MOLECULAR PHARMACOLOGY, 10, 1004–1008

Discrepancy in the Measurement of Reduced Triphosphopyridine Nucleotide Oxidized during Ethylmorphine N-Demethylation Due to the Presence of a Nucleotide Pyrophosphatase

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(Received August 26, 1974)

SUMMARY

JEFFERY, ELIZABETH, AND MANNERING, GILBERT J.: Discrepancy in the measurement of reduced triphosphopyridine nucleotide oxidized during ethylmorphine *N*-demethylation due to the presence of a nucleotide pyrophosphatase. *Mol. Pharmacol.* **10**, 1004–1008 (1974).

The failure to establish the required 1:1 stoichiometry for TPNH utilization and product formation for the hepatic drug-metabolizing system involving cytochrome P-450 has been attributed to TPNH utilization by endogenous substrates. Attempts to achieve stoichiometry by subtracting endogenous TPNH utilization from TPNH utilization in the presence of substrate have not proved satisfactory. When the disappearance of TPNH was monitored both enzymatically and by observing the decline in spectral absorbance at 340 nm in the presence and absence of the substrate, ethylmorphine, values were identical initially, but became increasingly divergent with incubation time. Less of the apparent TPNH was present when the enzymatic procedure was used. The high values obtained by the spectrophotometric method were shown to be due to reduced nicotinamide mononucleotide, which also gives a maximum absorption peak at 340 nm. NMNH is formed through the action of microsomal nucleotide pyrophosphatase. Moreover, ethylmorphine inhibits NMNH formation by competing for TPNH for its *N*-demethylation. A 1:1 stoichiometry of TPNH utilization to formaldehyde formation was possible when calculations were corrected to include rates of NMNH formation. EDTA (0.2 mM) inhibited NMNH formation and thereby enabled the calculation of a 1:1 stoichiometric relationship. ATP and 5′-AMP also inhibited nucleotide pyrophosphatase activity.

The cytochrome P-450-containing, drug-metabolizing system has been classified as a mixed-function oxidase, which by definition (1) means that the system must utilize equivalent amounts of substrate, oxygen, and TPNH (a 2-electron donor). Although this stoichiometry has been demonstrated for C_{21} hydroxylation of 17-hydroxyprogesterone by bovine adrenal microsomes (2) and for the *N*-demethylation of aminopyrine by hepatic microsomes from phenobarbital-treated rats (3), the stoichiometry of demethylation by hepatic microsomes from untreated rats is still unclear.

Cohen and Estabrook (4) found a stoichiometry of 2 TPNH:1 HCHO. Stripp *et al.* (5) suggested that an accurate measure of

This research was supported by USPH Grant GM 15477 from the United States Public Health Service.

TPNH utilized during drug metabolism might be obtained by subtracting the rate of oxidation in the absence of drug substrate from the rate in the presence of substrate (net TPNH utilized). Best results were obtained when basal rates were determined in an atmosphere of carbon monoxide, but a stoichiometry of unity was not obtained. In later studies (6), which employed phenobarbital-treated rats and 1 mM EDTA in the incubation medium, this formula gave results approximating unity. Rates of TPNH oxidation published by Cohen and Estabrook (4), as measured by the enzymatic estimation of TPNH (7), were much more rapid than those given by Stripp *et al.* (5), as measured by the disappearance of absorbance at 340 nm during metabolism (8). In the current study this difference between the two results is confirmed and explained. The explanation permits a corrected stoichiometry of unity for net TPNH utilized to formaldehyde formed.

Male Holtzman rats (200–300 g) were used to prepare hepatic microsomes daily in 1.15% KCl (9). Incubation mixtures (3 ml in 1.15% KCl) contained 3 mg of protein, 900 μmoles of phosphate buffer (pH 7.4), 67.5 μmoles of semicarbazide hydrochloride, 18 μmoles of magnesium chloride, and approximately 1 μmole of TPNH. The incubation medium was monitored continuously at 340 nm in an Aminco DW-2 spectrophotometer at 37°. Two-milliliter samples were removed into 0.5 ml of 5 N alcoholic KOH for TPNH estimation by the method of Estabrook and Maitre (7) or into 0.8 ml of 0.5% $ZnSO_4$ for formaldehyde estimation by the method of Nash (10).

Ethylmorphine, at the 2 mM concentration normally employed in this laboratory, inhibits lipid peroxidation to 15% of the value seen in the absence of the drug. Since lipid peroxidation is TPNH-dependent (11), it was decided that if the TPNH required for ethylmorphine demethylation was to be estimated as net TPNH utilized, lipid peroxidation should be allowed to occur at the same rate in experiments both with and without drug substrate present. Lowering the concentration of ethylmorphine to 1 mM decreased inhibition of lipid peroxidation to 11% without lowering rates of TPNH oxidation and formaldehyde production. Lipid peroxidation in both the presence and absence of 1 mM ethylmorphine was inhibited completely by 60 μM EDTA, but rates of TPNH oxidation and formaldehyde production were not altered. However, at a concentration of 200 μM, EDTA lowered TPNH oxidation but did not affect HCHO formation, thus resulting in a ratio of net TPNH utilized to formaldehyde formed of unity. At higher concentrations EDTA decreased both net TPNH utilization and ethylmorphine demethylation (Table 1). It thus becomes apparent that the effect of EDTA on the stoichiometry of demethylation is due to its effect on some mechanism other than lipid peroxidation. When TPNH was monitored by measuring the decrease in absorbance at 340 nm during incubation, it was

