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# PYRIDINE NUCLEOTIDE INVOLVEMENT IN RAT HEPATIC MICROSOMAL DRUG METABOLISM—IV

## INHIBITION OF AMINOPYRINE AND ETHYLMORPHINE-*N*-DEMETHYLASES BY 2,6-DIHYDROXY-ACETOPHENONE

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**Abstract**— Pyridine nucleotide kinetics for both aminopyrine and ethylmorphine-*N*-demethylases were evaluated in the presence of 2,6-dihydroxyacetophenone, (DiHAP) which inhibits certain microsomal mixed function oxidase reactions. The nature of the inhibition in the presence of varying NADPH concentrations was shown to be slope-linear, intercept-linear non-competitive. DiHAP was shown to combine preferentially with the ferric cytochrome $P_{450}$-substrate complex in the presence of aminopyrine, but had a greater affinity for the ferrous cytochrome $P_{450}$–substrate complex when ethylmorphine was present.

There were changes in the values of $K_{ii}$ and $K_{is}$ without changes in the type of inhibition of aminopyrine-*N*-demethylase in the presence of both NADH and DiHAP, whereas with ethylmorphine-*N*-demethylase, the nature of the inhibition kinetics changed to slope-hyperbolic, intercept-hyperbolic non-competitive inhibition in the presence of both NADH and DiHAP.

These results are consistent with the proposal that NADH interacts with the ferric cytochrome $P_{450}$–substrate complex prior to the reduction of this complex by an electron from NADPH.

Previous reports [1–3] on the involvement of the pyridine nucleotides in hepatic microsomal drug metabolism provided experimental evidence that the structure of NADH as well as its electron donating capacity were jointly responsible for the NADH synergistic increase in NADPH mediated product formation [4–6].

It was proposed [2] that the NADH molecule interacts with, and induces a conformational change in the ferric cytochrome $P_{450}$–substrate complex prior to the reduction of this complex by an electron from NADPH. The ferrous cytochrome $P_{450}$–substrate complex so formed then binds molecular oxygen and the oxygenated complex is further reduced by an electron from NADH.

2,6-Dihydroxyacetophenone (DiHAP) has been shown to inhibit aminopyrine-*N*-demethylase in rat liver microsomal preparations [7].

The present communication examines the DiHAP inhibition of aminopyrine and ethylmorphine-*N*-demethylase kinetics with NADPH alone and in the presence of NADH. This work provides further evidence for the interaction of NADH with the ferric cytochrome $P_{450}$–substrate complex in hepatic drug metabolism.

### MATERIALS AND METHODS

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. 2,6-Dihydroxyacetophenone was a gift from Dr. Adrian Ryan, University of Sydney, Australia.

*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 a.m. and 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 [1], 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 ethylmorphine or aminopyrine was identical to that described previously [1]. 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 three determinations employing eight substrate concentrations (in duplicate), ranging from 0.4 $K_m$ to 20 times $K_m$. Marbles were added to beakers to improve mixing [13].

*Enzyme assays.* NADPH cytochrome $P_{450}$ reductase activity was measured using a Gilford 2400S recording spectrophotometer by the method of Holtzman *et al.* [14] as modified by Stock and Fouts [15].

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 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 were calculated.

The kinetic constants ($K_m$,$V$) were computed using the iterative digital computer programme HYPER, written by Cleland [16] 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 then the reciprocal of the coefficient of variation (1/CV) at the substrate concentration. A selection of the values of the kinetic constants of the above computations were based upon criteria previously established [11].

Data were also fitted to the equation describing slope-linear, intercept-linear non-competitive inhibition using the iterative digital computer program NONCOM. The program was translated from FORTRAN to BASIC and run on a PDP 11/40 computer. The accuracy of the translation was verified using simulated data.

The unpaired Student's $t$ test was used to compare different experiments with a level of significance of at least $P < 0.05$.

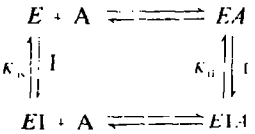
The values of all constants in this report are apparent values determined under the conditions detailed above.

