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PYRIDINE NUCLEOTIDE INVOLVEMENT IN RAT HEPATIC MICROSOMAL DRUG METABOLISM—I. FACTORS THAT INFLUENCE NADPH KINETIC ESTIMATIONS DURING MIXED FUNCTION OXIDASE REACTIONS

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Abstract—The localisation of significant amounts of nucleotide pyrophosphatase activity in the rat hepatic microsomal fraction results in erroneous values of apparent K_m (NADPH) for aminopyrine-*N*-demethylase. This was demonstrated by the inclusion of 20 mM pyrophosphate, which inhibits nucleotide pyrophosphatase activity and reduces the apparent K_m (NADPH) value from 27.9 μM to 7.92 μM. The apparent K_m (NADPH) values determined in the presence of three Type I substrates were not statistically different from each other, but the value in the presence of aniline was lower. The kinetic constants of NADPH for NADPH cytochrome P450-reductase in the presence of either aminopyrine, ethylmorphine or aniline, are also reported.

The hepatic microsomal suspensions used to study the *in vitro* metabolism of drugs and other xenobiotics have a dependence on both molecular oxygen and NADPH [1, 2]. While this dependence on NADPH is not absolutely specific, the substitution of NADH for NADPH as the sole electron source reduces the rate of product formation by approximately 90 per cent [3, 4].

Apart from the mixed function oxidase (MFO) system, hepatic microsomal suspensions also contain enzymes that catalyse other reactions utilizing reduced pyridine nucleotides. These reactions include lipid peroxidation [5], fatty acid desaturation [6], *w*-hydroxylation of long chained fatty acids [7] and steroid hydroxylations [8], all of which potentially-compete for the available NADPH and thus influence the concentration of this nucleotide available for drug hydroxylations.

In addition to these, microsomal suspensions also contain NAD glycohydrolase and nucleotide pyrophosphatase enzymes which degrade pyridine nucleotides. It is not surprising therefore that, while there has been considerable interest in the kinetics of the MFO system for various drug substrates in the presence of saturating concentrations of NADPH, relatively little attention has been devoted to the kinetics of NADPH itself for this system. The present report therefore describes the influence and control of these potentially competing reactions and provides numerical estimates of the kinetic constants of NADPH for the various steps in drug hydroxylation reactions.

MATERIALS AND METHODS

Materials

Pyridine nucleotides (both oxidised and reduced), isocitrate (mono potassium salt) isocitrate dehydrogenase (in 50% glycerin), and cytochrome *c* were obtained from Calbiochem. Tetrasodium pyrophosphate (LR) was obtained from Ajax Chemicals, all other reagents were commercially available reagent grade and were used without further purification.

Methods

Animals. Male hooded Wistar rats weighing 250–300 g were housed under controlled conditions of light and temperature. Animals were allowed both food and water to the time of sacrifice, which was between 7.30–8.00 a.m. to minimise any diurnal variation in enzyme activity. [9].

Tissue Preparation. The animals were killed by cervical dislocation, their livers quickly excised and chilled in 0.25 M sucrose containing 2 mM Tris buffer pH 7.5 (Buffered Sucrose). All subsequent procedures were performed at 4°. The livers were weighed, perfused, minced and then homogenised with 3 ml of buffered sucrose per gram of liver, using a motor driven Potter homogeniser. The microsomal fraction was isolated from the homogenate essentially according to Cinti *et al.* [10], with the following modifications: The microsomes were washed with buffered sucrose, resedimented and finally resuspended in 1.15% KCl buffered with 10 mM sodium phosphate pH 7.5. The protein content was determined by the method of Lowry *et al.* [11], using crystalline bovine serum albumin as standard.

Drug Metabolism. The *in vitro* incubation system consisted of 100 mM Tris–Cl buffer (pH 7.5) 10 μmoles of isocitrate, 20 μmoles of magnesium chloride, 0.6 i.u. isocitrate dehydrogenase, 10 μmoles semi-carbazide HCl, NADPH and NADH, at the concentrations indicated in the text, and 2.5 mg of microsomal protein in a final volume of 2.5 ml. Type I substrates aminopyrine, ethylmorphine HCl or benzphetamine, were present at 4 mM, 6 mM or 4 mM respectively, while the Type II substrate, aniline,

was present at 4 mM and for this substrate semicarbazide was omitted.