TABLE 1

Effects of EDTA on TPNH utilization, ethylmorphine N-demethylation, and lipid peroxidation by hepatic microsomes

Lipid peroxidation was estimated by the thiobarbituric acid method (12). Results are expressed as a percentage of the absorbance at 535 nm obtained when neither EDTA nor ethylmorphine was present in the incubation medium. Incubations were carried out in a shaking water bath at 37°. The incubation mixture was the same as described in the text, except that it contained 300 rather than 100 nmoles of TPNH, and the reaction was stopped after 10 min. TPNH was measured by the method of Estabrook and Maitre (7).

EDTA concentration	Net TPNH utilized	Formaldehyde formed	Lipid peroxidation
μM	*nmoles*	*nmoles*	
0[a]	70	31	15
0	71	32	89
20	70	30	55
40		31	52
60		31	4
80		30	1
100	32	30	0
200	32	31	
400	28	23	
1000	24	9	
5000	11	2	

[a] The ethylmorphine concentration was 2 mM; a concentration of 1 mM was used in all other incubation flasks.

TABLE 2

Comparison of spectrophotometric and enzymatic estimations of TPNH utilization by hepatic microsomes

Incubations were carried out in the spectrophotometer at 37° as described in the text. Samples were removed at 20, 40, 60, 120, 180, and 300 sec for TPNH estimation and at 120 sec for formaldehyde estimation.

Additions	TPNH present at zero time		TPNH utilization		Formaldehyde formation
	S[a]	E[b]	S	E	
	nmoles/ml		*nmoles/min/mg protein*		
A. None	98	97	11.5	31	
B. Ethylmorphine (1 mM)	98	98	17.0	45	2.8
B minus A			5.5	14	
C. EDTA (0.2 mM)	96	96	15.2	35	
D. Ethylmorphine (1 mM) + EDTA (0.2 mM)	95	96	18.0	38	2.8
D minus C			2.8	3	

[a] Spectrophotometric method.

[b] Enzymatic estimation.

noticed that the disappearance was far more gradual than that measured enzymatically by the method of Estabrook and Maitre (7), although zero-time samples gave the same value by both methods. Values obtained by the two methods are compared in Table 2. It was also observed that if incubations were allowed to continue until there was no further change in absorbance at 340 nm, there was still considerable absorption at 340 nm, although by enzymatic estimation there was no TPNH remaining in the medium. This raised the question whether the residual absorption at 340 nm represented TPNH or some product of TPNH metabolism.

Nicotinamide, formed by the cleavage of TPN^+ by microsomal DPNase (13), is not the interfering product because it does not absorb at 340 nm. Nicotinamide had no effect on the residual absorption at 340 nm. Reduced nicotinamide mononucleotide seemed a more likely candidate. Jacobson and Kaplan (14) showed the presence of a nucleotide pyrophosphatase (EC 3.6.1.9) in rat liver microsomal preparations. This enzyme splits nicotinamide dinucleotides as follows:

$$TPNH \rightarrow 2',5'\text{-AMP} + NMNH$$
$$DPNH \rightarrow 5'\text{-AMP} + NMNH$$

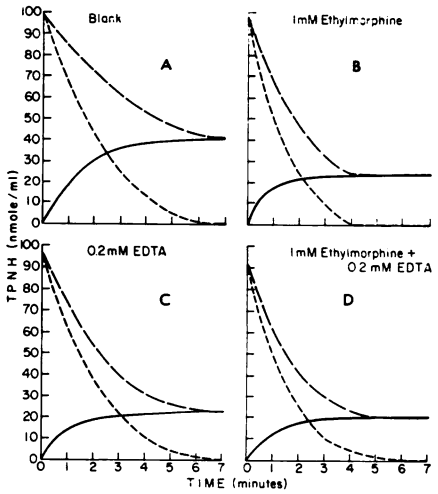

FIG. 1. *TPNH utilization monitored by disappearance of absorption at 340 nm* (- - -) *and by enzymatic estimation* (-----)

The difference between these two measurements is plotted as the appearance of NMNH (——), a product of TPNH metabolism with an absorption maximum identical with that of TPNH (340 nm).

Spectrophotometrically this activity would not be noticed, since 2',5'-AMP absorbs at 260 nm and NMNH absorbs at 340 nm, forming a combined spectrum identical with that of the parent compound. Sasame and Gillette (15) identified nico-

TABLE 3

Effect of EDTA on stoichiometry of TPNH utilization: formaldehyde formation during ethylmorphine N-demethylation by hepatic microsomes

TPNH was measured by the disappearance of absorption at 340 nm.

