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PYRIDINE NUCLEOTIDE INVOLVEMENT IN RAT HEPATIC MICROSOMAL DRUG METABOLISM—II. EVIDENCE FOR A CO-OPERATIVE INTERACTION BETWEEN NADPH AND NADH

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Abstract—There was a significant reduction in apparent K_m (NADPH) values for both aminopyrine and ethylmorphine demethylases when the kinetic constants for NADPH were determined in the presence of constant NADH concentrations. NADH was also shown to significantly stimulate apparent V for NADPH cytochrome P450-reductase (in the presence of aminopyrine) without changing the apparent K_m (NADPH) value. Further, NADH stimulated the initial rapid phase of the biphasic reduction kinetics of NADPH cytochrome P450-reductase in the presence of aminopyrine. These findings suggest that in the presence of both pyridine nucleotides, there has been a change in the rate limiting step which, with NADPH alone, is generally accepted to be the reduction of the cytochrome P450-substrate complex. It has been necessary to make certain modifications to a previously proposed mechanism to explain the results obtained in the present study.

It is now well established that NADPH is a more efficient electron donor than NADH during drug hydroxylation reactions catalysed by the hepatic microsomal cytochrome P450-enzyme complex [1, 2]. However a synergistic increase in product formation in the presence of both reduced pyridine nucleotides has been reported [3, 4].

A model [5, 6] to explain the increased product formation in the presence of both NADPH and NADH suggested that the oxidised cytochrome P450-substrate complex is first reduced by an electron from NADPH which is transferred by NADPH cytochrome-*c*-reductase. The ferrous cytochrome P450-substrate complex so formed binds molecular oxygen and is further reduced by another electron which is supplied from either NADPH or NADH via cytochrome b_5. The synergistic increase in product formation is postulated to occur because NADH is able to maintain a higher steady-state level of reduced cytochrome b_5 than NADPH.

The previous report from this laboratory [7] provided numerical estimates of the kinetic constants of NADPH for the various intermediate steps involved in drug hydroxylation reactions.

The present report considers the influence of NADH on these kinetics in an attempt to further characterise the involvement of both NADPH and NADH in drug hydroxylation reactions.

MATERIALS AND METHODS

Materials

Pyridine nucleotides (both oxidised and reduced), isocitrate (mono-potassium salt) and isocitrate dehydrogenase (in 50% glycerin), 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 [8].

Tissue preparation. The animals were killed by cervical dislocation and their livers quickly excised and chilled in 0.25 M sucrose containing 2 mM Tris–Cl buffer (pH 7.5). The microsomal fraction was isolated as previously described [7], based on the method of Cinti *et al.* [9]. The protein content of the microsomal suspension was determined by the method of Lowry *et al.* [10] using crystalline bovine serum albumin as standard.

Drug metabolism. The composition of the incubation medium used for the metabolism of either aminopyrine or ethylmorphine was identical to that described previously [7]. The amount of formaldehyde liberated was estimated by the method of Nash [11] as modified by Cochin and Axelrod [12].

Kinetic parameters were evaluated from at least three determinations which employed eight substrate concentrations (in duplicate), ranging from 0.4 K_m to 20–50 times K_m. Marbles were added to beakers to improve mixing [13].

Enzyme Assays. NADPH cytochrome P450-reductase activity was measured as previously described [7] using a Gilford 2400S recording spectrophotometer. In experiments concerned with the biphasic reduction kinetics of cytochrome P450, (Fig. 3) the reduction was monitored until asymptotic (4 min). The amount of unreduced cytochrome P450-CO complex at time t, sec was calculated thus:

$$A_{450,\infty} - A_{450,t}$$

where $A_{450,\infty}$ represents the absorbance at 450 nm at infinite time (4 min) and $A_{450,t}$ represents the

absorbance at time, t, seconds. This reading at time, t, was divided by $A_{450,}$, to give per cent unreduced cytochrome P450-CO complex, which was plotted logarithmically as a function of time.

NADPH and NADH solutions for kinetic experiments were standardized spectrophotometrically at 340 nm, using a molar extinction coefficient of 6220 and were cell corrected. Oxidised pyridine nucleotides were first reduced using isocitrate and isocitrate dehydrogenase and then standardised as above.

Computational methods. 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 iterative digital computer programme HYPER, written by Cleland [14] and run in BASIC on a PDP 11/40. The velocity readings at each substrate concentration were weighted with the reciprocal of the variance (1/VAR) and the reciprocal of the coefficient of variation (1/CV) at that substrate concentration. A selection of the values of the kinetic constants of the above computations were based upon criteria previously established [7]. The unpaired Students 't' test was used to compare different experiments with a level of significance of at least $P < 0.05$. All K_m and V values in this report are apparent values determined under the conditions detailed above.

