Biochemical Pharmacology, Vol. 27, pp. 979–983. Pergamon Press, 1978. Printed in Great Britain.

PYRIDINE NUCLEOTIDE INVOLVEMENT IN RAT HEPATIC MICROSOMAL DRUG METABOLISM—III. THE INFLUENCE OF THE 1,4,5,6-TETRAHYDRONICOTINAMIDE ANALOGUE OF NADH ON THE NADPH KINETICS OF AMINOPYRINE-*N*-DEMETHYLATION

GEOFFREY K. GOURLAY and BERESFORD H. STOCK

School of Pharmacy, South Australian Institute of Technology, North Terrace, Adelaide, S.A. 5000, Australia

(*Received* 12 *January* 1977; *accepted* 13 *September* 1977)

Abstract—1,4,5,6-Tetrahydronicotinamide adenine dinucleotide ($NADH_3$), a structural analogue of NADH, was unable to support the demethylation of aminopyrine or the reduction of the cytochrome P450-aminopyrine complex. However, the combination of $NADH_3$ with NADPH stimulated the NADPH dependent reduction of the cytochrome P450-aminopyrine complex. There was no significant alteration in the apparent K_m (NADPH) value, but there was an 80 per cent increase in apparent V of NADPH for NADPH-cytochrome P450-reductase (plus aminopyrine) when the kinetic constants were determined in the presence of 100 μM $NADH_3$. The inclusion of $NADH_3$ in the medium for aminopyrine demethylation also resulted in a significant increase in apparent V compared to the value obtained in the absence of $NADH_3$. The results suggest that the structure of NADH, as well as its capacity to donate the electron, is responsible for the NADH mediated increase in aminopyrine metabolism.

A previous report from this laboratory [1] examined the influence of NADH on the kinetic constants of NADPH during microsomal hydroxylation reactions. The results obtained suggested that in the presence of both reduced pyridine nucleotides, a co-operative interaction exists between NADPH and NADH for both aminopyrine and ethylmorphine-*N*-demethylases.

It was proposed that for the demethylation of either of these two Type I substrates in the presence of both reduced pyridine nucleotides, the NADH molecule associates with the oxidised cytochrome P450-substrate complex to induce a conformational change in this complex. This change then facilitates the reduction of the complex by an electron from NADPH, via NADPH cytochrome-*c*-reductase.

This proposal requires that the structure of NADH rather than its electron donating capacity is the major factor responsible for the conformational change in the cytochrome P450-substrate complex.

1,4,5,6-Tetrahydronicotinamide adenine dinucleotide ($NADH_3$)*, a structural analogue of NADH, while being incapable of substituting for NADH as a cofactor for lactic, malic and alcohol dehydrogenases, has been previously shown to be a competitive inhibitor of NADH with these enzymes [2]. The present communication reports the influence of $NADH_3$ on the kinetic constants of NADPH for aminopyrine demethylase and for NADPH cytochrome P450-reductase in the presence of aminopyrine.

* *Abbreviations.* $NADH_3$ 1,4,5,6-Tetrahydronicotinamide adenine dinucleotide. VAR Variance. CV Coefficient of variation.

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 [3].

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 [4], based on the method of Cinti *et al.* [5]. The protein content of the microsomal suspension was determined by the method of Lowry *et al.* [6] using crystalline bovine serum albumin as standard.

Drug metabolism. The composition of the incubation medium used for the metabolism of aminopyrine was identical to that described previously [4]. The amount of formaldehyde liberated was estimated by the method of Nash [7] as modified by Cochin and Axelrod [8].

Kinetic parameters were evaluated from three determinations employing eight substrate concentrations (in duplicate), ranging from 0.4 K_m to twenty

times K_m. Marbles were added to beakers to improve mixing [9].

Enzyme assays. NADPH cytochrome P450-reductase activity was measured as previously described [4] using a Gilford 2400S recording spectrophotometer.

In experiments concerned with the biphasic reduction kinetics of cytochrome P450, the reduction was monitored until asymptotic (4 min). The amount of unreduced cytochrome P450-CO complex at time, t, seconds 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 figure was divided by $A_{450,\infty}$ to give percentage of 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.