Kinetic parameters were calculated from values obtained over concentrations ranging from approx. 0.4 K_m to 20–50 times K_m. Marbles were added to beakers to improve mixing [12]. The microsomal enzyme suspension and the incubation beakers containing the remaining ingredients were separately preincubated at 37° for 5 min prior to initiating the reaction by mixing. The formaldehyde formed after a 5 min incubation period in a metabolic shaker was measured by the method of Nash [13] as modified by Cochin and Axelrod [14], while the parahydroxylation of aniline was measured by the method of Kato and Gillette [15] as modified by Gram *et al.* [16].

NADPH cytochrome P450 reduction was initiated by the addition of 50 μl of an appropriate NADPH standard solution into 2.5 ml of reaction medium in anerobic cuvettes according to Stock and Fouts [17], with the following modifications: The bulk buffer medium, which contained 125 mM Tris–Cl buffer (pH 7.5), 25 mM pyrophosphate (pH 7.5), and 25 μmoles of magnesium chloride was bubbled with dithionite scrubbed nitrogen at 37° for 60 min. The microsomal suspension was resuspended in 1.15% KCl buffered with 10 mM sodium phosphate (pH 7.5) to a protein concentration of 15 mg/ml and then diluted to 3 mg/ml with the buffer medium. The concentration of Tris buffer, magnesium chloride and pyrophosphate in the final assay medium were identical to that used for metabolism experiments as were the individual substrates when incorporated into this assay. The assay was performed at 26° to improve its reproducibility [18].

A molar extinction coefficient of 91000 [19] was used to calculate the number of nmoles of cytochrome P450 reduced/min/mg microsomal protein. NADPH and NADH solutions for kinetic experiments were standardised spectrophotometrically at 340 nm, using a molar extinction coefficient of 6220 and were cell corrected. Oxidised pyridine nucleotide was first reduced using isocitrate and isocitrate dehydrogenase and then standardised as above.

Computational Methods. The data for each kinetic experiment was obtained from at least three determinations using eight substrate concentrations (in duplicate). The variance (VAR) and the coefficient of variation (CV) of the velocity readings at each substrate concentration was calculated. The kinetic constants (K_m, V) were computed using the digital computer programme HYPER, written by Cleland [20] and run in BASIC on a PDP 11/40 computer. This programme was modified to calculate a correlation coefficient, r, once convergence had occurred [21]. The velocity readings at each substrate concentration were weighted with the reciprocal of the variance (1/VAR) and then the reciprocal of the coefficient of variation (1/CV) at that substrate concentration.

Three criteria were used to evaluate the results of different weight factors in any particular kinetic experiment.

(1) that the CV of any parameter be not more than 20 per cent.

(2) that the data points be equally distributed about the computer generated line of best fit, especially in the area where the rate of change in slope of the tangents to the curve is greatest.

(3) that the correlation, r, be greater than 0.98.

If any one of the above criteria were not satisfied, the experiment was repeated.

RESULTS AND DISCUSSION

The influence of pyrophosphate on NADPH kinetics. The validity of kinetic estimates for the involvement of NADPH in drug metabolism using hepatic microsomal suspensions will always be questionable. Apart from the MFO system these suspensions also contain several enzymes which have the capacity to utilize or degrade NADPH, and thus the reliability of any kinetic estimation will depend on how effectively these potentially competing reactions can be controlled if not eliminated.

The limited number of reports that have appeared in the literature using rat hepatic tissue list K_m (NADPH) values of 25 μM [4] and 28 μM [22] for aminopyrine demethylase, 15 μM for aniline hydroxylase [23] and 10 μM for cyclohexane hydroxylation [24], but none of the above reports acknowledged the potential influence of these contaminating enzymes on their kinetic estimates.