RESULTS AND DISCUSSION

Effect of NADH on NADPH kinetics during drug metabolism. The K_m and V values of NADPH for the mixed function oxidase system using either aminopyrine or ethylmorphine as substrate in the presence of three fixed NADH concentrations are shown in Table 1. The K_m (NADPH) value of 7.92 μM obtained in the absence of NADH (Table 1) [7] was reduced to 3.57 μM in the presences of 96.1 μM NADH with aminopyrine as substrate. A similar reduction from the control K_m (NADPH) value of 8.49 μM to 5.77 μM was obtained when ethylmorphine replaced aminopyrine as substrate. The reduction in K_m was also significant when the NADH concentration was reduced to either 19.2 or 12.0 μM.

In the presence of 96.1 μM NADH, there was a 5 and 41 per cent increase in apparent V value with aminopyrine and ethylmorphine respectively. It can be seen (Table 1) that V in the presence of either 19.2 or 12.0 μM NADH and with aminopyrine as substrate, was significantly lower than the V recorded in the absence of NADH. This contrasts with a lack of significant difference from control V found with these concentrations of NADH when ethylmorphine was the substrate.

The reduction in apparent K_m (NADPH) for both aminopyrine and ethylmorphine-*N*-demethylase in the presence of NADH could indicate one of two things. Either greater affinity of the mixed function oxidase for NADPH in the presence of NADH, or a change in the rate limiting step. The latter is generally accepted to be the reduction of the oxidised cytochrome P450-substrate complex by an electron from NADPH [15–19] via NADPH cytochrome-*c*-reductase [20, 21].

It has been suggested [22] that in multi-component systems, changes in K_m are more likely to represent changes in the rate limiting step than variation in enzyme substrate affinities. Such a proposal would therefore require that NADH stimulates NADPH cytochrome P450 reduction.

Effect of different NADH concentrations in the presence of a fixed NADPH concentration. Figure 1 shows the titration of aminopyrine and ethylmorphine demethylase as a function of NADH concentration in the presence of 104.7 μM NADPH. The curves are computer generated lines of best fit. The values obtained in the presence of NADPH alone were subtracted from those obtained in the presence of both pyridine nucleotides. These control values are 40 nmoles and 43.3 nmoles HCHO formed/5 min/mg microsomal protein for aminopyrine and ethylmorphine respectively. The sigmoidal nature of the curves with either aminopyrine or ethylmorphine suggest that NADH is exerting a homotropic co-operative effect. Sigmoidal binding kinetics have been reported for the binding of a drug substrate to rat liver cytochrome P450 [23]. This co-operative behaviour was interpreted to indicate a possible conformational change in cytochrome P450 when it complexes with the drug.

Effect of NADH on NADPH cytochrome P450-

Table 1. K_m and V values for NADPH in the presence of substrates and NADH

	Aminopyrine			Ethylmorphine		
NADH Conc., μM	96.1	19.2	12.0	96.1	19.2	12.0
K_m*	3.57 ± 0.12‖	3.31 ± 0.31‖	5.0 ± 0.86‖	5.77 ± 0.82¶	6.5 ± 0.92	5.7 ± 0.92¶
V†	52.7 ± 0.31	41.1 ± 0.63‖	36.0 ± 1.9‖	47.6 ± 1.7‖	36.7 ± 1.3	33.9 ± 1.3
r‡	0.999	0.991	0.982	0.986	0.99	0.986
N§	3	6	3	3	3	3

* K_m expressed as μmolar: mean ± S.D.
† V expressed as nmoles HCHO formed/5 min/mg microsomal protein: mean ± S.D.
‡ Correlation coefficient, as defined in the computational methods in [7].
§ Number of determinations.
‖ Significantly different from the value obtained in the absence of NADH at $P < 0.005$.
¶ Significantly different from the value obtained in the absence of NADH at $P < 0.05$.
The kinetic parameters obtained in the absence of NADH: (Table 1) [7].

Aminopyrine
K_m = 7.92 ± 0.82 μM.
V = 50.3 ± 1.48 nmoles HCHO formed/5 min/mg microsomal protein.

Ethylmorphine
K_m = 8.49 ± 1.48 μM.
V = 33.8 ± 2.25 nmoles HCHO formed/5 min/mg microsomal protein.

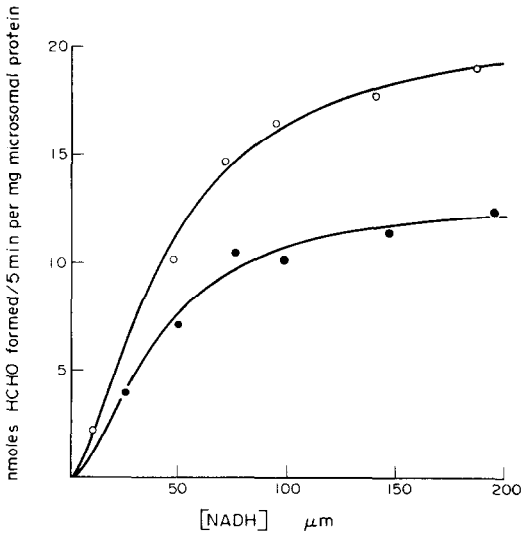

Fig. 1. The substrates, aminopyrine (●——●) and ethylmorphine (○——○) were included at a final concentration of 4 and 6 mM respectively. The activity obtained in the presence of NADPH alone was subtracted from all readings obtained in the presence of both reduced pyridine nucleotides. These control values were 40 nmoles and 43.3 nmoles HCHO formed/5 min/mg microsomal protein. The points are the mean values of three determinations while the lines are the computer generated lines of best fit by NONLIN using all the individual data points.