## RESULTS AND DISCUSSION

*2,6-Dihydroxyacetophenone inhibition of aminopyrine and ethylmorphine demethylation.* The double reciprocal plot (Fig. 1) of aminopyrine demethylase velocity against NADPH concentration alone and in the presence of three concentrations of DiHAP suggests that the inhibition is non-competitive. The data obtained at each inhibitor concentration were first fitted to the Michaelis–Menten equation using HYPER as previously described [11]. In Fig. 1, the lines are the computer generated lines of best fit, while the points represent the means of three duplicate determinations at each NADPH concentration.

Following the primary fitting procedure described above, secondary plots of slopes (obtained from HYPER output) versus inhibitor concentrations and the ordinate intercepts (HYPER output) versus inhibitor concentrations were constructed. These secondary plots of ethylmorphine (Fig. 2) and aminopyrine-*N*-demethylases (data not shown) were linear indicating that DiHAP may be classed as a slope-linear, intercept linear non-competitive inhibitor [16].

The following scheme and equation describe this type of inhibition.




and

$$v = \frac{VA}{K_m(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (1)$$

Where I represents the inhibitor concentration, $K_{is}$, $K_{ii}$, $v$, $V$, $K_m$ and $A$ represent the enzyme–inhibitor complex dissociation constant, the enzyme–inhibitor–substrate complex dissociation constant, velocity, maximum velocity, Michaelis–Menten constant and varied substrate (i.e. NADHP) concentration respectively.

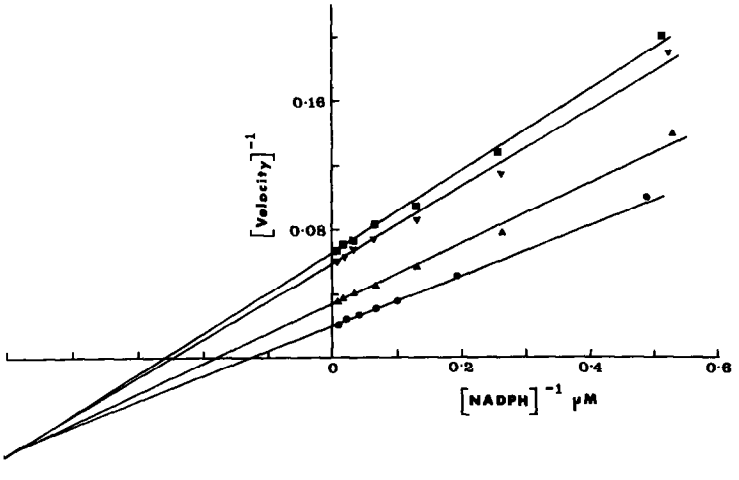



Fig. 1. Lineweaver–Burk plots for aminopyrine-*N*-demethylase in the presence of various DiHAP concentrations. The various DiHAP concentrations used were— ●——● No DiHAP (Data taken from Table 1, Reference 1); ▲——▲ 89.6 μM; ▼—— ▼194.8 μM; ■——■ 357.5 μM. Velocity readings are expressed as nmoles HCHO formed/5 min/mg microsomal protein and the NADPH concentrations as μM. The points are the means of three duplicate determinations while the lines are the computer generated lines of best fit.

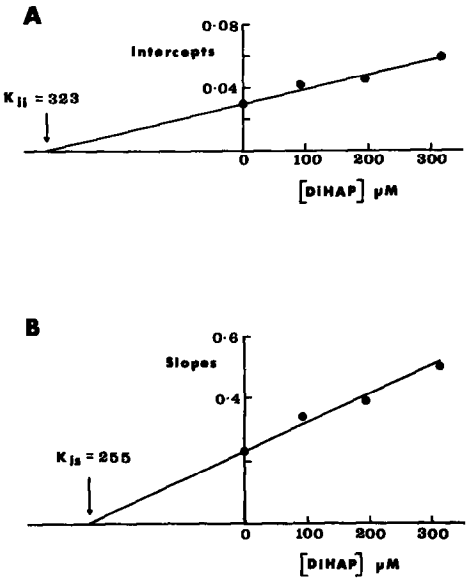



Fig. 2. Secondary plots of $1/V$ intercepts versus DiHAP concentration (Fig. 2A) and slopes versus DiHAP concentration (Fig. 2B) for ethylmorphine-*N*-demethylase.