EDTA (0.2 mM)	TPNH utilization			Formaldehyde formed	TPNH:HCHO
	Incubation with no EM[a]	Incubation with EM (1 mM)	Net	*nmoles/min/mg protein*	
	nmoles/min/mg protein				
−	10.1	15.8	5.7	3.0	1.9
−	7.4	15.4	8.0	4.2	1.9
−	9.6	15.7	6.1	2.9	2.1
+	12.3	15.4	3.1	3.0	1.03
+	8.5	12.8	4.3	4.2	1.02
+	15.2	18.6	3.4	2.9	1.03

[a] EM, ethylmorphine.

tinamide ribonucleoside (dephosphorylated NMNH) as a product of microsomal TPNH pyrophosphatase activity in rat liver. They also deduced, from phosphate determinations of the products of TPNH metabolism, that whereas the 5′-phosphate was cleaved from 2′,5′-AMP completely during metabolism, only 13% of the 2′-phosphate was hydrolyzed. However, they did not discuss the possibility that pyrophosphatase might interfere with the measurement of TPNH oxidation during drug metabolism. Alkaline phosphatase hydrolyzes 2′-AMP to adenosine (16). Tentative identification of 2′,5′-AMP was made by deaminating the adenosine moiety to inosine. Kalckar (17) has shown that when adenosine is deaminated, the spectral maximum shifts from 260 to 245 nm. Thus, by exposing 2′,5′-AMP to alkaline phosphatase (EC 3.1.3.1) and adenosine deaminase (EC 3.5.4.4), the spectral shift might be used to distinguish 2′,5′-AMP from TPN^+ and TPNH, which, although showing spectral peaks at 260 nm, cannot be deaminated by this system. Samples were incubated with alkaline phosphatase at pH 9.2, and no spectral shift was obtained. The same samples were then incubated with adenosine deaminase. TPNH and DPNH standards, or samples from zero-time incubations, exhibited no spectral shift from 260 nm to 245 nm, but a small peak at 245 nm was seen in samples after incubation. In comparison, samples that had been incubated in the presence of EDTA showed greatly reduced 245 nm peaks. Interference due to absorbance of TPN^+ and TPNH at 260 nm was too great to allow quantitative measurement of 2′,5′-AMP in these preliminary studies, but further work is planned along these lines.

Having established the presence of pyrophosphatase activity, we estimated NMNH formation by subtracting the enzymatically estimated amount of TPNH (in nanomoles) from $A_{340} \times 1000/6.22$ (TPNH + NMNH)[1] for the same sample. From the results shown in Fig. 1A and B, it can be seen that the initial rates of NMNH formation (15 nmoles/min/mg) were the same in the presence and absence of ethylmorphine. However, because of increased oxidation of TPNH by the mixed-function oxidase system in the presence of ethylmorphine (Fig. 1B), the rate of NMNH formation fell almost to zero by 2 min. EDTA (0.2 mM) inhibited the rate of NMNH formation by 40% (Fig. 1C and D), but NMNH formation was unaltered by the addition of ethylmorphine. The ratio of net TPNH utilized to formaldehyde formed after 2 min was 2:1 in the absence of EDTA and 1:1 in the presence of EDTA (Table 3). The figures for TPNH oxidation by both methods (Table 2) again show that a ratio of approximately 1:1 occurs in the presence of EDTA,

[1] E_{TPNH} at 340 nm = 6.22 mM^{-1} cm^{-1}.

whereas in its absence the discrepancy in stoichiometry can be explained entirely by NMNH formation. Thus it would seem that EDTA alters the stoichiometry of net TPNH to formaldehyde produced, by inhibiting nucleotide pyrophosphatase activity to the extent that competition by this enzyme for TPNH substrate is minimal, but more important for the purpose of calculating stoichiometry in the presence of EDTA, pyrophosphatase activity is no longer altered by ethylmorphine. Buening and Franklin (18) also found that pyrophosphatase activity interferes with the measurement of TPNH oxidation during drug metabolism.

Prior treatment of animals with phenobarbital causes induction of the mixed-function oxidase system. We have observed that this agent does not induce microsomal nucleotide pyrophosphatase activity, as determined by NMNH formation measured spectrally. The ratio of mixed-function oxidase activity to pyrophosphatase activity is so greatly increased by this inducing agent that the formation of NMNH does not become an important factor influencing the ratio of TPNH utilization to formaldehyde formation. Thus EDTA had no effect on the rates of TPNH oxidation or net TPNH utilized during ethylmorphine demethylation in microsomes from rats treated with phenobarbital. This would explain why Orrenius (3) and Sasame *et al.* (6) observed a stoichiometry of 1:1 for aminopyrine and ethylmorphine demethylation using microsomes from animals treated with phenobarbital. Sodium fluoride and ATP, known pyrophosphatase inhibitors, had effects similar to EDTA. 5′-AMP (0.5 mM) almost completely prevented NMNH accumulation. With 2 mM 5′-AMP in the medium, Buening and Franklin (18) obtained stoichiometry between TPNH and oxygen utilized during drug metabolism. In the absence of 5′-AMP they found that pyrophosphatase activity interferes with these results and stoichiometry is no longer obtained.

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