reductase kinetics. Figure 2 shows the titration of cytochrome P450-reductase as a function of NADPH concentration in the presence and absence of 119 μM NADH. The curves are the computer generated lines of best fit. Both reduced pyridine nucleotides were introduced simultaneously to initiate the reaction when the K_m for NADPH was measured in the presence of NADH and aminopyrine. NADPH cytochrome P450-reductase activity with NADH and either aminopyrine or ethylmorphine was approximately 10 per cent of that obtained with NADPH.

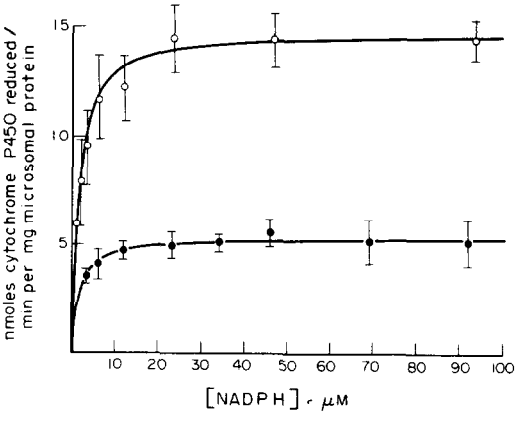

Fig. 2. The points are the means ±S.D. of three duplicate determinations, while the lines are the computer generated lines of best fit in the absence (●——●) and presence (○——○) of 119 μm NADH. The microsomal suspension used for each determination was obtained by pooling the livers of three control rats. NADPH cytochrome P450 reductase was assayed as described in the Methods in the presence of 4 mM aminopyrine. The velocity readings at each substrate concentration were weighted with I/CV obtained from the readings for that substrate concentration.

The V value increased from a control value of 5.28 to 14.6 nmoles cytochrome P450-reduced/min/mg microsomal protein in the presence of 119 μM NADH. There was, however, no significant change in K_m (NADPH) values. Both graphs are hyperbolic, suggesting that NADH exerts a heterotropic co-operative effect with respect to NADPH. The possibility that NADPH stimulates the NADH mediated reduction of cytochrome P450-substrate complex, although unlikely based on the low activity found with NADH alone, cannot be eliminated.

Similar results were obtained when the NADH concentration was reduced to 20.2 μM (Table 2). While there was no significant difference in V values

Table 2. K_m and V values of NADPH for NADPH cytochrome P450-reductase in the presence of NADH

	Aminopyrine		Ethylmorphine	
NADH Conc. ([91]) μM	119	20.2	98	20.4
Parameters				
K_m*	1.43 ± 0.23	1.9 ± 0.19	2.01 ± 0.22	1.54 ± 0.21§
V†‖	14.61 ± 0.32	14.53 ± 0.24	14.75 ± 0.49	12.43 ± 0.31
r‡	0.990	0.994	0.986	0.992

* K_m expressed a μmolar:mean ± S.D.

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

‡ Correlation coefficient.

§ Significantly different from the value obtained in the absence of NADH at $P < 0.05$.

‖ Significantly different from the value obtained in the absence of NADH at $P < 0.001$.

ᵉ Mean concentration: the coefficient at variation of NADH concentration was always < 4 per cent.

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

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

The kinetic parameters obtained in the absence of NADH (Table 2) [7].

Aminopyrine
$K_m = 1.56 \pm 0.28$.
$V = 5.28 \pm 0.13$ nmoles cytochrome P450 reduced/min/mg microsomal protein.

Ethylmorphine
$K_m = 2.5 \pm 0.5$.
$V = 8.92 \pm 0.38$ nmoles cytochrome P450 reduced/min/mg microsomal protein.

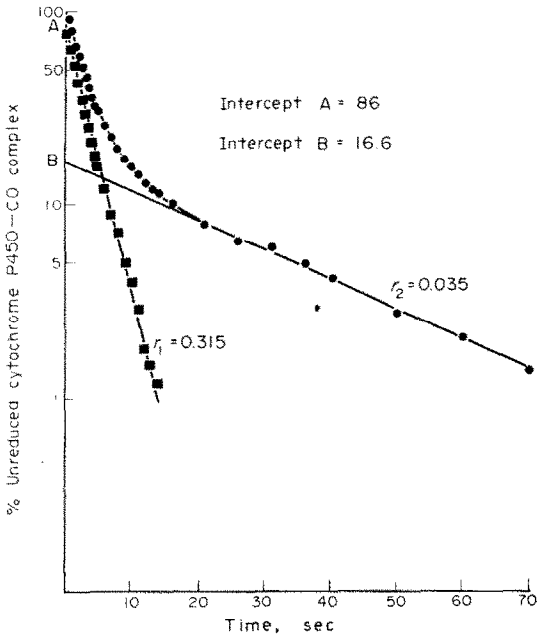

Fig. 3. The reduction of the cytochrome P450-aminopyrine complex by NADPH (107.4 μM) was measured as described in the Methods. The points (●) represent the amount of unreduced cytochrome P450-aminopyrine complex at a particular time, t. The straight line (■——■) was obtained by peeling the curve, i.e. at each time interval, t, the difference between the curve and the value at the same time of the straight line obtained by back projection of the terminal linear section of the curve is plotted as a function of time— r_1 and r_2 are the slopes of the straight lines. The graphical estimates of A, B, r_1 and r_2 were used as initial estimates in NONLIN for the data in Table 3.

