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METAL ION FUNCTION IN ALCOHOL DEHYDROGENASES—III

THE ENZYMIC REDUCTION OF PYRIDINE CARBALDEHYDES

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Abstract—The enzymic reduction of 2,3 and 4-PCA has been investigated using YADH with NADH as cofactor. All three aldehydes are effective substrates, with the V_{max} ratio for the 2:3:4 being 10:1:3, and the K_m ratio 1:4:2. This is interpreted in terms of substrate-metal ion interaction *via* the pyridine nitrogen, with no direct metal ion-NADH interaction.

INTRODUCTION

The behaviour of pyridine-2, 3- and 4-aldehydes as substrates in an enzymic reaction involving YADH is of interest in the light of the results obtained in the study of the non-enzymic reduction[1, 2]. It has been established conclusively[1] that the primary requirement in the non-enzymic reaction is preliminary complexation of the NADH analog (or NADH itself) by the metal ion centre, with complexation occurring *via* the amide oxygen[2] and that either this *or* the non-complexation of the aldehyde group precludes reaction with 3- and 4-PCA. Consequently any difference between enzymic and non-enzymic reaction with the 3- and the 4-aldehyde should provide an insight into the mode of metal ion involvement in the enzyme.

We give below the results of the reaction of 2-, 3- and 4-PCA with crystalline YADH using NADH as cofactor. All three aldehydes are effective substrates, with V_{max} ratios for the 2:3:4 being 10:1:3 and the K_m ratios 1:4:2. The results are interpreted in terms of substrate metal-ion interaction *via* the pyridine nitrogen, and it is concluded that there is no direct NADH:metal-ion interaction. A preliminary announcement of these results has been made[3].

MATERIALS AND METHODS

(a) *Product identification.* The identification of products was by NMR spectroscopy using a Perkin–Elmer RB12 spectrometer operating at 60 MHz. Spectra were recorded of each aldehyde in solution in D_2O, $_pH$ 7.5 (0.05 M Tris sulphate buffer). An equivalent (~0.1 M) amount of β-NADH, or alternatively ~100 μl of the enzyme solution prepared as described below, was added and the spectrum re-recorded. 100 μl of enzyme solution was then added to the NADH/aldehyde solution and the spectrum recorded once more.

The results were as follows. The characteristic aldehyde peak was unperturbed by the addition of NADH or enzyme, but the addition of enzyme *and* NADH caused loss of the aldehyde absorbance and concurrent appearance of the characteristic[4] low-field peaks due to NAD^+. As with the non-enzymic system[2] the field was scanned to 20 ppm downfield of TMS to ensure that the aldehyde peak had not disappeared as a result of enzyme-induced shifts.

(b) *Enzyme assay.* The assay procedure was as follows: buffer solution was 0.05 M Tris in deionised water containing 0.1% bovine serum albumin (BSA, ex Sigma). The pH was adjusted to 7.5 using AR H_2SO_4 (ex Fisons).

An arbitrary amount of crystalline enzyme was added to 20 cm^3 of buffer solution and the UV spectrum recorded in the region 350 to 250 nm using a Pye–Unicam SP800 spectrometer. The reference beam contained buffer solution alone. The amount of enzyme present was measured using the method of Racker[5], based on the fact that the optical density of a solution of 1 mg YADH/ml buffer at $\lambda = 280$ nm is 1.26. The concentration was adjusted until the value was ~1 mg/cm^3. The activity of such a preparation was measured by following the increase in absorbance at 340 nm when a small amount (10–100 μl) of the preparation was introduced to a stock buffer/ethanol/NAD^+ solution made up as follows.

(a) 10.0 g $Na_4P_2O_710H_2O$ and 0.5 g glycine were dissolved in distilled, de-ionised water, the pH adjusted to 9.0 by the addition of molar HCl, and the volume made up to 300 cm^3.

(b) 5.0 g semicarbazide hydrochloride was dissolved in 10 cm^3 2N NaOH, the pH adjusted to 6.4 with 5N NaOH and the volume made up to 20 cm^3.

