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Inhibition of Nicotinamide Phosphoribosyltransferase by Pyridine Nucleotides†

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ABSTRACT: The ability of various pyridine bases (nicotinamide nucleoside) and various NAD analogs to inhibit rat liver nicotinamide phosphoribosyltransferase was determined. All pyridine nucleotides in the oxidized state containing nicotinamide bound to ribose in the β configuration were found to be strong noncompetitive inhibitors of nicotinamide phosphoribosyltransferase when nicotinamide was the variable substrate. Under the same conditions, all the pyridine bases examined, nicotinamide riboside and β-$NMNH_2$, were found to be competitive inhibitors. Pyridine dinucleotide derivatives which are reduced, exist in the anti form of geometric distribution between the pyridine ring and the D-ribofuranose, or lack the β-ribosyl linkage, were found to be weak inhibitors of nicotinamide phosphoribosyltransferase activity. These kinetic data are correlated with known molecular configuration and geometric distributions of the inhibiting compounds. The nicotinamide and PRPP binding sites appear to be adjacent to one and the other since β-NMN, the product of the reaction, appears to occupy both sites simultaneously. The binding of the 5′-phosphate of the second substrate, PRPP, or the product, NMN, is apparently essential since loss of this binding group, as in the case of nicotinamide riboside, results in loss of "PRPP site" affinity but retention of "nicotinamide site" affinity. The configuration of the ribose of the pyridine nucleotide is critical since changes in configuration of the ribose, as occurs during pyridine nucleotide reduction, result in distinct changes in the ability to bind at the nonnicotinamide portion of the active site as measured by the K_i intercept. The geometric disposition between the pyridine base and the D-ribofuranose ring is also critical since compounds which exist in the anti form apparently do not bind at the nonnicotinamide portion of the active site yet have affinity for the nicotinamide binding site as measured by changes in K_i intercept and K_i slope, respectively.

Nicotinamide phosphoribosyltransferase (EC 2.4.2.12) has been purified from rat liver (Dietrich *et al.*, 1966). The enzyme requires ATP in addition to 5-phosphoribosyl 1-pyrophosphate (PRPP), the role of ATP being apparently that of a modifier (Powanda *et al.*, 1969). The reaction is as follows: In the absence of ATP, the apparent K_m for nicotinamide is 0.1 M (L. S. Dietrich, unpublished data) as compared to an apparent K_m for nicotinamide of 1×10^{-6} M in the presence of ATP (Dietrich *et al.*, 1966). Furthermore, ATP addition results in a 12-fold increase in V_{max} (Dietrich, unpublished data). These observations, together with those indicative that NAD as well as the product of the reaction, NMN, inhibit enzymatic activity (Dietrich and Muniz, 1966), led to an evaluation of the nature of the inhibition produced by these compounds. A preliminary report of these studies has appeared (Dietrich and Muniz, 1967).

$$\text{nicotinamide} + \text{PRPP} \xrightarrow{\text{ATP}} \text{NMN} + \text{PP}_i \qquad (1)$$

Materials and Methods

Enzymatic Material. A rat liver preparation (fraction B) reported previously (Powanda *et al.*, 1969) was used throughout. The preparation had a specific activity of *ca.* 0.18 μmole of NMN formed per hr per mg of protein, was free of inorganic pyrophosphatase, and contained no detectable NADase, NAD kinase, NAD pyrophosphorylase, nicotinic acid phosphoribosyltransferase, nicotinamide deamidase, or ATPase. No enzymatic degradation of NMN or PRPP could be observed under the conditions in which the experiments were carried out. The preparation was obtained by DEAE-cellulose chromatography of the $(NH_4)_2SO_4$ fraction described by Dietrich *et al.* (1966), and represented around a 300-fold purification. Recent studies utilizing enzymatic preparations having 20–30 times the specific activity of the preparation employed here have yielded identical results in all cases. Enzymatic activity was assayed as previously described (Powanda *et al.*, 1969). The assay involves the chromatographic separation in paper of the product, [^{14}C]NMN, from the substrate, [^{14}C]nicotinamide, and the subsequent quantitation of the product employing liquid scintillation spectrometry. This procedure is very reproducible. Duplicate values are normally within 1% of each other at all levels of substrate and inhibitor employed and values that varied more than 2% were generally discarded.