obtained in the presence of the higher NADH concentration with either aminopyrine or ethylmorphine, there was a difference in the V values of NADPH cytochrome P450-reductase for these two substrates in the absence of NADH (Table 2) [7]. The marked increase in V value was still apparent when the NADH concentration was lowered to 20.4 μM in the presence of ethylmorphine. In the absence of added substrates NADPH cytochrome P450-reductase activity rose from 3.6 ± 0.2 (N = 3) without NADH to 6.6 ± 0.7 nmoles cytochrome P450-reduced/min/mg microsomal protein (N = 3) in the presence of 123 μM NADH.

Hildebrandt and Estabrook [6] have previously reported that NADH did not stimulate the NADPH mediated reduction of the cytochrome P450-ethylmorphine complex. However, the present work differs from theirs in two respects. They used microsomes isolated from phenobarbital pretreated rats and the incubation mixtures used by them contained 150 mM KCl to stimulate NADPH cytochrome P450-reductase activity to a level that it would no longer be rate limiting [24]. Work in this laboratory has shown that 150 mM KCl abolishes the NADH stimulation and also that the NADH stimulation does not occur in microsomes isolated from phenobarbital pretreated rats.*

* S. Penglis, G. K. Gourlay and B. H. Stock, manuscript in preparation.

Reduction kinetics of the cytochrome P450-aminopyrine complex by NADPH. A semi-logarithmic plot of the percent unreduced cytochrome P450-aminopyrine complex as a function of time is shown in Fig. 3. The reaction was initiated by the introduction of NADPH to give a final concentration of 107.3 μM. The graph consists of two phases and therefore can be expressed in mathematical form thus:

$$P = Ae^{-r_1 t} + Be^{-r_2 t} \quad (1)$$

where P represents the amount of unreduced cytochrome P_{450}-aminopyrine complex at time t, and A, r_1, B and r_2 are constants. The data obtained from three separate duplicate experiments were fitted to the above equation using NON-LIN [25].

Equation 1, which describes the reduction kinetics, was supplied in the sub-routine D FUNC. The data from each individual experiment was fitted to the above equation using a weight factor of 1. Then, at each time interval, t, the data from the three duplicate experiments was bulked and a mean and S.D. calculated. Weight factors of unity, reciprocal of the variance (1/VAR) and reciprocal of the coefficient of variation (1/CV) at each time interval were used to weight the bulked data for that time interval. The numerical values assigned to A, r_1, B and r_2 by NONLIN are shown in Table 3. No significant differences were found in the values of the constants using the various weight factors. The excellent fit of the data to equation 1 is shown by the small S.D. of all constants and the high value of the correlation coefficient, cor.

Reduction of the cytochrome P450-aminopyrine complex by NADPH in the presence of NADH. The reduction of the cytochrome P450-aminopyrine complex as a function of time when the reaction was initiated by the addition of NADPH and NADH was also biphasic and the data was therefore fitted to equation 1 as described above (Table 4). The final concentrations of NADPH and NADH were 107.3 μM and 101.1 μM respectively. The half life ($0.691/r_1$) of the initial rapid phase (r_1 of equation 1) determined in the presence of both reduced pyridine nucleotides (1.4 sec) was significantly lower than that obtained in the absence of NADH (1.7 sec, $P < 0.001$).

Original reports [16, 26, 27] on the *in vitro* reduction of the cytochrome P450-substrate complex from hepatic microsomes showed the system to exhibit biphasic kinetics in both the intact, and subsequently in the resolved and reconstituted system [28]. Gillette [29, 30] has proposed that the reduction of the cytochrome P450-substrate complex can give polyphasic kinetics under certain conditions. Nevertheless, individual determinations in the present work (Tables 3 and 4) show that, under the experimental conditions used, the reduction of the cytochrome P450-aminopyrine complex fits a biphasic model. The present work shows that when the reduction of the cytochrome P450-aminopyrine complex is initiated by the simultaneous addition of NADPH and NADH, the half lives of both phases are significantly lower than the values obtained with NADPH alone. However, the difference in half lives for the slow phase could be due to variations in residual oxygen concentration [29, 30].