The values for the $1/V$ intercept and slope at each DiHAP concentration were obtained from the HYPER output for that DiHAP concentration.

Numerical estimates of the constants of equation 1 for both aminopyrine and ethylmorphine-*N*-demethylases in the presence of DiHAP were obtained using the iterative digital computer program NONCOM (Table 1).

The numerical values of $K_m$ (NADPH) and $V$ obtained in the presence of the inhibitor and either aminopyrine or ethylmorphine (Table 1) are not significantly different from the values obtained in the absence of the inhibitor (Table 1, reference 1). The agreement between the numerical values of $K_m$ and $V$ in the presence and absence of the inhibitor indicates that NONCOM has converged on realistic estimates of the constants in equation 1 from the data supplied to it.

The numerical values of $K_{ii}$ and $K_{is}$ obtained with aminopyrine as the substrate suggest that DiHAP associates more readily with the ferric cytochrome $P_{450}$–aminopyrine complex ($E$) than with the ferrous cytochrome $P_{450}$–aminopyrine complex ($EA$, where $A$ rep-

Table 1. The numerical estimates of the constants of equation 1 for aminopyrine and ethylmorphine demethylases in the presence of 2,6-dihydroxyacetophenone

| Parameter* | Aminopyrine | Ethylmorphine |
|---|---|---|
| $K_m$+ | 7.93 ± 0.56 | 8.35 ± 0.9 |
| $V$‡ | 50.28 ± 1.01 | 33.5 ± 1.36 |
| $K_{is}$ | 650.0§ | 172.4 ± 84.7 |
| $K_{ii}$ | 128.1 ± 8.5 | 336.0 ± 72.6 |
| $r$ | 0.985 | 0.972 |

* Each parameter mean ± S.D.

+ Expressed as μM.

‡ nmoles HCHO formed/5 min/mg microsomal protein.

§ When the data for aminopyrine-*N*-demethylase obtained in the presence of various DiHAP concentrations was fitted to equation 1 using NONCOM, the value of $K_{is}$ became negative after the first iteration and increasingly more negative after each subsequent iteration. It appears that NONCOM is unable to converge on positive values of all constants when the value $K_{is}$ is much greater than $K_{ii}$ or vice versa. The accuracy of the translation into BASIC was verified using simulated data when the ratio of $K_{is}/K_{ii}$ was 1.2. Further, in other cases where NONCOM was used to fit data to equation 1, the program—

(a) quickly converged on positive values of $K_{ii}$ and $K_{is}$ which were in good agreement with graphical estimates, and,

(b) the actual values of $K_m$ and $V$ in the presence of DiHAP were not significantly different from the values obtained in the absence of the inhibitor [2, 3], suggesting that when the values of $K_{ii}$ and $K_{is}$ are not greatly different, NONCOM can converge on realistic estimates for these constants. Therefore, the value of $K_{is}$ for aminopyrine demethylase in this table was obtained by a linear regression of the slopes versus DiHAP concentration secondary plot.

Table 2. The influence of 2,6-dihydroxyacetophenone on NADPH cytochrome $P_{450}$ reductase activity in the presence of either aminopyrine or ethylmorphine

| | Aminopyrine | | Ethylmorphine | |
|---|---|---|---|---|
| | –DiHAP | +DiHAP* | –DiHAP | +DiHAP* |
| NADPH alone‖ | 5.3 ± 0.13+ | 1.4 ± 0.7§ | 8.9 ± 0.38+ | 4.6 ± 1.02§ |
| NADPH + NADH‖ | 14.6 ± 0.3‡ | 2.5 ± 0.9§ | 14.8 ± 0.5‡ | 7.2 ± 1.7§ |

* Final DiHAP concentration was 210 μM.