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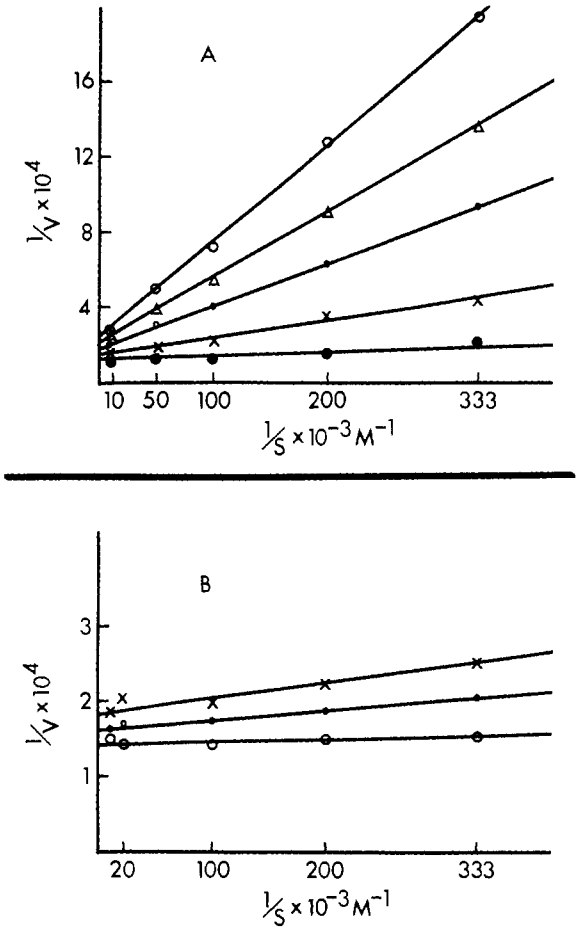

FIGURE 1: Double-reciprocal plots of nicotinamide phosphoribosyltransferase activity varying the concentration of nicotinamide in the presence of various concentrations of NAD and NADH. Each reaction flask contained: 0.7 μmole of PRPP, 2.0 μmoles of ATP, 10 μmoles of $MgCl_2$, 50 μmoles of Tris (pH 8.0), and enzyme in a total volume of 1 ml. The specific activity of the [^{14}C]nicotinamide was kept constant (8.0 μCi/μmole). Incubation was carried out at 37° for 1 hr. [S] = nicotinamide; V = cpm/reaction vessel per hour. The concentrations of NAD shown in part A are: (●) none, (×), 2.2×10^{-4} M, (○) 4.5×10^{-4} M, (△), 7.2×10^{-4} M, and (○) 9.0×10^{-4} M. The concentrations of NADH shown in part B are: (○) none, (○) 5.0×10^{-4} M, and (×) 1.0×10^{-5} M.