Table 3. Numerical values of the constants of the biexponential equation when the reaction was initiated by NADPH alone

Experiment	Parameters*				
	A	r_1	B	r_2	cor†
1.	87.8 ± 1.2	0.39 ± 0.01	21.3 ± 1.05	0.043 ± 0.003	0.999
	80.1 ± 1.4	0.42 ± 0.01	22.7 ± 1.0	0.036 ± 0.002	1.000
2.	80.1 ± 1.26	0.47 ± 0.013	24.3 ± 0.6	0.023 ± 0.001	0.999
	73.0 ± 1.54	0.41 ± 0.016	25.0 ± 1.1	0.033 ± 0.002	0.999
3.	77.6 ± 1.05	0.36 ± 0.01	23.4 ± 0.8	0.031 ± 0.002	1.000
	79.8 ± 0.74	0.41 ± 0.01	22.1 ± 0.6	0.039 ± 0.001	1.000
Bulked	80.2 ± 1.17	0.41 ± 0.01	23.0 ± 0.8	0.033 ± 0.002	0.996

* Mean ± S.D.

† Correlation coefficient as defined in the computational methods [7].

The data was obtained from the reduction of cytochrome P450-aminopyrine-CO complex as a function of time when the reaction was initiated by NADPH (107.3 μM).

Aminopyrine was included in the reaction at a final concentration of 4 mM. The weight factor used for the computations was the reciprocal of the variance.

Recently, it has been reported [31, 32] that NADPH-cytochrome P450-reductase activity in microsomes isolated from rats that had been previously treated with phenobarbital also exhibited biphasic reduction kinetics which, it was suggested, was most probably composed of two concurrent first order reactions. This particular study utilised a dual wavelength stopped flow spectrophotometer to assay NADPH-cytochrome P450-reductase activity.

It may well be true that the absolute values of the rate constants determined with the more sophisticated stopped flow spectrophotometer are different from those determined with the more conventional spectrophotometric technique used in this study. Nevertheless, meaningful comparisons can still be made using the spectrophotometric technique between the results obtained when NADPH cytochrome P450-reductase activity was initiated by the addition of NADPH alone or NADPH and NADH together. The present study places emphasis on the changes in apparent V values or the change in slope of the initial rapid phase of the biphasic reduction kinetics rather than the absolute values of these parameters.

Steady state cytochrome b_5 involvement in demethylation reactions. The generally accepted model of microsomal electron transport during drug hydroxylation [5] was detailed in the Introduction to this report. This mechanism has been re-affirmed in a subsequent communication [6] which implicated cytochrome b_5 as the donor of the second electron from either NADPH or NADH. While there are unresolved questions regarding the exact mechanism of cytochrome b_5 involvement in electron transfer, [33–36] the bulk of the experimental evidence currently available supports its involvement in transferring electrons from NADH, but not NADPH.

Table 5 shows the steady state levels of reduced cytochrome b_5 during the metabolism of aminopyrine or ethylmorphine. The incubation medium for these experiments was identical to that used for *in vitro* demethylation reactions except for the final protein concentration was adjusted to 3 mg/ml. The microsomal suspension used for each determination was obtained by combining the microsomal fraction from three rats.

NADPH reduced approximately 0.25 nmole cytochrome b_5/mg microsomal protein in the presence of either aminopyrine or ethylmorphine, while the

Table 4. Numerical values of the constants of the biexponential equation when the reaction was initiated by the simultaneous addition of NADPH and NADH

Experiment	Parameters*				
	A	r_1	B	r_2	cor†
1.	70.2 ± 0.47	0.47 ± 0.004	26.7 ± 0.16	0.039 ± 0.001	1.000
	71.7 ± 1.07	0.489 ± 0.01	25.3 ± 0.36	0.039 ± 0.001	1.000
2.	62.8 ± 0.66	0.50 ± 0.006	27.5 ± 0.2	0.039 ± 0.001	0.998
	61.7 ± 0.66	0.49 ± 0.006	29.3 ± 0.27	0.037 ± 0.001	0.999
3.	71.2 ± 0.64	0.5 ± 0.006	24.2 ± 0.21	0.04 ± 0.001	0.999
	66.8 ± 0.74	0.47 ± 0.007	28.6 ± 0.25	0.037 ± 0.001	0.999
Bulked	67.5 ± 0.7	0.49 ± 0.006‡	26.9 ± 0.22	0.039 ± 0.001§	0.998

* Mean ± S.D.

† Correlation coefficient.

‡ Significantly different compared to r_1 (Table 3) at $P < 0.001$.

§ Significantly different compared to r_2 (Table 3) at $P < 0.01$.

The data was obtained from the reduction of the cytochrome P450-aminopyrine–CO complex as a function of time when the reaction was initiated by the simultaneous addition of NADPH (107.3 μM) and NADH (100.1 μM).

The data was weighted with the reciprocal of the coefficient variation. Aminopyrine was included in the reaction at a final concentration of 4 mM.