(c) 100 cm^3 of the solution made in (a) above, and 4 cm^3 of that made in (b) were mixed with 4 cm^3 of 95% ethanol. The final pH was 8.8.

(d) 0.055 g of β-NAD^+ (monosodium salt, ex Sigma) was dissolved in 25 cm^3 of the solution prepared in (c). The resulting solution was ~3×10^{-3} M in NAD^+.

The change in optical density was measured using a Pye–Unicam SP1800 spectrometer thermostatted at 25°C, with chart recording. Typically 3 cm^3 of solution prepared as in (d) were introduced into a quartz cuvette and the reaction initiated by addition of a certain quantity of enzyme solution. The quantity added, the chart speed and the range of absorbance over which the spectrometer was operating were varied so that a trace with a slope as near as possible to 45° was obtained. The rate of change was measured as the initial rate when curvature was observed. All assays were repeated at least three times. Enzyme solutions were then diluted to produce a solution with 200 units of activity/cm^3.

(c) *Enzyme kinetics.* For a sequential bireactant enzyme mechanism of the form

$$E + NADH \rightleftharpoons E'NADH$$

$$E'NADH + S \rightleftharpoons E''NADH\,S \rightarrow PRODUCTS$$

(where S = substrate)

Cleland has shown[6] that the rate of reaction is governed by an equation of the form

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \qquad (1)$$

where $V = V_{max}$; K_a = Michaelis (dissociation) constant for substrate A; K_b = Michaelis (dissociation) constant for substrate B; A, B = concentrations of substrate A or B; K_{ia} = self-inhibition constant for substrate A, and v = initial velocity.

Inversion of (1) yields

$$\frac{1}{v} = \frac{K_{ia}K_b}{VAB} + \frac{K_a}{VA} + \frac{K_b}{VB} + \frac{1}{V} \quad (2)$$

if B is held constant, and A varied, then

$$\frac{1}{v} = \left(\frac{K_{ia}K_b}{VB} + \frac{K_a}{V}\right)\frac{1}{A} + \frac{K_b}{VB} + \frac{1}{V}. \quad (3)$$

A plot of $1/v$ against $1/A$ therefore yields a line of slope $(K_{ia}K_b/VB + K_a/V)$ and intercept $(K_b/VB + 1/V)$.

If the initial rates measured at a number of different values of A with a constant value of B are repeated at several *different* constant values of B, with each slope and intercept measured, then a plot of the value of the *slopes* against $1/B$ yields a line of slope $K_{ia}K_b/V$, intercept K_a/V. A plot of the *intercepts* of the lines against $1/B$ yields a line of slope K_b/V, intercept $1/V$. Thus all the parameters are accessible.

In the studies reported here, the procedure for a series of runs was as follows. A stock solution was prepared of β-NADH in 0.05 M Tris sulphate, pH 7.5, together with 0.1% BSA, and this stock solution was diluted with buffer-BSA solution to produce solutions which were 2×10^{-4}, 10^{-4}, 7.5×10^{-5}, 4×10^{-5} M respectively. Molar stock solutions of each aldehyde in acetonitrile were also prepared. To 3 cm^3 of one of the NADH solutions in a quartz cuvette thermostatted at 30 ± 0.5°C were added microlitre quantities of an aldehyde/acetonitrile solution. The formal concentrations of aldehyde used in the reaction were 3, 5, 10 and 15 millimolar. Reaction was initiated by the introduction of 100 microlitres of an enzyme solution prepared as described previously with 200 units of activity/cm^3. The final reaction volume was 3.1 cm^3, and as a result of this the formal concentrations of NADH and aldehyde described above are reported in the figures after being reduced by a dilution factor of 0.9677.

To establish that the presence of acetonitrile had no effect on the activity of the enzyme the assay procedure described above was repeated after the addition of various amounts of acetonitrile, up to a concentration equal to five times the maximum used in the kinetic experiments. No effect was observed.