+ Value shown in the table is the maximum velocity for the appropriate substrate (Table 2, Reference 1).

‡ Value shown in the table is the maximum velocity for the appropriate substrate in the presence of 119 μM NADH (aminopyrine) and 98 μM NADH (ethylmorphine) (Table 2, Reference 2).

§NADPH cytochrome $P_{450}$ reductase activity obtained in the presence of either NADPH (125 μM) alone or NADPH (125 μM) + NADH (130 μM) together.

‖ For either substrate, values obtained in the presence of DiHAP are significantly different from the values obtained in the absence of DiHAP at $P < 0.001$.

$N = 3$ for all determinations.

All values in the table are expressed as nmoles cytochrome $P_{450}$ reduced/min/mg microsomal protein.

resents NADPH, the varied substrate). However, the reverse appears to be true when ethylmorphine is the substrate, i.e. the inhibitor appears to combine more readily with the ferrous cytochrome $P_{450}$–ethylmorphine complex.

Gigon *et al.* [17, 18] have suggested that Type 1 substrates combine with and induce conformational changes in cytochrome $P_{450}$ such that the cytochrome $P_{450}$–substrate complex is reduced at a greater rate than cytochrome $P_{450}$ alone. Previous results [1] reported that the reduction rates of the cytochrome $P_{450}$–aminopyrine complex, cytochrome $P_{450}$–ethylmorphine complex and cytochrome $P_{450}$ alone were 5.28, 8.92 and 3.7 nmoles cytochrome $P_{450}$ reduced/min/mg microsomal protein respectively.

It would therefore appear that the extent of this confirmational change is influenced by the structure of the Type 1 substrate. Such a difference in the extent of the conformational change could explain why DiHAP combines more readily with the ferrous cytchrome $P_{450}$–ethylmorphine complex than with the ferric cytochrome $P_{450}$–aminopyrine complex.

Based upon these results, DiHAP would be expected to inhibit the reduction of the cytochrome $P_{450}$–substrate complex which is generally accepted as the rate limiting step of mixed function oxidase reactions. The results shown in Table 2 support this hypothesis where, in the presence of 210 $\mu$M DiHAP, marked inhibition of NADPH cytochrome $P_{450}$ reductase activity is observed with either aminopyrine or ethylmorphine.

*The effect of 2,6-dihydroxyacetophenone on aminopyrine or ethylmorphine-N-demethylase in the presence of NADH.* The secondary plots of the data from

Table 3. The numerical estimates of the constants of equation 1 for aminopyrine in the presence of NADH and 2,6 dihydroxyacetophenone

| Parameter | Numerical estimates* |
|---|---|
| $K_m$† | 3.54 ± 0.14 |
| $V$‡ | 52.52 ± 0.37 |
| $K_{is}$ | 234.2 ± 50.0 |
| $K_{ii}$ | 185.7 ± 7.0 |
| $r$ | 0.994 |

* Mean ± S.D.
† $K_m$ expressed as $\mu$M.
‡ $V$ expressed as nmoles HCHO formed/5 min/mg microsomal protein.

DiHAP inhibition of NADPH kinetics for aminopyrine-*N*-demethylase in the presence of 120 $\mu$M NADH are shown in Fig. 3. The nature of the inhibition still appears to be slope-linear, intercept-linear, non-competitive with the numerical values of the constants of equation 1 given in Table 3.

If the sole function of NADH in the synergistic increase in drug hydroxylation is to donate the second electron via cytochrome $b_5$ after the first has been supplied by NADPH is correct [3–6], there should not be any significant changes in $K_{ii}$ and $K_{is}$ values when the inhibition experiments were repeated in the presence of a constant NADH concentration. However, the numerical value of $K_{is}$ was reduced from 650 (in the