Table 5. Steady state b_5 levels during the metabolism of aminopyrine and ethylmorphine

Pyridine nucleotide	Conc. of NADH μM	Reduced cytochrome b_5 levels during metabolism (nmoles/mg microsomal protein)	
		Aminopyrine	Ethyl-morphine
NADPH 93.1 μM		0.261	0.234
NADPH + NADH	10.6	0.468	0.459
(93.1 μM)	21.2	0.477	0.459
	106.1	0.459	0.45
Levels of cytochrome	10.6	0.324	0.252
b_5 remaining after addition of NADH	21.2	0.342	0.27
(5 min at 37°)	106.1	0.414	0.432

The incubation medium was identical to that used in metabolism studies except that the protein concentration was 3 mg/ml. The cuvette contents were adjusted to 37° and a baseline established. The spectrum was re-recorded after NADPH (25 μl, final concentration 93.1 μM) was added to one cuvette and an equivalent volume of buffer to the reference cuvette. 25 μl of appropriate NADH solution was added to the test cuvette and the spectrum recorded immediately and after 5 min at 37°.

further addition of 10.6 μM NADH increased the steady state level of reduced cytochrome b_5 to 0.46 nmole/mg microsomal protein. However, after incubation at 37° for 5 min, the level of reduced cytochrome b_5 had almost returned to that obtained with NADPH alone. On the other hand, when 106.1 μM NADH was added to the 93.1 μM NADPH, almost complete reduction of cytochrome b_5 was maintained furing the 5 min incubation, in the presence of either drug substrate. The addition of 21.2 μM NADH following the NADPH gave a level of reduced cytochrome b_5 intermediate between these two values. There appears to be a constant relationship between the maximum velocity of *N*-demethylation (Table 1) and the steady state concentration of reduced cytochrome b_5 after the 5 min incubation at 37°. (Table 6). The concentration of reduced cytochrome b_5 is in turn apparently dependent on the concentration of added NADH.

Correia and Mannering [37, 38] have however proposed an alternative mechanism to explain NADH synergism which is based on an examination of the role of cytochrome b_5 in microsomal suspensions. These investigators proposed that NADH acts by providing electrons to the fatty acid desaturase system thereby sparing NADPH from this electron drain. While this proposition appears plausible with the saturating NADH concentrations used (1 mM), it would be difficult to accept that this mechanism would operate with 10 μM NADH which has been shown (Table 1) to reduce the apparent K_m (NADPH) value for aminopyrine or ethylmorphine-*N*-demethylase. Further, the stimulatory effects of NADH on the *V* value and the rate constant for the initial rapid phase of the biphasic reduction kinetics for NADPH cytochrome P450-reductase are inconsistent with their proposal.

Modified proposal for NADH interaction with cytochrome P450. The present results indicate that, in addition to its role in supplying the second electron NADH also interacts with, and induces a perturbation in, the cytochrome P450-substrate complex, allowing the electron from NADPH to be supplied at a much greater rate. This concept is supported by the following:

(a) The K_m value of both aminopyrine and ethyl-

Table 6. Ratio of *V* values for the demethylation of aminopyrine and ethylmorphine to the steady state concentration of reduced cytochrome b_5 in the presence of various NADH concentrations

Approximate NADH conc. μM	*V* value (Table 1)	Steady state cytochrome b_5 levels during metabolism (Table 5)	A/B Ratio
	A	B	
	Aminopyrine		
100	52.7	0.414	127
20	41.1	0.342	120
10	36.0	0.324	113
	Ethylmorphine		
100	47.6	0.432	111
20	36.7	0.27	136
10	33.9	0.252	135

NADH concentrations have been approximated to 100, 20 and 10 μM. The actual concentrations are 96.1 μM (metabolism) and 106.1 μM (b_5 levels) for the highest NADH concentrations (i.e. 100 μM). The actual concentrations are given in Tables 1 and 5.

morphine-*N*-demethylase, determined in the presence of three NADH concentrations, is almost half the value determined in the absence of NADH. This suggests that the reduction of the cytochrome P450-substrate complex is no longer rate limiting;

(b) The significant increase in V over control values for NADPH cytochrome P450-reductase (in the presence of either aminopyrine or ethylmorphine), when the reaction is initiated by NADPH plus NADH. This is in spite of the relative inefficiency of NADH in reducing cytochrome P450. The presponse to NADH must be essentially instantaneous because both pyridine nucleotides are introduced simultaneously.

It was not possible to show the NADH induced perturbation of the cytochrome P450-substrate complex spectrally because of the different steady state levels of reduced cytochrome b_5 obtained with NADPH or NADH [39]. However, a similar proposal has been advanced for both horse liver alcohol dehydrogenase [40, 41] and octopine dehydrogenase [42] where NADH, in addition to donating an electron also induces a conformational change in the enzyme. Lenk [43] has proposed that the integration of the mixed function oxidase complex into a protein membrane does not appear to hinder any possible conformational change in the enzyme complex.

Any significant contribution due to the interaction of NADH with NADPH cytochrome-*c*-reductase can be excluded because the rate at which electrons can be transported through this flavoprotein with cytochrome *c* as the electron acceptor is at least an order of magnitude above the maximum rate of cytochrome P450-reduction.