Preparation of 1,4,5,6-tetrahydronicotinamide analogue of NADH. NAD (150–200 mg) was hydrogenated using 1 per cent Palladium suspended on barium carbonate at 4° and atmospheric pressure according to Dave *et al.* [10]. The hydrogenation was performed in a 100 ml Quick-fit flask with a side arm sealed with an air-tight rubber closure. This apparatus allowed the removal of samples to monitor the extent of the reduction during hydrogenation. The hydrogenation was stopped before all the NAD was reduced at a point when the 289/265 nm absorbance ratio reached 0.75. The catalyst was removed by centrifugation at 4° and the solution further clarified by passage through a 0.22 μ membrane filter* in a Swinney adaptor†.

$NADH_3$ was purified essentially according to Stock [2]. The solution was applied to an 8 mm × 20 cm DEAE cellulose column (bicarbonate form) at 4°. Samples of 6 ml were collected when the column was eluted with a linear gradient of 0–0.2 M ammonium bicarbonate. Samples with a 289/265 nm ratio of greater than 0.85 were bulked and lyophilised.

The residue was reconstituted with 10 mM Tris buffer (pH 7.5) and clarified by filtration using a 0.22 μ membrane filter in a Swinney adaptor and stored at 4° until required. A molar extinction coefficient of 17,700 at 289 nm [2] was used to calculate the concentration of $NADH_3$ in solution. Although previous work [2] has shown that $NADH_3$ lost only 7 per cent of the 289 nm chromophore over 34 days at 4°, all samples were used within 1 week of preparation.

Computational methods. The variance (VAR) and the coefficient of variation (CV) of the velocity readings at each substrate concentration were calculated.

The kinetic constants (K_m, V) were computed using the iterative digital computer programme HYPER, written by Cleland [11] and run in BASIC on a PDP 11/40.

The velocity readings at each substrate concentration were weighted with the reciprocal of the variance (I/VAR) and then the reciprocal of the coefficient of variation (I/CV) at that substrate concentration. A selection of the values of the kinetic constants of the above computations were based upon criteria previously established [4].

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

NADPH Cytochrome P450-reductase activity in the presence of $NADH_3$ analogue. Figure 1 shows the reduction of the cytochrome P450-aminopyrine complex by NADPH, $NADH_3$ alone and NADPH in the presence of $NADH_3$. While $NADH_3$ by itself was incapable of reducing the cytochrome P450-aminopyrine complex, the simultaneous addition of $NADH_3$ and NADPH increased the reduction rate compared to the rate for NADPH alone.

It has been demonstrated [1] that the reduction of the cytochrome P450-aminopyrine complex by either NADPH alone or both NADPH and NADH together, could successfully be fitted to a two exponential equation.

$$P = Ae^{-r_1 t} + Be^{-r_2 t}$$

where P represents the percent unreduced cytochrome P450-substrate complex at time, t, and A, r_1, B, and r_2 are constants.

The results of similar studies where the $NADH_3$ analogue was added with NADPH were also fitted to the above equation and are shown in Table 1. The concentrations of reduced pyridine nucleotides and $NADH_3$ were determined spectrophotometrically at

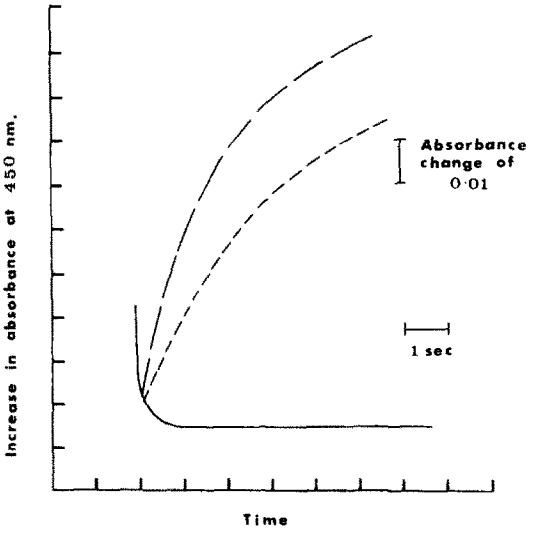

Fig. 1. The reduction of the cytochrome P450-aminopyrine complex was initiated by the introduction of $NADH_3$ to give a final concentration of 100 μM (——), NADPH, 93.6 μM (-----) or both $NADH_3$ (100 μM) and NADPH (93.6 μM) together (– – –).

