication includes substrates other than ethylmorphine, and microsomes from species other than the rat, different strains of rat, and rats of both sexes.

MATERIALS AND METHODS

Chemicals. [N-methyl-^{14}C] codeine HCl was purchased from Amersham-Searle Corp., ^{14}C-formaldehyde was obtained from International Chemical and Nuclear Corp., and catalase, TPNH, 5′AMP, 2′AMP and 2′5′ADP from Sigma. Codeine and ethylmorphine came from Mallinckrodt, and aminopyrine from K & K Laboratories, Inc. Benzphetamine was a gift from Upjohn Co.

Animals. Male rats, mice, guinea pigs and rabbits weighed 200–270 g, 10–12 g, 250–400 g, and about 2000 g, respectively. Female rats weighed 250–270 g. Holtzman rats were used except in specified experiments which used Sprague-Dawley or Simonsen rats. Phenobarbital administration was 40 mg of sodium phenobarbital in saline/kg of rat/day for 4 days; 3-methylcholanthrene administration was 20 mg of 3-methylcholanthrene in corn oil/kg of rat/day for 3 days. Animals were killed about 24 hr after the last injection of these agents.

Liver preparation. Hepatic microsomes

were prepared as described previously (12).

Determination of the ratio of the rate of TPNH oxidation to the rate of product formation. The rate of oxidation of TPNH was measured by monitoring the rate of disappearance of absorbance at 340 nm. The incubation medium (3 ml) contained 60 mM phosphate buffer (pH 7.4), 0.154 M KCl, 0.5 mM KCN, 0.2 mM EDTA (disodium salt), 2 mM $MgCl_2$, 7.5 mM semicarbazide and 3 mg of microsomal protein. EDTA was included to inhibit lipid peroxidase and nucleotide pyrophosphatase activities (10). Cyanide was included to inhibit the fatty acyl CoA desaturation system; 0.5 mM cyanide inhibits the desaturase system almost completely, but has little or no effect on the monooxygenase system when TPNH is the sole source of electrons (13). Three milliliters of the incubation medium with or without substrate (ethylmorphine, codeine, aminopyrine, or benzphetamine) was placed in two cuvettes contained in the heated chamber (37°) of an Aminco DW-2 spectrophotometer. The cuvettes were allowed to stand for 4 min. This warming period was necessary because an initial swelling of microsomes due to the presence of Mg^{++} interferes with absorption at 340 nm if swelling is not complete prior to addition of TPNH. The contents of the cuvettes were balanced spectrally at 340 nm. The reaction was started by rapidly injecting 10 μl of an aqueous solution containing about 300 nmoles of TPNH from a Hamilton syringe into the sample cuvette. The disappearance of absorption at 340 nm was recorded for exactly 2 min at which time 2.0 ml of medium was immediately removed for formaldehyde analysis. The initial rate of disappearance of TPNH was derived from the initial linear portion of the curve. Calculation of the rate of TPNH oxidation was based on an absorbance extinction coefficient at 340 nm of 6.22 mM^{-1} cm^{-1}. The formation of reduced nicotinamide mononucleotide (NMNH) does not interfere with the measurement of disappearance of TPNH due to oxidation because the molar absorbance of NMNH at 340 nm is the same as that for TPNH. The stoichiometry of TPNH oxidation relative to product formation was calculated by dividing the difference between the rates of TPNH oxidation in the absence and presence of substrate by the rate of HCHO formation.

Determination of the rate of nicotinamide mononucleotide (NMNH) formation from TPNH. The previously described incubation medium with or without substrate in the presence or absence of EDTA was spectrally balanced at 37° as described for the determination of microsomal TPNH oxidation. Forty seconds after the addition of varying amounts of TPNH (210–900 nmoles), 0.1 ml of a mixture of glutamic dehydrogenase (5 units), α-ketoglutarate (0.12 M), ammonium chloride (0.12 M) and 5′AMP (30 mM) was added rapidly. The 40 sec incubation time was used because the reaction was found to proceed linearly for only 80–100 sec when TPNH was not in excess. The glutamic dehydrogenase and α-ketoglutarate convert all of the TPNH to TPN within 10 to 20 sec; 5′AMP prevents further conversion of TPNH to NMNH. Absorption at 340 nm levels off within 10 to 20 sec after the addition of the mixture. The plateau level of absorption at 340 nm was used to calculate the amount of NMNH formed in 40 sec ($\epsilon = 6.22$ mM^{-1} cm^{-1}).

Assays. ^{14}C-N-demethylation of [N-methyl-^{14}C] codeine was measured by the method employed by Poland and Nebert (14) to measure ^{14}C-N demethylation of [N-methyl-^{14}C] aminopyrine except that the pH was adjusted to 8.0 with 0.1 N $NaHCO_3$ before the chloroform extraction to insure that morphine, an amphoteric product of the reaction, would remain with the substrate in the chloroform phase. Recovery studies employing [N-methyl-^{14}C] codeine and ^{14}C-formaldehyde showed that 99.47% of the substrate was extracted into the combined chloroform extracts and 82.3% of the HCHO remained in the aqueous phase. Calculations took into account this distribution of HCHO between aqueous and chloroform phases.