If the addition of both pyridine nucleotides in *in vitro* demethylation studies stimulates NADPH cytochrome P450-reductase to such an extent that it is no longer the rate limiting step, the question arises as to which subsequent step assumes this role? NADPH cytochrome P450-reductase activity can also be stimulated by the prior treatment of rats with phenobarbital [44, 45] and Estabrook *et al.* [46] using microsomes isolated from phenobarbital pretreated rats, have suggested that in this case the introduction of the second electron becomes rate limiting. In the present work the actual magnitude of the apparent V values were similar at each NADH concentration (Table 1) when the kinetic constants of NADPH cytochrome P450-reductase activity can also ethylmorphine demethylases. This finding together with the constant ratio between V values and the steady state reduced cytochrome b_5 levels (Table 6) supports the suggestion that the introduction of the second electron is rate limiting regardless of the drug substrates present.

On the basis of the experimental evidence presented in this report, the following sequence of events are proposed to occur during either aminopyrine or ethylmorphine demethylation in the presence of both reduced pyridine nucleotides. In this it is assumed that cytochrome b_5 mediates the introduction of the second electron from NADH but not NADPH and it is further assumed that in the presence of both reduced pyridine nucleotides, the second electron is derived from NADH via cytochrome b_5 rather than from NADPH via its, as yet, unidentified carrier. It is proposed that:

(a) The substrate combines with ferric cytochrome P450 to form a ferric cytochrome P450-substrate complex.

(b) The NADH molecule binds to and induces a conformational change in the ferric cytochrome P450-substrate complex thereby allowing the electron from NADPH to be transferred more efficiently. NADPH cytochrome P450-reductase is stimulated by the binding of NADH to the extent that it is no longer rate limiting.

(c) The NADH molecule that binds to cytochrome P450 also donates one electron via cytochrome b_5 reductase to reduce cytochrome b_5.

(d) Molecular oxygen binds to the ferrous cytochrome P450-substrate complex either before or after cytochrome b_5 reduction.

(e) The second electron required for the activation of the bound oxygen is transferred from reduced cytochrome b_5. This step could involve, if necessary, the translational diffusion of reduced cytochrome b_5.

(f) The active hydroxylating species is introduced into the substrate.

(g) The complex dissociates liberating hydroxylated substrate, water, and regenerates ferric cytochrome P450.

Both cytochrome b_5 [47, 48] and cytochrome b_5 reductase [49] have been postulated to undergo translational diffusion within microsomal membranes. Further, the reduction of cytochrome b_5 by cytochrome b_5 reductase is much more rapid than the re-oxidation of the cytochrome by the donation of an electron to the oxygenated ferrous cytochrome P450-substrate complex [5, 6, 47].

It is therefore possible that cytochrome b_5 and the flavoprotein reductase could be situated close to the NADH binding site on the cytochrome P450-substrate complex. This postulate requires that the reduction of cytochrome b_5 by an electron from the cytochrome P450 bound NADH molecule occurs after the reduction of the cytochrome P450-substrate complex by an electron from NADPH.

The sequence of events above proposes that a single molecule of NADH is involved in both the stimulation of cytochrome P450-reductase and also the donation of the second electron to the system. However the results, shown in Fig. 1, raise the possibility that two molecules of NADH are involved and that there is a homotropic co-operative interaction between them. Further examination of the data of Fig. 1 showed that it could not be successfully fitted to the Michaelis–Menten equation using HYPER, based upon previously established criteria [7], so it was therefore fitted to the Hill equation using the digital computer programme NONLIN [25].

$$v = (VS^n/K + S^n) \qquad (2)$$

where v is the initial velocity, V is the maximum velocity, S is the substrate concentration and n and K are constants. This equation, which is applied to the kinetics of allosteric enzymes reverts to the normal Michaelis–Menten equation, when $n = 1$.

The value of n is a function of two factors:

(a) The number of binding sites, and

(b) The strength of interaction between the sites.

The values of the Hill equation constants using the above data are given in Table 7. With both substrates,

Table 7. Hill equation parameters of NADH determined in the presence of NADPH

	Aminopyrine		Ethylmorphine	
NADPH Conc., μM	104.7	379	104.7	347.6
K	453 ± 116.6	100 ± 23.4	338.6 ± 21.3	80 ± 12.0
V*	10.2 ± 0.5	10.2 ± 0.8	21.3 ± 2.6	20 ± 3.9
n	1.74 ± 0.1	1.38 ± 0.12	1.53 ± 0.1	1.0 ± 0.1
r	0.971	0.96	0.94	0.95

* V expressed an nmoles HCHO formed/5 min/mg microsomal protein.

The data (obtained as previously described in the legend of Fig. 1) was fitted to the Hill equation using the digital computer programme NONLIN [25].

Initial estimates required by NONLIN were obtained by first fitting the data to the Hill equation using Atkins [50] programme. The initial estimates were:

	Aminopyrine		Ethylmorphine	
NADPH Conc., μM	104.7	379	104.7	346.9
K	437	131.6	338.7	105.1
V	10.1	9.6	21.33	15.95
n	1.74	1.49	1.53	1.2

the n values are approaching a value of two with an NADPH concentration of 104.7 μM suggesting that there are at least two NADH binding sites.