* Cellulose Acetate Oxoid, U.K.

† 25 mm Sartorius Membrane Filter Holder, Germany.

Table 1. Numerical values of the constants of the biexponential equation determined in the presence of NADPH and $NADH_3$

Experiment	Parameter* A	r_1	B	r_2	cor†
1.	74.7 ± 0.53	0.42 ± 0.005	23.2 ± 0.005	0.035 ± 0.002	1.000
	67.4 ± 0.4	0.47 ± 0.006	29.2 ± 0.26	0.035 ± 0.001	0.999
2.	70.5 ± 0.39	0.431 ± 0.005	26.7 ± 0.27	0.045 ± 0.001	1.000
	67.6 ± 0.27	0.42 ± 0.004	29.4 ± 0.2	0.042 ± 0.001	1.000
3.	72.1 ± 0.36	0.486 ± 0.006	25.0 ± 0.25	0.039 ± 0.001	1.000
	72.9 ± 0.23	0.456 ± 0.004	24.2 ± 0.17	0.045 ± 0.001	1.000
Bulked	70.2 ± 0.64	0.433 ± 0.007‡	26.9 ± 0.32	0.044 ± 0.001§	0.997

* Mean ± S.D.
† Correlation coefficient as defined in the Methods [4].
‡ Significantly different from r_1 (Table 3) [1] at $P < 0.05$.
§ Significantly different from r_2 (Table 3) [1] at $P < 0.005$.
$NADH_3$ (64 μM) was preincubated in the reaction medium for 5 min prior to initiating the reaction with NADPH. Weight factor used in computations was reciprocal of the coefficient of variation.

340 and 289 nm respectively as described in the Methods.

An r_1 value (the slope of the initial rapid phase) of 0.426 ± 0.016 was obtained for the reduction of the cytochrome P450-aminopyrine complex when the reaction was initiated by the simultaneous addition of NADPH and $NADH_3$. This value was not statistically different from the value of 0.41 ± 0.01 obtained in the presence of NADPH alone (Table 3) [1]. However, as $NADH_3$ has been shown to be unable to reduce the cytochrome P450-aminopyrine complex, the experiment was repeated and the $NADH_3$ was added 5 min prior to initiating the reaction with NADPH (Table 1). Under these conditions, there was a statistically significant increase in the value of r_1. Table 2 shows the kinetic constants of NADPH for NADPH cytochrome P450-reductase determined in the presence and absence of $NADH_3$. The inclusion of the $NADH_3$ analogue did not change the apparent K_m (NADPH) value, but increased the apparent V by approximately 80 per cent to 9.05 nmoles cytochrome P450 reduced/min/mg microsomal protein.

This suggests that while $NADH_3$ was incapable of reducing the complex, it was capable of inducing a conformational change in the cytochrome P450-substrate complex, similar to that produced by NADH. However, $NADH_3$ appears to bind more slowly and to be less efficient than NADH in inducing this conformation change.

Other workers have previously proposed, on the basis of both crystallographic and spectroscopic evidence, that $NADH_3$ induces the same conformational change as NADH for horse liver alcohol dehydrogenase [12] and octopine dehydrogenase [13].

Although the molecular conformation of $NADH_3$ would be similar to the folded boat conformation of NADH [14], the more fully hydrogenated analogue without the nicotinamide C_5–C_6 double bond, would not be identical. This slight difference in shape of the nicotinamide ring could alter the strength of interaction between the pyridine nucleotide and the oxidised cytochrome P450-substrate complex and hence explain the reduced efficiency of $NADH_3$ relative to NADH in inducing a conformational change in the

Table 2. K_m and V values of NADPH for NADPH cytochrome P450-reductase and aminopyrine-*N*-demethylase in the presence of $NADH_3$ alone and both $NADH_3$ and NADH together