A particularly serious contaminant in rat liver microsomes is nucleotide pyrophosphatase which splits both oxidised and reduced pyridine dinucleotides to their respective mononucleotides. The K_m (NADPH) of 27.9 μM obtained during the demethyl-

Table 1. K_m and V values for NADPH using aminopyrine, ethylmorphine, benzphetamine or aniline as substrates

	Aminopyrine		Ethylmorphine	Benzphetamine	Aniline
	− pyrophosphate	+ pyrophosphate	+ pyrophosphate	+ pyrophosphate	+ pyrophosphate
K_m*	27.9 ± 1.8‖	7.92 ± 0.82‖¶	8.49 ± 1.48	8.78 ± 1.75	5.86 ± 0.4¶
V†	59.95 ± 1.23	50.3 ± 1.48	33.8 ± 2.25	24.9 ± 1.5	7.16 ± 0.11
N‡	3	6	3	3	3
r§	0.99	0.985	0.989	0.98	0.997

* K_m expressed as μM: mean ± S.D.

† V expressed an nmoles HCHO formed/5 min/mg microsomal protein for aminopyrine, ethylmorphine, benzphetamine and nmoles *p*-aminophenol formed/15 min/mg microsomal protein for aniline: mean ± S.D.

‡ Number of experiments.

§ Correlation coefficient.

‖ Significantly different at $P < 0.005$.

¶ Significantly different at $P < 0.01$.

Wt. factor used in computations was 1/CV.

Table 2. K_m and V values of NADPH for NADPH cytochrome P450-reductase

	No substrate	Aminopyrine	Ethylmorphine	Aniline
K_m*§	10.93 ± 2.15	1.56 ± 0.28‖	2.5 ± 0.5‖	3.7 ± 0.71
V†§	3.7 ± 0.23	5.28 ± 0.13¶	8.92 ± 0.38¶	2.2 ± 0.1
r‡	0.980	0.991	0.98	0.982

* K_m expressed as μM: mean ± S.D.

† V expressed as nmoles cytochrome P450 reduced/min/mg microsomal protein: mean ± S.D.

‡ Correlation coefficient as defined in computational methods.

§ Values obtained in the presence of a substrate are significantly different from "No Substrate" values at $P < 0.005$.

‖ Significantly different at $P < 0.05$.

¶ Significantly different at $P < 0.05$.

The values assigned to the kinetic parameters were obtained from three duplicate independent determinations.

The final concentrations of aminopyrine, ethylmorphine and aniline in the reaction were 4 mM, 6 mM and 4 mM respectively.

ation of aminopyrine in the absence of pyrophosphate (Table 1) agrees with previously reported values for *N*-demethylation reactions [4, 22]. However, in the presence of 20 mM pyrophosphate, the K_m value was reduced to 7.92 μM, suggesting that pyrophosphate afforded significant protection to the NADPH present. There was no significant differences between the three Type I substrates in K_m (NADPH) values in the presence of pyrophosphate, but there was a significant difference between aniline and aminopyrine. 20 mM pyrophosphate reduced the K_m (NADPH) value for aniline hydroxylase by approximately 60 per cent from 15 μM [23] to 5.86 μM (Table 1). This reduction in K_m (NADPH) is of the same order as that obtained for aminopyrine demethylase (68 per cent reduction).

The K_m (NADPH) values for the three Type I substrates obtained in the presence of pyrophosphate are in good agreement with the value of 7.04 μM reported by Lu and West [25] using a resolved and reconstituted microsomal hydroxylating system which would be free of nucleotide pyrophosphatase activity. The above results suggest that the K_m (NADPH) values obtained with Type I substrates are independent of the substrate undergoing demethylation although the variation in V value reflects the different intrinsic rates of metabolism of these compounds.

Kinetics of NADPH for NADPH cytochrome P450-reductase. It is evident that various investigators consider different sites in the overall hydroxylation sequence to be rate limiting. At the present time, however, the bulk of the experimental evidence suggests that the rate of reduction of the cytochrome P450-substrate complex is the rate limiting step in untreated rats [26–28]. The kinetic constants of NADPH for NADPH cytochrome P450-reductase determined in the presence of various drug substrates are shown in Table 2.

The hyperbolic nature of the rate of reduction of the cytochrome P450-aminopyrine complex versus NADPH concentration curve would suggest the classical Michaelis–Menten equation can be used to describe this reaction (results not shown).