Formaldehyde was determined by the method of Nash (15). Protein was estimated by the method of Lowry *et al.* (16). The method of Omura and Sato (17) was used for the estimation of microsomal cytochrome P-450. Hydrogen peroxide was determined by the method of Gillette *et al.* (18), which measures HCHO formed

through the action of catalase and H_2O_2 on methanol, or by the colorimetric assay of Hildebrandt and Roots (19) using ferrous ammonium sulfate and potassium thiocyanate.

RESULTS

Stoichiometry of TPNH utilization and HCHO formation of N-demethylase reactions by hepatic microsomes from rabbits, mice, guinea pigs and three strains of rat. Rates of codeine metabolism (O- + N-demethylation) by microsomes from male untreated rats, rabbits, mice and guinea pigs using the measurement of HCHO formed and the concomitant disappearance of TPNH, monitored by the loss of absorbance at 340 nm, are given in Table 1. Ratios of TPNH oxidized to HCHO formed ranged between 0.90 and 1.22 with a mean value of 1.04. With the possible exception of the 1.22 value obtained with the rabbit, the ratios are within experimental error of being unity. Similar stoichiometry was observed with microsomes from rats which had been treated with phenobarbital or 3-methylcholanthrene. Comparable TPNH:HCHO ratios were observed with microsomes from Holtzman and Sprague-Dawley rats when ethylmorphine was the substrate, but a ratio of 1:1.3 was seen with Simonsen rats. Stoichiometry of unity was also observed with aminopyrine and benzphetamine.

TPNH utilization and HCHO formation by substrate-limited microsomal demethylation reactions. It is conceivable that TPNH-dependent microsomal reactions other than that responsible for the formation of HCHO may be stimulated or inhibited by the substrate. If this is the case, the possibility must be considered that the favorable stoichiometry shown in Table 1 may have been achieved through a fortuitous balance between stimulation and inhibition of these other reactions. Degrees of stimulation and inhibition of these reactions would presumably relate to substrate concentration; thus, any balance that may have existed when saturating concentrations of substrate were used (Table 1) would be altered at less than saturating substrate concentrations. With this in mind, the ratios of TPNH utilization to HCHO formation were determined using varying concentrations of aminopyrine, benzphetamine, codeine or ethylmorphine. These data were used to calculate apparent kinetic constants (Table 2). The ratio of the apparent maximum velocity (V) obtained by measuring TPNH to that obtained by measuring HCHO formation was near unity when microsomes from male rats were used to demethylate aminopyrine, benzphetamine, codeine or ethylmorphine. Unity was observed when codeine was demethylated by microsomes from female rats, but not when ethylmorphine was the substrate. This discrepancy can be explained on the basis that microsomes from female rats contain more O-demethylase activity relative to N-demethylase activity than microsomes from male rats. When codeine is the substrate, the rate of TPNH disappearance equals the rate of HCHO formation regardless of the relative rates of O- and N-dealkylation because both O- and N-positions are substituted with methyl groups. On the other hand, ethylmorphine has an ethyl group in the O-position and a methyl group in the N-position. The relative rates of O- and N-dealkylation now become important because the Nash method measures the HCHO from N-demethylation, but not the CH_3CHO from O-deethylation although TPNH is required for both dealkylations. If the ratio of N-dealkylation to O-dealkylation is high, the rate of disappearance of TPNH will not differ greatly from the rate of HCHO formation, but if it is not, the rate of TPNH disappearance will be accordingly higher than the rate of HCHO formation. We have in fact shown recently that the rates of O- to N-dealkylation of ethylmorphine in the male Simonsen rat are about 4:1, whereas those in the female are about 1:1 (22). Thus, when microsomes from female rats are used, only about half of the TPNH consumed during the O- and N-dealkylation of ethylmorphine can be attributed to the formation of HCHO, i.e., the ratio of TPNH utilization to HCHO formation becomes 1.0:1.1 (0.91) rather than 2.0:1.1 (1.82). While the relative amount of TPNH consumed in the O-dealkylation of ethylmorphine by male rats is much less than that seen with female rats, it cannot be ignored. If the hepatic microsomes from the Holtzman rats used in the current study