	NADPH cytochrome P450-reductase		Aminopyrine-*N*-demethylase			
	$NADH_3$ absent‡	$NADH_3$ present, 100 μM	$NADH_3$ absent§	$NADH_3$ present, 68 μM	NADH, 96.1 μM‖	NADH, 108.4 μM and $NADH_3$, 100 μM
K_m	1.56 ± 0.28	2.0 ± 0.4	7.92 ± 0.82	7.1 ± 0.53**	3.57 ± 0.12††	4.66 ± 0.52**††
V	5.28 ± 0.13¶	9.05 ± 0.4¶	50.3 ± 1.48‡‡	78.6 ± 1.3‡‡	52.7 ± 0.31	79.7 ± 1.9
r*	0.991	0.981	0.985	0.996	0.999	0.994
N†	3	3	6	3	3	3

K_m expressed as μM: mean ± S.D.
V expressed as nmoles cytochrome P450 reduced/min/mg microsomal protein for NADPH cytochrome P450-reductase and nmoles HCHO formed/5 min/mg microsomal protein for aminopyrine-*N*-demethylase: mean ± S.D.
* Correlation coefficient.
† Number of determinations.
‡ Data (Table 2) [4].
§ Data (Table 1) [4].
‖ Data (Table 1) [1].
¶ Significantly different at $P < 0.001$.
** Significantly different at $P < 0.005$.
†† Significantly different at $P < 0.005$.
‡‡ Significantly different at $P < 0.001$.
Aminopyrine was included in the assay for NADPH cytochrome P450-reductase at a final concentration of 4 mM.

complex. Olomucki *et al.* [13] have also postulated that the conformation adopted by the nicotinamide ring of $NADH_3$ is not identical to that adopted by the same ring in NADH.

The term, conformational change, suggests that there is an alteration of the tertiary structure of the cytochrome P450-substrate complex upon the binding of NADH or $NADH_3$. However, it should be noted that this may not necessarily be the case. The binding of NADH or $NADH_3$ may modify the site for the introduction of the electron from NADPH without any change to the tertiary structure of the complex. It would be difficult to show which of the two possibilities actually occurs in the heterogeneous microsomal suspension used for these studies, so that although the following discussion suggests a conformational change, it is equally possible that the latter of the above two proposals could operate. Nevertheless, the results obtained with $NADH_3$ provides evidence that the structure of NADH rather than its capacity to donate an electron is responsible for this change.

Cytochrome P450-aminopyrine complex reduction by the simultaneous addition of NADPH (94 μM), NADH (121.2 μM) and $NADH_3$ (139.4 μM) was 13.05 $\pm$ 0.87 nmoles reduced/min/mg microsomal protein ($N = 3$), which was significantly lower than the V value of 14.61 $\pm$ 0.32 nmoles cytochrome P450 reduced/min/mg microsomal protein obtained in the presence of NADPH and NADH (Table 2) [1]. This suggests that NADH and $NADH_3$ may be competing for the same site on the cytochrome P450-substrate complex.

This possibility was tested by the pre-incubation of $NADH_3$ (89 μM) with the reaction medium for NADPH-cytochrome P450-reductase (plus aminopyrine) for 5 min prior to initiating the reaction by the simultaneous addition of NADPH (92.4 μM) and NADH (98 μM). 10.9 $\pm$ 0.97 nmoles of cytochrome P450 was reduced/min/mg microsomal protein which was significantly lower than the value obtained by the simultaneous addition of NADPH, NADH and $NADH_3$ (13.05 $\pm$ 0.87 nmoles cytochrome P450 reduced/min/mg microsomal protein) but not significantly different from the reduction rate in the presence of NADPH and $NADH_3$ (Table 2).

This lack of stimulation by NADH suggests that the NADH binding site has been either partially or completely blocked by $NADH_3$. Further, it suggests that in the time taken for the cytochrome P450-substrate complex to be reduced by an electron from NADPH, NADH cannot effectively displace bound $NADH_3$ from this site.

Kinetic constants of NADPH in the presence of $NADH_3$ during drug hydroxylations. K_m and V values of NADPH for aminopyrine-*N*-demethylase determined in the presence of $NADH_3$ alone and with NADH are also given in Table 2. $NADH_3$ by itself was unable to support the demethylation of aminopyrine. There was no change in the K_m (NADPH) in the presence of $NADH_3$, compared to the values obtained in its absence, but there was a significant increase in V (Table 1) [4].