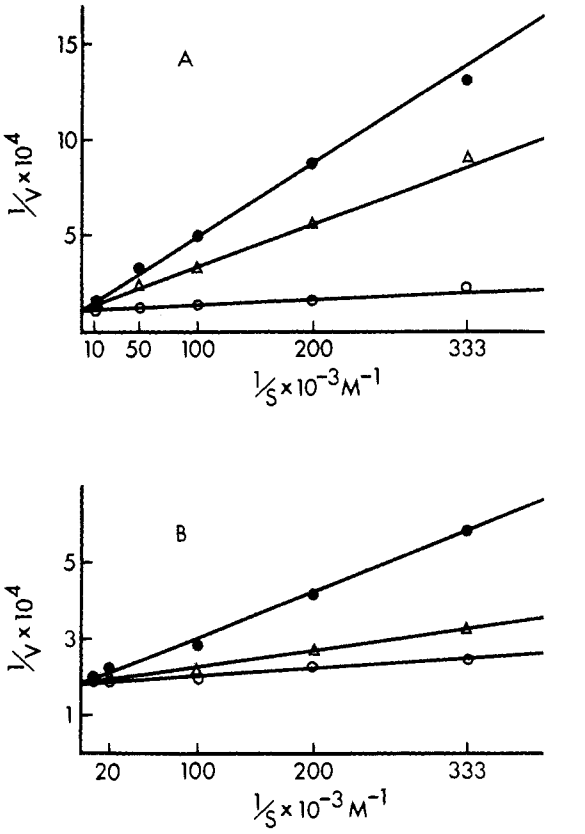

FIGURE 2: Double-reciprocal plots of nicotinamide phosphoribosyltransferase activity varying the concentration of nicotinamide in the presence of various concentrations of $NMNH_2$ and 3-acetylpyridine–AD. See Figure 1 for experimental details. The concentrations of $NMNH_2$ shown in part A are: (○) none, (△) 2.5×10^{-4} M, (●) 5.0×10^{-4} M. The concentrations of 3-acetylpyridine-adenine dinucleotide shown in part B are: (○) none, (△) 5.0×10^{-4} M, and (●) 1.0×10^{-3} M.

Analysis of Kinetic Data. All kinetic data were processed according to Cleland (1963a) employing an IBM 7040 computer. The nomenclature of reaction mechanisms and description of kinetic constants employed here are those proposed by Cleland (1963b). After preliminary plots were made of the data in the double-reciprocal form ($1/V$ *vs.* $1/[S]$), iterative least-square fits were made to eq 2. Since in all cases

$$v = \frac{V[S]}{K + [S]} \qquad (2)$$

these data fit a straight line, least-square fits to other equations were unnecessary. Replots of slopes and intercepts obtained from this analysis were made against inhibitor concentration to determine the numerical value of the K_i slope and K_i intercept. The standard errors of the K_i values were determined by utilizing eq 2 in an analysis of the replot data.

[^{14}C]Nicotinamide was purchased from New England Nuclear Corp. Immediately upon receipt, the compound was dissolved in water and stored frozen. Periodic analysis of this compound by comparison of its R_F value in three different chromatographic solvents indicated that under these conditions no detectable deterioration occurs. PRPP, thionicotinamide–adenine dinucleotide, 3-acetylpyridine–adenine dinucleotide, nicotinamide–hypoxanthine dinucleotide, and NMN were purchased from P-L Biochemicals, Milwaukee, Wis. α-NAD and ATP were purchased from Sigma Chemical Co., St. Louis, Mo. Nicotinate–adenine dinucleotide was the generous gift of Dr. N. O. Kaplan, University of California at San Diego.

NMN was reduced chemically employing a modification of the procedure used for reducing NAD (Lehninger, 1957). Aqueous solutions of $NMNH_2$ were stored at −20° and used within 48 hr. Storage under these conditions for longer periods of time resulted in the decomposition of $NMNH_2$.

Results

When nicotinamide was used as the variable substrate and NAD was the inhibitor varied, the double-reciprocal plots (Figure 1A) were linear. Replots of values obtained from fits to eq 2 against the fixed levels of NAD were also linear and demonstrated noncompetitive inhibition. The K_i slope and K_i intercept values obtained from these replots are shown in Table I. Similar results have previously been obtained employing NMN (Powanda *et al.*, 1969). K_i slope and K_i intercept values for NMN calculated from these data are pre-

TABLE I: Summary of Replot Data Obtained from Computer Analysis Employing Nicotinamide as the Variable Substrate.