TABLE 1

Stoichiometry of TPNH utilization and HCHO formation of N-demethylase reactions

Substrate[a]	Species	Strain	Treatment of animals	TPNH Disappearance[b]			HCHO[b] Formation	NADPH/HCHO[c]
				−Substrate (1)	+Substrate (2)	(2)-(1)		
			None	12.5	14.8	2.3 ± 0.6	2.4 ± 0.9	0.97 ± 0.09
			PB	30.1	34.4	4.3 ± 2.0	4.9 ± 2.3	0.90 ± 0.03
Codeine (5 mM)	Rat	Holtzman	3-MC	14.0	16.6	2.6 ± 1.0	2.3 ± 1.0	1.10 ± 0.08
	Rabbit	New Guinea White	None	7.7	9.5	1.8 ± 0.13	1.4 ± 0.15	1.22 ± 0.08
	Mouse	Swiss Webster	None	15.0	18.8	3.8 ± 0.5	3.9 ± 0.06	1.00 ± 0.15
	Guinea Pig	English Hartley	None	7.9	9.3	1.4 ± 0.2	1.4 ± 0.3	1.07 ± 0.08
		Holtzman	None	11.9	15.1	3.2 ± 0.8	3.0 ± 0.7	1.06 ± 0.09
Ethylmorphine		Sprague-Dawley	None	15.3	20.0	4.7 ± 0.03	4.2 ± 0.2	1.10 ± 0.10
(2 mM)	Rat	Simonsen	None	16.9	22.5	5.6 ± 0.2	4.3 ± 0.3	1.30 ± 0.03[d]
Aminopyrine (5 mM)	Rat	Holtzman	None	12.9	15.4	2.5 ± 0.9	2.4 ± 0.9	1.03 ± 0.03
Benzphetamine (5 mM)	Rat	Holtzman	None	10.1	13.2	3.1 ± 0.3	3.0 ± 0.9	1.03 ± 0.04

[a] Saturating concentrations.

[b] TPNH disappearance and HCHO formation (nmoles/mg of protein/min) are the means of 3 experiments.

[c] Means of 3 ratios calculated individually.

[d] See text for an explanation for this discrepancy in stoichiometry.

± Values are SE.

TABLE 2

Apparent kinetic constants for the demethylation of drug substrates and the concomitant oxidation of TPNH by hepatic microsomes

The experimental procedure was the same as that given in MATERIALS AND METHODS except that 5 concentrations (0.1–1.0 mM) of each substrate were employed. Michaelis constants were analyzed by the method of Wilkinson (20) using a Fortran program written by Cleland (21). Values are means ± S.E. obtained from 3 experiments. No statistical difference ($p > 0.10$) between TPNH utilized and HCHO formed was observed with any of the substrates except at one concentration of benzphetamine (0.2 mM), where p was <0.1, but > than 0.05.

Substrate	Sex	K[a] or V[b]	TPNH disappearance	HCHO formation	$\frac{V_{(TPNH)}}{V_{(HCHO)}}$
Aminopyrine	M	K	0.13 ± 0.02	0.11 ± 0.02	1.05
		V	6.4 ± 0.2	6.1 ± 0.3	
Benzphetamine	M	K	0.18 ± 0.03	0.15 ± 0.04	0.93
		V	2.8 ± 0.3	3.0 ± 0.5	
Codeine	M	K	0.71 ± 0.05	0.65 ± 0.07	1.04
		V	5.6 ± 0.1	5.4 ± 0.1	
	F	K	0.27 ± 0.03	0.31 ± 0.02	1.10
		V	2.1 ± 0.2	1.9 ± 0.1	
Ethylmorphine	M	K	0.28 ± 0.05	0.34 ± 0.02	1.07
		V	4.5 ± 0.5	4.2 ± 0.7	
	F	K	0.21 ± 0.07	0.34 ± 0.05	1.82[c]
		V	2.0 ± 0.1	1.1 ± 0.1	

[a] Apparent Michaelis constant ± S.E. (mM).

[b] Apparent maximum velocity ± S.E. (nmole/mg of protein/min).

[c] See text for explanation for this disparity in stoichiometry.

possessed 4 times more N- than O-demethylase activity, as was the case with the Simonsen rats used in the previous study (22), the TPNH:HCHO ratio of 1.05 (Table 2) becomes 0.85 when adjusted to accommodate the amount of TPNH used for O-deethylation. However, this extrapolation should be viewed with caution because rats were used in the previous study at a different time of the year; the same ratio of O- to N-dealkylation may not be constant throughout the months or years. An experiment conducted during the course of the current study using N-methyl-^{14}C codeine (1.0 mM) as the substrate showed 12.0 ± 0.5 and 6.7 ± 1.3 nmoles of total HCHO formed/nmole of P-450/min by microsomes from male and female Holtzman rats, respectively, and corresponding values of 10.8 ± 0.8 and 4.3 ± 0.7 nmole of ^{14}C-HCHO formed/nmole of P-450. It can be calculated from these data that the ratio of N-:O-dealkylation of codeine was 8.3 and 1.8 for males and females, respectively. These results would suggest that either the ratio may differ from time to time or that the substrate specificities of the O- and N-dealkylating systems are different for ethylmorphine and codeine. In any event, the ratios were close to unity for the demethylation of codeine and two other substrates which do not offer the possibility of O-dealkylation (Tables 1 and 2).

If the ratio of N- to O-dealkylation of ethylmorphine by Simonsen rats was 4:1 when the data for Table 1 were collected, as was the case when the animals were observed earlier (22), the 1.30 stoichiometry of TPNH utilization:HCHO formation seen with that strain of rat can be explained. When recalculated to accommodate the TPNH utilized for O-dealkylation, the ratio of 1.30 becomes 1.04.