The reaction was followed by monitoring the loss of absorbance at 340 nm, using the SP1800 spectrometer. Each rate was measured at least twice, usually three times, in each series. Each NADH solution was submitted to each aldehyde successively to minimize the possibility of spurious rate differences arising through slow loss of enzyme activity during the course of the experiments. All runs in a series were performed on the same day using the same solutions, i.e. all aldehydes at all concentrations were submitted to all NADH concentrations with reaction being initiated by the same stock enzyme solution. The whole series was repeated three times using fresh solutions. Rates were measured manually from the chart, and are subject to an estimated error of 10%. The standard deviation of repeated runs from the mean value was generally better than 10%.

The slopes and intercepts of primary and secondary plots of the measured rates were calculated using a least-squares program written by Dr. M. G. Segal for the University's IBM 370/165 computer.

Abbreviations

2-PCA, pyridine 2-carboxaldehyde; YADH, alcohol dehydrogenase from yeast; LADH, alcohol dehydrogenase from liver, E.C.(1.1.1.1.).

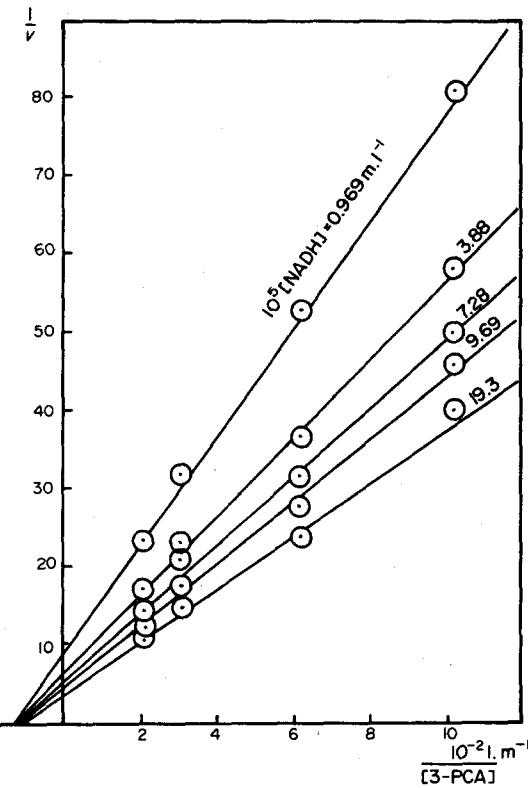

Fig. 1. Reciprocal plot of variation of initial rate of 3-PCA reduction with concentration of 3-PCA at different constant concentrations of NADH (units of v are absorption units/min/cm^3 in this and subsequent figures).

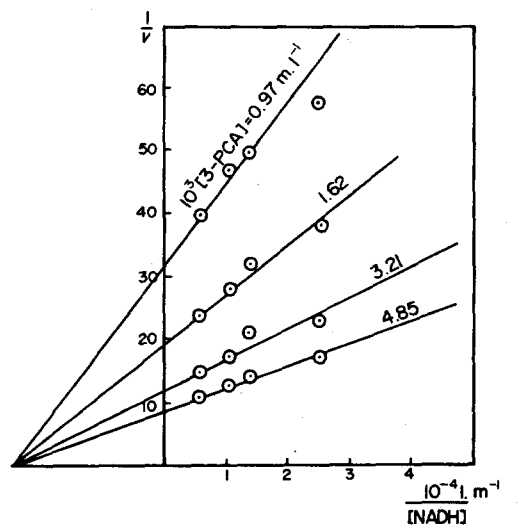

Fig. 2. Reciprocal plot of variation of initial rate of 3-PCA reduction with concentration of NADH at different constant concentrations of aldehyde.

RESULTS

(a) *The kinetics of the enzyme reduction of* 3- *and* 4-PCA

Typical results are given in Figs. 1–4. Figures 1 and 2 contain the double-reciprocal plots for the variation of concentration of 3-PCA at different NADH concentrations and variation of NADH at different 3-PCA concentrations respectively.