However, when the experiments were repeated in the presence of approximately 350 μM NADPH, the n value was reduced from 1.74 to 1.38 for aminopyrine and from 1.53 to 1.0 for ethylmorphine. Similar numerical values of K, V and n were obtained when the experiments were repeated, but the correlation coefficient r, was not significantly improved. NONLIN was used to obtain numerical estimates for the parameters of the Hill equation in Table 7, because this programme gives an estimate of the variation in each parameter and also a correlation between the data and the model.

Variations in n values with different effector concentrations has been reported for sheep liver pyruvate carboxylase.* It was suggested that sigmoidal curves in the absence of binding studies are not necessarily indicative of homotropic co-operative kinetics, i.e. only one NADH molecule could be responsible for the sigmoidal kinetics obtained in Fig. 1 and Table 7. The kinetic analyses involving the Hill equation have been performed using microsomal membranes rather than purified enzymes. In such a heterogeneous system, it is not readily possible to examine the binding of the pyridine nucleotides exclusively to the mixed function oxidase system. Therefore, at the present time, the number of NADH molecules involved in the NADH synergistic effect is not resolved. The variation in n value with different NADPH concentrations would probably suggest that a single NADH molecule is responsible for the observed effects. It should be noted, that this interpretation does not alter the NADH heterotropic effect with respect to NADPH for cytochrome P450-reductase.

While the available evidence favours the involvement of a single NADH molecule in both NADH influenced sequences, only a minor modification to the previously proposed mechanism is necessary if the homotropic interaction between two NADH molecules is shown to be correct. In such a modified mechanism, it is suggested that the first NADH molecule binds to and induces a conformational change in the cytochrome P450-substrate complex as previously proposed. It does not, however, donate any electrons to cytochrome b_5, but rather influences the site for the introduction of the second electron via cytochrome b_5. The second NADH molecule binds in a co-operative manner with the first NADH molecule and donates the electron to reduce cytochrome b_5. A homotropic co-operative interaction between the two NADH binding sites is observed because the NADH reduction of cytochrome b_5 is very rapid compared to the rate at which it is re-oxidised by donating an electron to the oxygenated ferrous cytochrome P450-substrate complex.

The lack of stimulation of aminopyrine demethylation in the presence of 96 μM NADH and the reduced levels of activity at the lower NADH concentrations (Table 1) suggests that if the above hypothesis is correct, then in the presence of NADH, the second electron can only come from NADH and that the rate of transfer of the second electron from NADH is dependent on the concentration of reduced cytochrome b_5. Thus for aminopyrine demethylation in the presence of both reduced pyridine nucleotides, the

Table 8. Kinetic parameters for NADPH obtained by refitting the data of Table 1 to the Hill equation

Substrate	NADH Conc., μM	K	V*	n
Aminopyrine	96.1	4.68	51.35	1.19
	19.2	3.97	43.35	1.06
	12.0	4.39	38.83	0.80
Ethylmorphine	96.1	7.23	45.48	1.17
	19.2	7.51	35.79	1.1
	12.0	6.4	33.0	1.1

* V expressed an nmoles HCHO formed/5 min/mg microsomal protein.

The data was fitted to the Hill equation using the digital computer programme of Atkins [50], run in BASIC on a PDP 11/40.

* D. B. Keech, personal communication.

Table 9. Kinetic parameters of NADPH for NADPH Cytochrome P450-reductase obtained by refitting the data of Table 2 to the Hill equation

Substrate	NADH Conc., μM	K	V*	n
Aminopyrine	119	1.27	15.29	0.74
	20.2	1.91	15.76	0.72
Ethylmorphine	98	2.31	15.62	0.86
	20.4	1.6	14.7	0.56

* V expressed as nmoles cytochrome P450-reduced/min/mg microsomal protein.

The data used in Table 2 was refitted to the Hill equation, using the digital computer programme of Atkins [50], run in BASIC on a PDP 11/40.

steady state levels of reduced cytochrome b_5 which gives the same rate of product formation as that obtained with saturating concentrations of NADPH alone requires the presence of at least 100 μM NADH. When the levels of NADH fall below this, the steady state level of reduced cytochrome b_5 fall and the obligatory donation of the second electron from the associated NADH molecule reduces the rate of the new rate limiting step below that of the original cytochrome P450 reduction in the absence of NADH.

The data used to calculate the apparent K_m and V values for NADPH in the presence of three fixed NADH concentrations using either aminopyrine or ethylmorphine as substrate (Table 1) was similarly fitted to the Hill equation (Table 8) using Atkin's programme.

As the number of binding sites can never be less than n [51], two NADPH binding sites must be considered. However, with either substrate, n values of 1.1, would indicate little interaction between sites. Further the results obtained for NADPH cytochrome P450-reductase in the presence of both pyridine nucleotides, when fitted to the Hill equation (Table 9) indicate that there is only a single NADPH binding site on this enzyme. Therefore, the above results cannot be explained by the co-operative binding of NADPH molecules.

Finally, if the proposed interaction of NADH with the cytochrome P450-substrate complex is correct, the actual structure of NADH would be expected to be a significant factor responsible for the proposed NADH induced conformational change. Further experimental evidence in support of this concept is given in the accompanying communication [52].

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