All three substrates lowered the apparent K_m from the value obtained in their absence. There was, however, a significant difference in the K_m values between the two Type I substrates, aminopyrine and ethylmorphine, which significantly increased the apparent V and the Type II substrate, aniline, which lowered the V value. This change in V, which has been reported previously [27], suggests that the substrate in the cytochrome P450-substrate complex influences the electron transfer from NADPH via NADPH cytochrome P450-reductase.

The other degradative enzyme, NAD glycohydrolase, which has previously been shown to cleave the oxidised [29] but not the reduced forms [30] of the pyridine nucleotides, appeared not to be a serious problem in our rat microsomal suspensions as the inclusion of 10 mM nicotinamide which inhibits NAD glycohydrolase activity, did not improve the stability of NADPH when in contact with microsomal preparations. This is in agreement with the results of Buening and Franklin [31].

Enzymes competing for the available NADPH include lipid peroxidase and fatty acid desaturase. The former, which increases with age of microsomal preparations [5], has been shown to be undetectable in calcium aggregated microsomes when incubated with aminopyrine and negligible with ethylmorphine [32]. In the present study where all microsomal preparations were used within 5 hr of killing the rats, malondialdehyde, the end product of microsomal lipid peroxidation, could not be detected after incubation in the complete assay medium used for demethylation reactions.

Fatty acid desaturase, unlike lipid peroxidation, can only be demonstrated in a substrate fortified incubation medium [23]. While the endogenous levels of fatty acyl CoA compounds in microsomal suspensions were not established, it is known that degradative hydrolase and transacylase enzymes are present and are thus in contact with the substrates required for desaturase activity for at least 4 hr. Under such conditions any fatty acyl CoA compounds present should be effectively removed thereby eliminating the drain of electrons from the pyridine nucleotides by this pathway.

Perhaps the most effective method of estimating the net effects of all these contamination enzymes on NADPH utilisation is to examine the stoichiometry between NADPH oxidised (2 electron donor), oxygen

consumed, and product formed by drug hydroxylation reactions. The desired stoichiometry of 1:1:1 has been shown for the C-21 hydroxylation of 17-hydroxyprogesterone by bovine adrenal microsomes [33], the aminopyrine N-demethylation by liver microsomes isolated for phenobarbital pretreated rats [4], the N-demethylation of benzphetamine by a solubilised and reconstituted microsomal enzyme system [34] and the hydroxylation of cyclohexane [35]. However, a stoichiometry of 2 NADPH oxidised–1 formaldehyde formed was obtained for Type I substrates using microsomes isolated from phenobarbital pretreated rabbits [36]. This finding has led Stripp *et al.* [37] and Sasame *et al.* [38] to propose various methods of estimating and correcting for endogenous NADPH oxidation in an attempt to re-establish stoichiometry, none of which is completely satisfactory.

Recently reports have indicated that a 1:1 stoichiometry exists between NADPH oxidised–oxygen consumed [31] and also between NADPH oxidised and product formed from ethylmorphine [39] when endogenous nucleotide pyrophosphatase activity is controlled. In view of this demonstrated stoichiometry, it is probable that these other reactions that can oxidise NADPH, utilize only a small amount of the available NADPH and that their individual and collective significances can be over-estimated in drug hydroxylations. Nevertheless, these other reactions must exert some influence and it is probable that the true K_m(NADPH) values will be slightly lower than those obtained in the present work.

However, the basic kinetic parameters reported in this present work represent the best estimates available at this time* and have been used as base lines for the following studies which examine the mechanism by which NADH influences NADPH mediated drug hydroxylations [40].

* Since the submission of this manuscript, an article has appeared in the literature by Jansson and Schenkman (*Archs Biochem. Biophys.* **178**, 89 (1977)), which reports a K_m(NADPH) value of 7 μM for aminopyrine-N-demethylase in the presence of 10 mM sodium pyrophosphate using microsomes isolated from untreated rats. This value is in excellent agreement with the value of 7.92 $\pm$ 0.82 μM in the presence of 20 mM pyrophosphate reported in this communication.

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