Figure 3 contains the plots of the slopes of analogous results relating to 4-PCA against reciprocal 4-PCA and NADH concentrations while Fig. 4 shows the analogous situation for the intercepts.

A consideration of the rate equation described earlier shows that the *slopes* of the two plots in Fig. 3 should be the same, both being equal to $K_{ia}K_b/V$, while the intercepts should be different (one equalling K_a/V, and one K_b/V). Similarly, the *intercepts* of the plots in Fig. 4 should be the same, since both are equal to $1/V$, while the slopes (one of which equals K_b/V and the other

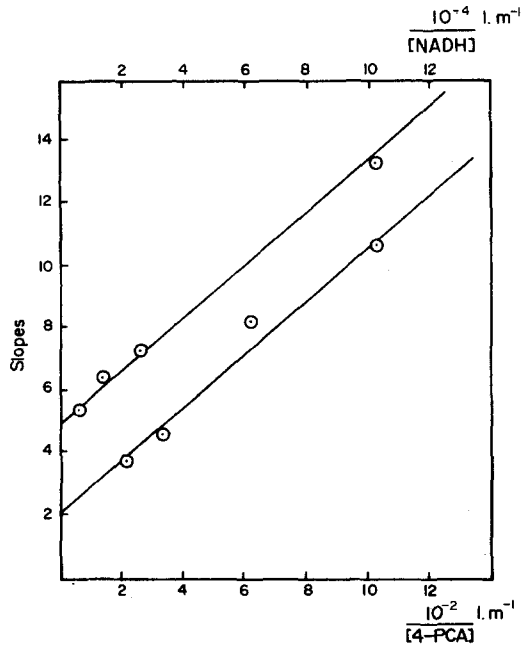

Fig. 3. Slopes of reciprocal rate plots for 4-PCA plotted against reciprocal NADH and 4-PCA concentrations.

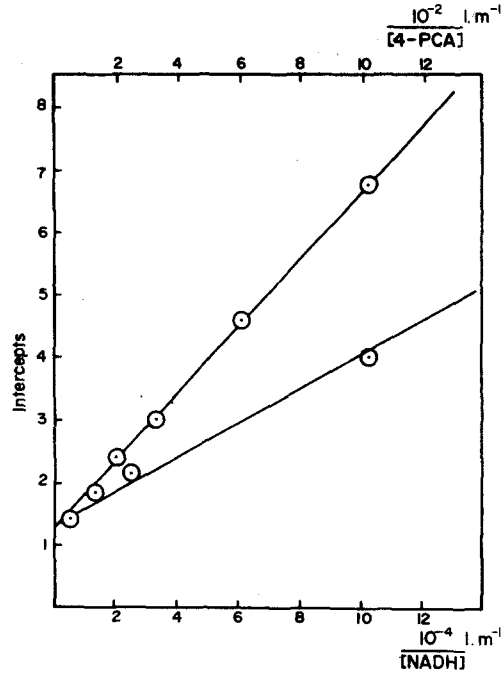

Fig. 4. Intercepts of reciprocal rate plots for 4-PCA plotted against reciprocal NADH and 4-PCA concentrations.

K_a/V) should be different. The values of all the parameters measured for both 3- and 4-PCA are contained in Table 1, and there is seen to be excellent agreement where such agreement is predicted: it is seen to be within the experimental error of 10%.

(b) *The kinetics of the enzymic reduction of* 2-PCA

Interestingly, the kinetics of reaction of 2-PCA with YADH are qualitatively different from those reported above for 3- and 4-PCA. The difference is that under identical conditions the rate of reduction of 2-PCA was found to be practically independent of NADH concentration, i.e. the variation in initial rate with concentration of 2-PCA was the same whether the concentration of NADH was 10^{-4} or 10^{-5} M.