This increase in V in the presence of $NADH_3$ was greater than that obtained in the presence of NADH (Table 1) [1]. However, unlike NADH, $NADH_3$ did not reduce the apparent K_m (NADPH) value obtained during the demethylation of aminopyrine. This latter finding would suggest that, while $NADH_3$ is able to significantly stimulate NADPH cytochrome P450-reductase activity, the stimulation is not sufficient to change the rate limiting step from the reduction of the cytochrome P450-substrate complex. The observation that there are parallel increases in V values for aminopyrine-*N*-demethylase (56%) and NADPH cytochrome P450-reductase (71%) in the presence of both NADPH and $NADH_3$ also supports the proposal, that the reduction of the cytochrome P450-aminopyrine complex is still the rate limiting step in the presence of $NADH_3$.

The finding that $NADH_3$ gave greater stimulation of aminopyrine demethylation than NADH in spite of its inferior stimulation of NADPH cytochrome P450-reductase can also be explained within the framework of the present proposal [1].

In the presence of NADPH and $NADH_3$, the $NADH_3$ associates with the oxidised cytochrome P450-substrate complex in the same manner as NADH. The first electron for the reduction of the ferric cytochrome P450-substrate complex would originate, as before, in NADPH. However, the second electron which is required for the complete reduction of the oxy-ferrous cytochrome P450-substrate complex must necessarily come from the second molecule of NADPH.

In the presence of NADH it was proposed [1] that the second electron must come from this reduced pyridine nucleotide and that the reduction of the oxygenated cytochrome P450-substrate complex is controlled by the level of reduced cytochrome b_5. As a result of providing this electron, the NADH molecule is oxidised and its conformation changes from a boat form to that of a planar aromatic ring. It is suggested that this conformational change is sufficient to dissociate the NAD^+ molecule from the oxygenated cytochrome P450-substrate complex.

However, in the presence of the associated $NADH_3$ molecule which is not oxidisable, this pyridine nucleotide analogue will retain its original non-planar conformation and thus stay associated with the oxygenated cytochrome P450-aminopyrine complex during the addition of the second electron from NADPH.

Under these circumstances the rate of transfer of this second electron is not now dependent on the level of reduced cytochrome b_5 and thus increases in cytochrome P450 reduction will be directly reflected by increases in the overall demethylation rate (Table 2).

Finally, the results presented in this paper lend support to the proposal that NADH influences aminopyrine demethylation both by a structurally mediated stimulation of NADPH cytochrome P450-reductase and also by provision of the second electron required in the two electron transfer sequence.

REFERENCES

1. G. K. Gourlay and B. H. Stock, *Biochem. Pharmac.* **27**, 969 (1978).
2. B. H. Stock, *Ph.D. Thesis*, University of Adelaide (1969).
3. F. M. Radzialowski and W. F. Bousquet, *J. Pharmac. exp. Ther.* **163**, 229 (1968).
4. G. K. Gourlay and B. H. Stock, *Biochem. Pharmac.* **27**, 965 (1978).

5. D. L. Cinti, P. Moldeus and J. B. Schenkman, *Biochem. Pharmac.* **21**, 3249 (1972).
6. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
7. T. Nash, *Biochem. J.* **55**, 416 (1953).
8. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
9. J. R. Fouts, *Toxic. appl. Pharmac.* **16**, 48 (1970).
10. K. G. Dave, R. B. Dunlap, M. K. Jain, E. H. Cordes and E. Wenkert, *J. biol. Chem.* **243**, 1073 (1968).
11. W. W. Cleland, *Adv. Enzymol.* **29**, 1 (1967).
12. J. F. Biellman and M. J. Jung, *Eur. J. Biochem.* **19**, 130 (1971).
13. A. Olomucki, F. Thome-Beau, J. F. Biellman and G. Branlant, *Eur. J. Biochem.* **56**, 109 (1975).
14. N. J. Oppenheimer, L. J. Arnold and N. O. Kaplan, *Proc. natn. Acad. Sci. U.S.A.* **68**, 3200 (1971).