Effects of EDTA and zinc on microsomal nucleotide pyrophosphatase activity. Nucleotide pyrophosphatase activity was observed to vary considerably in microsomes prepared on different days. However, $ZnCl_2$ (0.05 mM) elevated low levels of activity to the high levels (Table 3); high levels of activity were not altered by Zn^{++}. This suggests that the observed day to day variation of pyrophosphatase activity may relate to the amount of contaminating zinc

TABLE 3

Effects of EDTA and zinc on the activity of nucleotide pyrophosphatase

Day	Control		+ EDTA (0.2 mM)		+ Zinc (.05 mM)	
	V[a]	K[b]	V	K	V	K
1	18.2	150	8.3	151	23.0	151
2	26.3	154	13.0	154	26.3	149
3	16.5	154	8.3	151	24.0	158
4	24.0	141	12.1	140	24.0	141

[a] Apparent maximum velocity (nmoles NMNH formed/mg of protein/min) as derived in Table 2 (n = 1); 5 concentrations of TPNH (70–300 μM) were used.

[b] Apparent Michaelis constant (μM of TPNH).

contained in the incubation medium. EDTA inhibited the reaction non-competitively by about 50%. Zinc is a cofactor for nucleotide phosphatase (23); EDTA is presumed to inhibit this enzyme by chelating zinc. The apparent Michaelis constant (about 150 μM TPNH) was not altered by EDTA or Zn^{++}.

Inhibition of microsomal ethylmorphine N-demethylation by 2'5'ADP and 2'AMP. 2'5' ADP is a product of the hydrolysis of TPNH by nucleotide pyrophosphatase; 2'5' ADP is converted to 2'AMP by alkaline phosphatase. Both enzymes are present in hepatic microsomal preparations (11, 24). 2'AMP inhibits monooxygenase activity by inhibiting TPNH-cytochrome c reductase (24). 5'AMP inhibits nucleotide pyrophosphatase activity without inhibiting monooxygenase activity. Its inclusion in the incubation medium enabled the evaluation of the inhibitory effects of added 2'5' ADP and 2'AMP without interference from additional formation of these nucleotides through the action of pyrophosphatase on TPNH. 2'5' ADP is seen to be a more potent inhibitor of ethylmorphine N-demethylase than 2'AMP (Fig. 1). The apparent inhibition constants of 2'AMP for the inhibition of ethylmorphine N-demethylase and NADPH oxidase activities were 20 to 40 times larger than those for 2'5' ADP (Table 4). The similarity of the apparent Michaelis constants for the inhibition of TPNH oxidase and monooxygenase activities suggests that inhibition occurs at a common site, presumably at the level of

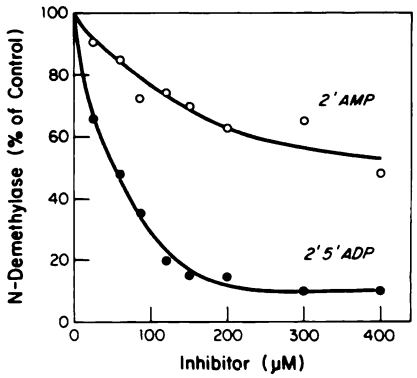

FIG. 1. *Inhibition of microsomal ethylmorphine N-demethylase by 2'AMP and 2'5' ADP*

Microsomes were incubated for 2 min with ethylmorphine using the medium described in Table 6 plus 1 mM 5'AMP and indicated concentrations of 2'AMP or 2'5' ADP. HCHO was determined by the method of Nash (15). Each point represents the mean of two experiments.

TABLE 4

Apparent inhibition constants (K_i) for the inhibition of TPNH oxidase and ethylmorphine (EM) N-demethylase by 2'AMP and 2'5' ADP

Inhibitor	Concentration (μM)	K_i (mM)	
		EM N-demethylase	TPNH oxidase
2' AMP	50	0.30	0.43
	100	0.29	0.53
2'5' ADP	50	0.015	0.015
	100	0.015	0.010

Experimental procedures were the same as those given in Table 2.

TPNH-cytochrome c reductase (23).

Effect of ethylmorphine on nucleotide pyrophosphatase and TPNH oxidase activities. When nucleotide pyrophosphatase is inhibited by EDTA or 5'AMP, the rate of TPNH oxidation by microsomes is increased (10). When ethylmorphine is present, the rate of TPNH oxidation is the same regardless of whether or not EDTA or 5'AMP is present (10). This could be explained if EDTA, 5'AMP and ethylmorphine increased TPNH oxidation, or if each of these compounds inhibited the formation of 2'5' ADP and 2'AMP from TPNH. Experiments were designed to test the latter possibility and to determine whether 2'5' ADP and 2'AMP produced by microsomes