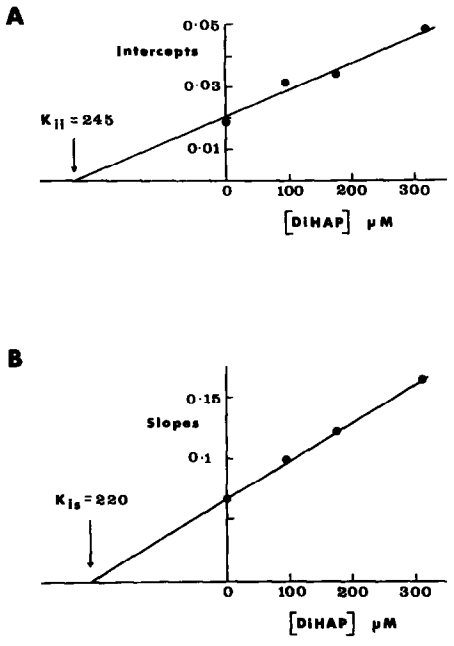



Fig. 3. Secondary plots of $1/V$ intercepts versus DiHAP concentration (Fig. 3A) and slopes versus DiHAP concentration (Fig. 3B) for aminopyrine-*N*-demethylase in the presence of 120 $\mu$M NADH.

The values for the $1/V$ intercept and slope at each DiHAP concentration were obtained from the HYPER output for that DiHAP concentration.

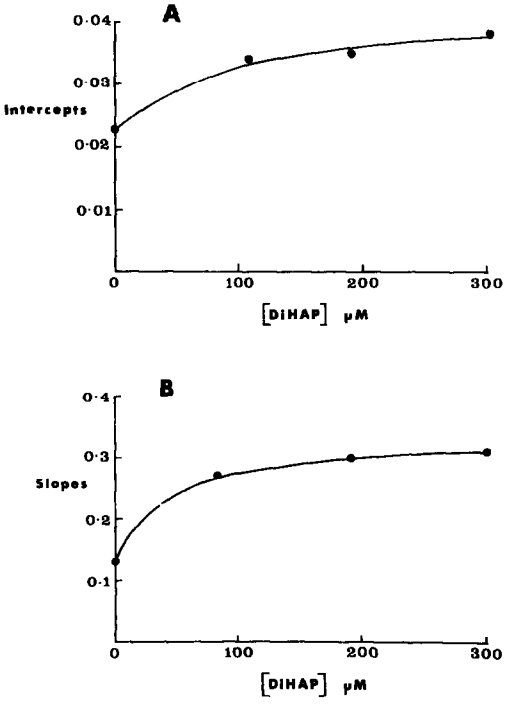



Fig. 4. Secondary plots of $1/V$ intercepts versus DiHAP concentration (Fig. 4A) and slopes versus DiHAP concentration (Fig. 4B) for ethylmorphine-*N*-demethylase in the presence of 96.1 $\mu$M NADH.

The values for the $1/V$ intercept and slope at each DiHAP concentration were obtained from the HYPER output for that DiHAP concentration.

absence of NADH) to 234 (in the presence of 120 $\mu$M NADH). At the same time, the value of $K_{ii}$ was increased from 128 to 186. These results suggest that the NADH molecule interacts with the ferric cytochrome $P_{450}$–substrate complex to induce a conformational change thereby altering the DiHAP inhibition kinetics.

The secondary plots obtained in the presence of NADH and DiHAP for ethylmorphine-*N*-demethylase (Fig. 4) indicate that the inhibition was changed from slope-linear, intercept-linear, non-competitive to slope-hyperbolic, intercept-hyperbolic, non-competitive, a finding which is also consistent with the above concept.

The addition of DiHAP (305 $\mu$M) to the incubation medium did not alter the steady state level of reduced cytochrome $b_5$ upon the addition of either NADPH (210 $\mu$M) alone or NADPH (210 $\mu$M) and NADH (244 $\mu$M) together compared to the values obtained in the absence of the inhibitor with either aminopyrine or ethylmorphine as the drug substrate. This latter finding suggests that the addition of DiHAP to the incubation medium does not influence the introduction of the second electron from NADH via cytochrome $b_5$ and cytochrome $b_5$ reductase. Overall the results provided by the inhibitor studies further support the proposed interaction of NADH with the ferric cytochrome $P_{450}$–substrate complex prior to the reduction of this complex by an electron originating in NADPH.

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