That there *is* interaction between 2-PCA and NADH is evident for the following reasons: the reaction was followed by the loss of the NADH absorbance—therefore there is interaction between NADH and some species present in the medium. The NMR results show that (a) 2-PCA and enzyme are stable in the absence of NADH; (b) 2-PCA and NADH are stable in the absence of

Table 1. Calculated enzyme parameters for 3- and 4-PCA. The top row names the substance originally varied

	Initial substance NADH				Initial substance PCA			
Data source	Slope of slopes	Intercept of slopes	Slope of intercepts	Intercept of intercepts	Slope of slopes	Intercept of slopes	Slope of intercepts	Intercept of intercepts
Function measured	$\frac{K_{ia}K_b}{V}$	$\frac{K_a}{V}$	$\frac{K_b}{V}$	$\frac{1}{V}$	$\frac{K_{ia}K_b}{V}$	$\frac{K_b}{V}$	$\frac{K_a}{V}$	$\frac{1}{V}$
(Value)	1.1×10^{-7}	1.3×10^{-4}	2.89×10^{-2}	3.5	1.09×10^{-7}	2.6×10^{-2}	1.36×10^{-4}	3.5
Parameter calculated	K_{ia}	K_a	K_b	V	K_{ia}	K_b	K_a	V
(Value)	3.93×10^{-5}	3.7×10^{-5}	8×10^{-3}	0.286	4.20×10^{-5}	7.4×10^{-3}	3.88×10^{-5}	0.286
Mean values	3-PCA: $V_{max} = 2.86\times10^{-1}$ Abs min^{-1} ml E. $K_b = 7.7\pm0.3$ mM, $K_a = 3.79\pm0.09\times10^{-5}$ ml^{-1}, $K_{ia} = 4.06\pm0.14\times10^{-5}$ ml^{-1}							
Function measured	$\frac{K_{ia}K_b}{V}$	$\frac{K_a}{V}$	$\frac{K_b}{V}$	$\frac{1}{V}$	$\frac{K_{ia}K_b}{V}$	$\frac{K_b}{V}$	$\frac{K_a}{V}$	$\frac{1}{V}$
(Value)	8.42×10^{-8}	2.2×10^{-5}	5.38×10^{-3}	1.25	8.4×10^{-8}	5×10^{-3}	2.81×10^{-5}	1.3
Parameter calculated	K_{ia}	K_a	K_b	V	K_{ia}	K_b	K_a	V
(Value)	1.56×10^{-5}	1.76×10^{-5}	4.304×10^{-3}	0.8	1.68×10^{-5}	3.84×10^{-3}	2.16×10^{-5}	0.769
Mean values	4-PCA: $V_{max} = 7.8\pm0.2\times10^{-1}$ Abs min^{-1} ml E. $K_b = 4.0\pm0.3$ mM, $K_a = 1.96\pm0.2\times10^{-5}$ ml^{-1}, $K_{ia} = 1.62\pm0.04\times10^{-5}$ ml^{-1}							

enzyme; and (c) in the presence of enzyme, NADH is converted to NAD^+ at the same time as the aldehyde group of 2-PCA is reduced. We conclude that NADH reduces 2-PCA, the reduction being catalysed by YADH.

The K_m determined for NADH in the experiments on 3- and 4-PCA is of the order of 10^{-5} M, which agrees well with other published values[7]. Thus, if the K_m for NADH in reaction with 2-PCA is of the same order, there should be a similar dependence of initial rate on NADH concentration: one possible reason why no such dependence is observed is that K_m for this substrate is less than 10^{-5} M, perhaps as low as 10^{-6}. If this is the case then the enzyme would be saturated at a concentration of 10^{-5} M NADH—let alone 10^{-4}—and no rate dependence would be observed. In this case it is necessary to explain how 2-PCA is affecting the affinity of the enzyme for NADH. One possibility is that binding of 2-PCA causes a conformational change in the enzyme, which affects the affinity of the enzyme for NADH and brings about saturation. (It is not inconceivable that this could occur for 2-PCA and not for 3- and 4-, since 2-PCA *may* chelate, while 3- and 4- cannot).