from TPNH are more or less effective than 2′5′ ADP and 2′AMP added to the medium. Two samples of the same microsomal preparation were incubated with 100 nmole of TPNH/ml for exactly 3 min, at which time one of the samples was used for the determination of NMNH. 5′AMP was added to the second sample to stop nucleotide pyrophosphatase activity; this was followed by the addition of enough TPNH to restore the concentration of 100 nmole/ml. The rate of oxidation of TPNH was then measured by monitoring the disappearance of absorbance at 340 nm for about one minute. This procedure enabled the determination of the inhibitory effect of a measurable amount of endogenously produced nucleotides (2′5′ ADP produced by nucleotide pyrophosphatase + 2′AMP produced from 2′5′ ADP by alkaline phosphatase = NMNH = 30 nmole/ml). Three samples of the same microsomes were also incubated for 3 min with 100 nmole of TPNH/ml in the presence of 5′AMP. TPNH was added to the first sample to restore its concentration to 100 nmole/ml; the same amount of TPNH was added to the other two samples and either 2′5′ ADP or 2′AMP was added in a concentration equimolar to the amount of NMNH formed in the first part of the experiment (30 nmole/ml). The rate of oxidation of TPNH by each of the three samples was then measured by monitoring the disappearance of absorption at 340 nm for about one minute. The entire experiment was repeated with an incubation medium containing ethylmorphine (2 mM). The amount of NMNH formed in the presence of ethylmorphine during the 3 min incubation period was 15.2 nmole/ml. Results are presented in Table 5. 5′AMP increased the rate of TPNH oxidation by 50% (Condition 1 vs. Condition 2), ethylmorphine by 85% (Condition 6 vs. Condition 2). The combination of 5′AMP and ethylmorphine doubled the rate of TPNH oxidation (Condition 5 vs. Condition 2). Endogenously formed nucleotides (2′5′ ADP + 2′AMP) inhibited TPNH oxidation to a lesser degree than an equivalent concentration of added 2′5′ ADP (Condition 4 vs. Condition 2), but to a greater degree than an equivalent concentration of 2′AMP (Condition 3 vs. Condition 2). The greater inhibitory effect on TPNH oxidation seen with exogenous 2′5′ ADP over that seen with nucleotides produced endogenously through the activities of nucleotide pyrophosphatase and alkaline phosphatase can best be explained by differences in the activity of alkaline phosphatase under the two conditions. Under the condition where 2′5′ ADP was added, 5′AMP, a substrate inhibitor of

TABLE 5

Effect of 2′AMP, 2′5′ ADP and ethylmorphine (EM) on microsomal TPNH oxidase activity

Two samples of hepatic microsomes (1 mg of protein/ml) were incubated for exactly 3 min in a medium (3 ml) containing 60 mM phosphate buffer (pH 7.4), 0.154 M KCl, 2 mM $MgCl_2$, 7.5 mM semicarbazide and 0.1 mM TPNH. One sample was used for the determination of NMNH. 5′AMP (1 mM) and an amount of TPNH which restored the TPNH concentration to about 0.1 mM was added to the second sample and the rate of TPNH oxidation was measured by monitoring the disappearance of absorbance at 340 nm for about 1 min. Three samples of the same microsomes were also incubated for 3 min in the presence of 5′AMP (1 mM) and 0.1 mM TPNH. TPNH was added to the first sample to restore its concentration to 0.1 mM; the same amount of TPNH was added to the other two samples and either 2′5′ ADP or 2′AMP was added in a concentration equimolar to the amount of NMNH formed, as determined earlier. The rate of oxidation of TPNH by each of the three samples was then measured.

Conditions	Rate of TPNH oxidation
	nmoles/min/mg
1. 5′AMP added at 0 min	14.9
2. 5′AMP added at 3 min	10.0
3. 5′AMP added at 0 min and 2′AMP[a] added at 3 min	13.1
4. 5′AMP added at 0 min and 2′5′ ADP[a] added at 3 min	7.5
5. EM and 5′AMP added at 0 min	20.2
6. EM added at 0 min and 5′AMP[b] added at 3 min	18.6
7. EM and 5′AMP added at 0 min; 2′AMP[b] added at 3 min	18.8
8. EM and 5′AMP added at 0 min; 2′5′ ADP[b] added at 3 min	13.9

[a] 30 nmole (the amount of NMNH formed in the absence of EM in 3 min).

[b] 15.2 nmole (the amount of NMNH formed in the presence of EM in 3 min).

alkaline phosphatase, prevented the conversion of the highly inhibitory 2'5' ADP to the much less inhibitory 2'AMP. Moreover, under Condition 2 the alkaline phosphatase was active for 3 min, whereas under Condition 4, the alkaline phosphatase was permitted to react with 2'5' ADP for only 1 min. Under the condition where nucleotides were produced endogenously, 5'AMP was not present, and some of the highly inhibitory 2'5' ADP was converted to the less inhibitory 2'AMP. This could also account for the unidentified "other inhibitors" of TPNH-cytochrome c reductase observed by Sasame and Gillette (24) in extracts of microsomal preparations that had been incubated with TPNH for 20 min. They showed that when TPNH was incubated with microsomes for 60 min all of the phosphorous that did not appear as inorganic phosphorus could be accounted for as 2'AMP, which did not yield inorganic phosphorus. An extract of the mixture made after 20 min of incubation had an inhibitory constant which was an order of magnitude lower than that of 2'AMP. They concluded that "other inhibitors" were present in the extract. It would seem likely, however, that in their experiment, not all of the 2'5' ADP, which has a K_i about 20 times lower than that of 2'AMP for the inhibition of ethylmorphine N-demethylation (Table 4), was converted to 2'AMP in 20 min and that "other inhibitors" is in fact 2'5' ADP.

In the presence of ethylmorphine, the rate of TPNH oxidation was only about 8% faster when 5'AMP was added than when it was omitted (Condition 5 vs. Condition 6). Since ethylmorphine does not prevent the inhibition of TPNH oxidation by 2'5' ADP (Condition 8), this suggests that ethylmorphine largely prevents the formation of inhibitory nucleotides from TPNH. Because there is no rationale for the direct inhibition of nucleotide pyrophosphatase by ethylmorphine, another explanation is needed to account for the inhibition of nucleotide pyrophosphatase by ethylmorphine. The apparent K_m TPNH for TPNH oxidase (data not shown), monooxygenase (benzpyrene, 24; aminopyrine, 25) and TPNH-cytochrome c reductase (24) is 1 μM or less; that for nucleotide pyrophosphatase is about 150 μM (Table 3). A rate limitation of nucleotide pyrophosphatase activity imposed by the increased utilization of TPNH that occurs when ethylmorphine is being demethylated would best explain the effect.

Stoichiometry of TPNH utilization and HCHO formation by microsomes with different levels of nucleotide pyrophosphatase activity. For a period of several weeks, our rats yielded microsomes which contained only about one-third of the nucleotide pyrophosphatase activity usually observed. Activity was not increased by zinc. While this unexplained event interrupted our studies temporarily, it offered an opportunity for a more direct observation of the role of nucleotide pyrophosphatase activity on stoichiometry. Microsomes from these animals demethylated ethylmorphine at a rate of 6.8 nmole of HCHO formed/mg of protein/min and used TPNH at rates of 7.15, 7.10 or 7.10 nmole/mg of protein/min with the addition of 5'AMP (1 mM), the addition of EDTA (0.2 mM) or no additions, respectively. These data indicate that under certain conditions, the nucleotide pyrophosphatase content of microsomes may be low enough as not to interfere with stoichiometric relationships.

Phenobarbital induces demethylase activity without causing an increase in pyrophosphatase activity (10). This enables the determination of TPNH:HCHO ratios in the presence of varying levels of pyrophosphatase activity. Rats given phenobarbital for 0, 2 or 4 days yielded microsomes with pyrophosphatase activities of 17.3, 9.1 and 3.02 nmole NMNH formed/min/mg of protein, respectively, in one experiment, and corresponding values of 14.4, 12.3 and 5.25 in a second experiment. Fig. 2 shows the direct relationship between TPNH:HCHO ratios and nucleotide pyrophosphatase activity.

Effect of substrate on microsomal hydrogen peroxide generation. TPNH is utilized in the generation of hydrogen peroxide by cytochrome P-450 (8). If the generation of hydrogen peroxide and the demethylation reactions are linked to the same system, the possibility exists that hydrogen peroxide may be formed at the expense of monoox-

ygenase activity. While the stoichiometry of demethylation reactions considered in the current study indicate that this is not the case, measurement of the effect of the substrates on microsomal peroxide generation was undertaken to provide direct evidence for this conclusion. In Table 6 it can be seen that during the time that the oxidation of TPNH was measured (within 2 min after initiation of the reaction), neither ethylmorphine, aminopyrine, benzphetamine nor codeine had a statistically significant ($p < 0.05$) effect on the rate of generation of hydrogen peroxide. This was the case regardless of whether or not EDTA and cyanide were present. Rates were lower in the presence of EDTA and cyanide; this degree of inhibition is sometimes seen with 0.5 mM cyanide.

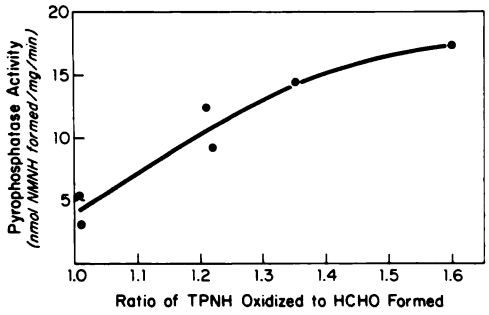

FIG. 2. *Effect of nucleotide pyrophosphatase concentration on the ratio of the rate of TPNH oxidation to HCHO formation during the microsomal N-demethylation of ethylmorphine*

Hepatic microsomes from male rats that had received phenobarbital for 0, 2, or 4 days were incubated with 0.1 mM TPNH as described in MATERIALS AND METHODS except that EDTA and cyanide were omitted. The rate of TPNH oxidation in the presence and absence of ethylmorphine was measured and the difference was compared to the HCHO formed in the presence of ethylmorphine. Nucleotide pyrophosphatase activity was measured as described in MATERIALS AND METHODS. Data represent results obtained from individual rats.