As a result of insensitivity to [NADH], the parameters K_{ia} and K_a could not be determined as they were for 3- and 4-PCA. The values K_m and V_{max} were obtained, however, from the slope and intercept of Fig. 5, i.e. by the Lineweaver–Burk method[8]. The values are $K_m = 1.8 \pm 0.2$ mM, $V_{max} = 2.85 \pm 0.3$ abs. units/min cm^{-3}. The K_m and V_{max} values for each substrate are summarised in Table 2.

DISCUSSION

The nitrogen atom in a pyridine molecule may be regarded as sp^2 hybridised, with the lone pair directed away from, but in the plane of, the aromatic ring. As a result of the π-system of the molecule, the nitrogen attracts π-electrons and confers positive charges on the other ring atoms[7], as calculated electron densities show (Fig. 6). The effect of this charge depletion at the ring atoms on substituents at those positions is well illustrated by the decarboxylation of pyridine 2,3,4-tricarboxylic acid, which proceeds in the order 2 > 4 > 3[10]. Thus the effect of a nitrogen on the carbon of a carbonyl substituent may be regarded as being one of electron depletion, and the magnitude of such depletion is in the order 2 > 4 > 3.

The coordination of the pyridine nitrogen to a zinc ion will be *via* the sp^2 lone pair orbital. Since zinc is d^{10}, there cannot be any nitrogen π-density donation to the zinc—while the reverse (zinc to pyridine π^* system) although possible is unlikely in view of the effect of added zinc on the ligand NMR spectrum[2]. This is not to say, however, that coordination of the zinc ion cannot enhance the existing π-electron shift, since depletion of charge on the nitrogen might lead to an electrostatically-induced enhancement of shifts in the π-system[11], which then results in a greater depletion of charge on the ring atoms.

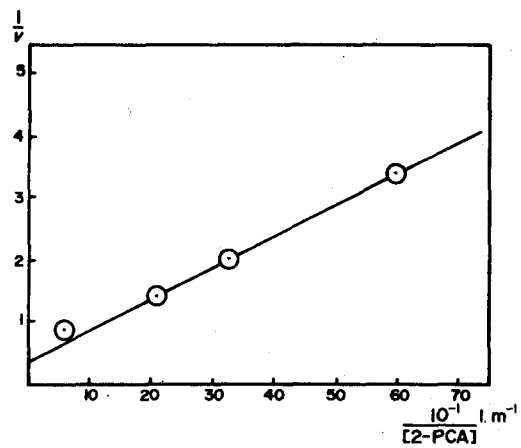

Fig. 5. Lineweaver Burke plot for the reduction of 2-PCA.

Table 2. K_m and V_{max} values for 2-, 3- and 4-PCA

Substrate	K_m (mM)	V_{max} (abs u min^{-1} ml^{-1})
2-PCA	1.8 ± 0.2	2.85 ± 0.3
3-PCA	7.7 ± 0.3	$2.86 \pm 0.1 \times 10^{-1}$
4-PCA	4.0 ± 0.3	$7.8 \pm 0.2 \times 10^{-1}$

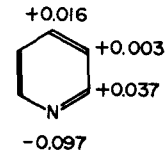

Fig. 6. Calculated electron densities on the ring atoms of pyridine from Ref. [9].

The above facts are of significance in considering the mechanism of the enzymic reduction of the three aldehydes reported here, where there are *a priori* three distinctly different possibilities to consider in regard to the function of the metal ion at the active site:

(1) *Both substrate and cofactor bind to the metal ion.* The results of the non-enzymic reduction[2] show conclusively that the binding of a pyridine aldehyde to a metal-ion centre occurs *via* the nitrogen in a free metal-ion-PCA interaction. Unless the protein groups at the active site exert a highly specific effect it is difficult to envisage a mechanism whereby such a situation should be reversed in an enzymic environment to cause ligation to occur *via* the aldehyde oxygen and we shall assume that, if substrate-metal interaction occurs, the binding is the same. Furthermore, the relative K_m's support this assumption. If the binding in the enzyme was *via* the aldehyde oxygen, it would be expected that the K_m order would be the opposite of the order of electron densities at the oxygen—which would reflect that at the relevant pyridine carbon. Figure 6 shows this order would be 3 > 4 > 2. Table 2 gives the relative K_m's as 2 < 4 < 3—the opposite of that required for binding *via* the oxygen. Similarly, bearing in mind the binding of NADH (or NBDN) to a free metal centre, the assumption must be that *if* there is any NADH/metal ion interaction in the enzyme it is *via* the amide oxygen.