Hydrogen peroxide generation by microsomes from control or phenobarbital-treated animals, measured by the method of Gillette *et al.* (18), was linear throughout a 10 min incubation period and was not affected by the presence of ethylmorphine (data not shown). When the method of Hildebrandt and Roots (19) was used, no effect of ethylmorphine on H_2O_2 production was observed, but there was a severe digression of apparent H_2O_2 production from linearity after 3 min of incubation. When the method of Gillette and associates is used, H_2O_2 does not accumulate during incubation because added catalase rapidly converts it to an amount of HCHO equivalent to the H_2O_2 formed. The method of Hildebrandt and Roots measures the H_2O_2 that accumulates during the incubation period (contaminating catalase is inhibited by azide). The apparent decline in the rate of H_2O_2 generation with time, observed when the method of Hildebrandt and Roots is

TABLE 6

Effect of microsomal N-demethylation on microsomal hydrogen peroxide generation

Microsomes (1 mg protein/ml) were incubated (37°) with a medium (7.5 ml) containing 60 mM phosphate buffer (pH 7.4), 0.154 M KCl, 2 mM $MgCl_2$, 7.5 mM semicarbazide and 0.1 mM TPNH. When H_2O_2 alone was determined (HCHO formed from H_2O_2), catalase (60 units/ml) and methanol (100 mM) were added to the medium. When demethylase activity alone was measured, substrate was added to the medium in the indicated concentrations and catalase and methanol were not added. When both H_2O_2 formation and demethylase activity were measured (HCHO formed from both H_2O_2 and substrate) catalase, methanol and substrate were added to the medium. Samples were taken for HCHO analysis after 0 (blank) and 2 min of incubation.

Source of HCHO	EDTA (0.2 mM) + CN^- (0.5 mM)	HCHO Formation				
		No Substrate	Ethylmorphine (2 mM)	Aminopyrine (5 mM)	Benzphetamine (2 mM)	Codeine (5 mM)
Substrate (1)	−	−	5.44 ± 0.3	10.30 ± 0.7	7.52 ± 0.9	6.64 ± 0.4
	+	−	4.86 ± 0.5	8.51 ± 0.5	6.60 ± 0.5	5.82 ± 0.5
Substrate + H_2O_2 (2)	−	10.65 ± 0.7	14.85 ± 1.0	22.62 ± 0.8	18.97 ± 0.7	16.61 ± 1.2
	+	8.24 ± 1.1	12.56 ± 1.2	16.66 ± 0.5	15.21 ± 0.8	12.67 ± 1.5
H_2O_2 (2-1)[a]	−	10.65 ± 0.7	9.32 ± 1.0	12.31 ± 1.0	11.14 ± 1.0	9.95 ± 1.2
	+	8.24 ± 1.1	8.00 ± 1.0	8.15 ± 0.5	8.58 ± 1.0	6.85 ± 1.0

[a] By subtraction of individual experimental results.

used, is probably due to destruction of H_2O_2, possibly by cytochrome P-450 (26), as significant amounts of H_2O_2 accumulate in the medium.

DISCUSSION

The current studies implicate the presence of nucleotide pyrophosphatase in microsomes as the major cause of the failure to obtain a 1:1 relationship of TPNH utilization and product formation when cytochrome P-450 linked N-demethylase systems have been studied. The following evidence supports this conclusion:

1. TPNH:HCHO stoichiometry predictive of a monooxygenase reaction is achieved in the presence of EDTA, an inhibitor of nucleotide pyrophosphatase (10 and Table 1).

2. TPNH:O_2 stoichiometry predictive of a monooxygenase reaction is achieved in the presence of 5′AMP, an inhibitor of pyrophosphatase (27).

3. A 1:1 stoichiometry of TPNH oxidation and product formation is achieved in the absence of nucleotide pyrophosphatase inhibitors when the ratio of monooxygenase activity to nucleotide pyrophosphatase activity is high, as is the case in hepatic microsomes from rats treated with phenobarbital (Fig. 2) and in certain rats. A direct relationship between TPNH:HCHO ratios and nucleotide pyrophosphatase activity exists (Fig. 2).

4. 2′5′ ADP, a product of the hydrolysis of TPNH by nucleotide pyrophosphatase, inhibits TPNH oxidase. The inhibitory effect on TPNH oxidase of an amount of 2′5′ ADP produced by microsomes in which nucleotide pyrophosphatase had not been inhibited by 5′AMP was about equal to the inhibition produced by approximately the same amount of 2′5′ ADP added to microsomes to which 5′AMP had been added (Table 5).

The distortion of stoichiometry observed when inhibitors of nucleotide pyrophosphatase are not employed is caused by a substrate-induced increase in endogenous TPNH oxidase activity. This increase is due to a substrate-induced amelioration of the formation of 2′5′ ADP, which inhibits TPNH oxidase (Table 5). This occurs only because the apparent K_m TPNH for nucleotide pyrophosphatase is much higher than that for the oxidase reactions. The initial concentration of TPNH in the medium used for the determination of the rate of TPNH oxidation was 100 μM. The apparent Michaelis constant for the hydrolysis of TPNH by nucleotide pyrophosphatase is 150 μM; thus TPNH is rate-limiting for the nucleotide pyrophosphatase reaction at 0 time and the degree of rate limitation increases rapidly as TPNH is oxidized. As the oxidation of TPNH proceeds, an increasingly smaller amount of 2′5′ ADP is generated. When substrate is present, the rate of loss of TPNH via oxidation is increased, the degree of rate limitation of TPNH for the nucleotide pyrophosphatase reaction is enhanced, and less 2′5′ ADP is formed. Because less 2′5′ ADP is formed in the presence of substrate than in its absence, there is less inhibition of TPNH oxidase in the presence of substrate than in its absence. This 2′5′ ADP-induced disparity in the amount of TPNH oxidation that occurs in the presence and absence of substrate causes a false (higher) assessment of the increase in TPNH oxidation due to N-demethylation when the value for TPNH oxidation in the absence of substrate is substracted from that obtained in the presence of substrate. The very low apparent Michaelis constant of about 1 μM for TPNH oxidation (24, 25) insures that TPNH is never rate-limiting during the period when measurements of TPNH oxidation are made.

This explanation for the role of nucleotide pyrophosphatase in the distortion of stoichiometric relationships is even more plausible when one considers the possibility that the systems involved in the endogenous oxidation of TPNH do not compete with the cytochrome P-450-linked monooxygenase systems involved in the oxidation of exogenous substrates. In a recent study (28), we observed that when linoleic acid hydroperoxide is added to microsomes, as much as 75% of the cytochrome P-450 and more than 75% of the ethylmorphine N-demethylase activity was destroyed, but that TPNH oxidase activity was not affected. This suggests that the same systems

are not involved in the oxidation of endogenous and exogenous substrates and that any effect of exogenous substrate on TPNH oxidase activity would have to be indirect. The alteration of endogenous TPNH oxidation by a substrate-induced rate limitation of an inhibitor produced by nucleotide pyrophosphatase is an example of such an indirect effect. The failure of the substrates used in the current study to alter microsomal hydrogen peroxide generation suggests that the same cytochrome P-450-linked system is not involved in hydrogen peroxide production as that involved in the N-demethylation of these substrates.

Our observation of a 1:1 stoichiometric relationship between TPNH utilization and product formation when aminopyrine, benzphetamine, codeine and ethylmorphine were oxidized by hepatic microsomes shows that the uncoupling phenomenon described by Staudt *et al.* (6) is not a feature of the N-demethylation of these drugs. These investigators studied the stoichiometric relationship between product formation, TPNH utilization and oxygen utilization using hepatic microsomes from phenobarbital-treated rats with cyclohexane, n-hexane and perfluoro-n-hexane as substrates. With cyclohexane, the stoichiometry for product formed:TPNH used:O_2 used was 1:1:1. With perfluoro-n-hexane, a relationship of 0:2:1 was observed. The stoichiometry of n-hexane suggested that partial uncoupling had occurred. These studies have been interpreted to mean that uncoupling may occur in various degrees with most substrates for the hepatic monooxygenase system and that this would explain the well-known synergistic effect of DPNH on TPNH-supported reactions. According to this concept, active oxygen which is not used for monooxygenation is reduced to water by the DPNH-cytochrome b_5 system and this sparing effect accounts for DPNH synergism (6). Because NADH synergism occurs when aminopyrine, benzphetamine, codeine and ethylmorphine are substrates (29), and because the current study shows that appreciable uncoupling cannot occur with these substrates, we conclude that the explanation for DPNH synergism given by Staudt and associates cannot apply to monooxygenase reactions involving these substrates.

When nucleotide pyrophosphase was inhibited by EDTA, rates of N-demethylase activity were very similar when measured by the determination of HCHO formed or by disappearance of absorbance at 340 nm (Table 1). Comparable results were obtained with microsomes from several animal species, different strains of rat, rats of both sexes, and rats which had been induced with phenobarbital or 3-methylcholanthrene. The assay of the activities of hepatic cytochrome P-450-linked monooxygenase systems which measure the disappearance of absorbance at 340 nm offers certain advantages over assays which measure product formation. 1) The assay is dynamic; this may have advantages in certain studies. 2) Measurement of product often requires innovative methodology, which may also be tedious. 3) Assays of drug metabolism which must rely on substrate disappearance are frequently lacking in sensitivity and reliability because the substrate disappears in accordance with first order kinetics and a large amount of substrate must disappear before a significant loss can be measured. The spectrophotometric method measures the disappearance of TPNH, but the oxidation of TPNH proceeds in accordance with zero order kinetics throughout the period of measurement even though a large percentage of the TPNH has been oxidized. Because of the very low K_m TPNH, a large percentage of the TPNH can be measured without it becoming rate-limiting. 4) The method measures all of the TPNH-dependent reactions that may be involved simultaneously in the oxidation of the substrate. This was illustrated when the dealkylation of ethylmorphine was studied using microsomes from both male and female rats. An apparent sex-related discrepancy in the stoichiometry of ethylmorphine N-demethylation, which did not exist when codeine was the substrate, was explained when it was discovered that O-dealkylation is a relatively more prevalent reaction in the dealkylation of ethylmorphine and codeine when microsomes from female rats are used than when the source of microsomes is the male rat.

Thus the apparent discrepancy not only revealed the sex difference in relative O- and N-dealkylase activities in the rat, but also tested the validity of the method.

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