Admitting these two assumptions, it is difficult to understand how 3- and 4-PCA are substrates in the enzymic, and not in the non-enzymic reaction. It is, of course, possible that there is on the enzyme some moiety which can stabilise the increasing negative charge on the aldehyde oxygen as reaction proceeds *while* that aldehyde is locked in position by coordination *via* the nitrogen. This would undoubtedly facilitate reaction in

the enzymic case, and would be a satisfactory explanation of the observed facts were it not for the other difficulty imposed by steric requirements—viz. that it is impossible (without invoking exotic hydride-shuffling mechanisms) to explain how a metal-bound NADH can offer direct hydride transfer to a metal-bound 3-PCA and 4-PCA, being already in a position to do so with 2-PCA.

Consequently, since all three aldehydes are substrates, it is probable that NADH and the aldehyde do not bind to the same metal ion centre.

(2) *The cofactor binds to the metal; the substrate binds elsewhere.* Assuming that NADH interacts with the metal ion via the amide oxygen, and is thus in a position to offer the hydride to the substrate, the question of where and how the substrate binds is a matter of conjecture. If the binding site is a form of hydrophobic pocket—not involving a formal positive charge—then it is unlikely that the relative K_m's for the three substrates should be $2<4<3$—rather $2<3<4$, since this is the relative order of numbers of aromatic unsubstituted carbons available to bind in such a hydrophobic site. Furthermore, the relative V_{max}'s would not be $2>4>3$, but either $2>3>4$, or $4>3>2$, since whichever of 2- or 4- is the most accessible in such a binding scheme, 3- *must* be the next most accessible.

If the substrate binding is to some other positive centre rather than a hydrophobic pocket, then again the assumption must be that the binding is *via* the nitrogen. This being so, and since NADH is assumed to be "locked", it is difficult to understand how all three aldehydes are substrates for the same reasons as discussed in part 1 of this discussion.

(3) *Substrate binds to metal ion; cofactor binds elsewhere.* Figure 6 shows that the relative sizes of electron depletion at the 2-, 3- and 4-positions of a pyridine molecule are $2>4>3$. The NMR results[2] show that binding of a zinc ion to the pyridine nitrogen in 2-PCA effectively enhances this situation (if not each to the same extent). The relative K_m's indicate a positive centre: pyridine nitrogen interaction, since the electron density at the nitrogen in each case will be $2>4>3$, since the aldehyde at the 3-position will withdraw electrons inductively and therefore reduce the electron density at nitrogen.

The ratio of the electron densities in Fig. 6 is 10:1:5 for the 2:3:4, which compares well with the V_{max} ratio reported above of 10:1:3.

The consequence of the observation is that the dihydro moiety of enzyme-bound NADH must be regarded as being able to move freely in order to reduce each aldehyde while that aldehyde is coordinated *via* a remote atom to the enzymic zinc. Therefore the NADH does not interact with the zinc ion (since it cannot be the nicotinamide ring that is involved in binding, and since[1, 2] the preferred binding site of a zinc ion to NADH is that nicotinamide ring). The conclusions reached here regarding the remote binding of NADH—since this would allow the greatest freedom of movement of the nicotinamide moiety of the coenzyme—are consistent with other evidence on the YADH/NADH interaction which is reviewed elsewhere[12].

To summarise, we conclude that the substrate probably interacts with the zinc ion in YADH, and the NADH cofactor does not.

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