Inhibitors	μM ± Std Dev			
	K_i Slope	t^a	K_i Intercept	t^a
NAD	40 ± 12		340 ± 12	
NADP	5 ± 30	<3	470 ± 40	3.1
NADH	300 ± 84	4.3	3400 ± 22	122
NADPH	350 ± 55	5.5	2800 ± 40	58
α-NAD	90 ± 20	<3	5550 ± 32	153
Nicotinate–AD	4500 ± 87	51	1000 ± 60	11
Nicotinamide–hypoxanthine dinucleotide	25 ± 20	<3	340 ± 40	<3
Thionicotinamide–AD	6 ± 10	<3	500 ± 59	<3
3-Acetylpyridine–AD	100 ± 40	<3	$\rightarrow\infty$	>200
NMN	9 ± 8	<3	45 ± 20	8.7
$NMNH_2$	22 ± 17	<3	$\rightarrow\infty$	>200
Nicotinamide riboside	4 ± 25	<3	$\rightarrow\infty$	>200
Thionicotinamide	0.7 ± 3	3.3	$\rightarrow\infty$	>200
5-Fluoronicotinamide	120 ± 54	<3	$\rightarrow\infty$	>200
3-Acetylpyridine	7 ± 41	<3	$\rightarrow\infty$	>200
6-Aminonicotinamide	20 ± 10	<3	$\rightarrow\infty$	>200

[a] A t value of 4 is significant at the 1% level. t values were calculated according to Cleland (1967).

sented in Table I. Further studies were carried out to determine the degree and type of inhibition produced by pyridine nucleotide derivatives. Compounds were compared where there were modifications of the AMP and NMN moieties of the dinucleotide. In addition, nicotinamide nucleoside and various pyridine bases were studied.

Modification of the AMP Moiety of NAD. Double-reciprocal plots obtained employing various concentrations of hypoxanthine–adenine dinucleotide, when nicotinamide was used as the variable substrate, were linear. Replots of values demonstrated a linear noncompetitive inhibition very similar to that observed with NAD. K_i slope and K_i intercept values thus obtained are shown in Table I. Similar results were obtained employing several fixed levels of NADP. K_i slope and K_i intercept values thus obtained are shown in Table I.

Modification of the NMN Moiety of NAD. Double-reciprocal plots obtained employing various concentrations of thionicotinamide–adenine dinucleotide, when nicotinamide was used as the variable substrate, were linear. K_i slope and K_i intercept values calculated from replots of the slope and intercept values obtained employing thionicotinamide–adenine dinucleotide are presented in Table I. The substitution of sulfur for oxygen on the carbon in position 7 of the nicotinamide molecule had little effect on the type or degree of inhibition.

When NADH was used as the inhibitor, the double-reciprocal plots were linear (Figure 1B). Replots of the slope and intercept values were linear within the precision of the data. K_i slope and K_i intercept values calculated from these data are shown in Table I. NADH is such a weak antagonist that one cannot, on the basis of the data, clearly define the type of inhibition. Similar results were obtained with NADPH (Table I). $NMNH_2$, on the other hand, is a strong competitive inhibitor of nicotinamide phosphoribosyltransferase (Figure 2A), exhibiting a K_i slope similar to that of NMN (Table I). The K_i intercept, however, was significantly higher than that observed with NAD.

Similar studies employing nicotinate–adenine dinucleotide indicate that substituting a carboxyl grouping for an amide grouping in the pyridine moiety produces a very weak antagonist of nicotinamide phosphoribosyltransferase activity, the K_i slopes and K_i intercepts observed being significantly higher than those obtained with NAD (Table I).

Double-reciprocal plots of the data obtained employing 3-acetylpyridine–adenine dinucleotide are shown in Figure 2B. The double-reciprocal plots were linear and indicated a linear competitive inhibition (Table I). When the geometric disposition between the pyridine base and the D-ribofuranose ring is changed from syn to anti, as occurs when 3-acetylpyridine–adenine dinucleotide is substituted for NAD, the degree of inhibition observed is markedly altered (Table I).

Alteration of the configuration of the pyridine–ribose bond, as in the case of α-NAD, markedly affects the degree and probably the type of inhibition.[1] Double-reciprocal plots of the data are presented in Figure 3A. As in the case of NADH, the K_i intercept is significantly higher than that observed with NAD.

The double-reciprocal plots obtained with nicotinamide nucleoside are presented in Figure 3B. Removal of the phosphate from the pyridine nucleotide NMN produces a competitive inhibition in contrast to the noncompetitive inhibition produced by NMN or NAD. The K_i slope and K_i intercept calculated from replots of double-reciprocal plots are shown in Table I.

All the pyridine bases investigated, 6-aminonicotinamide, 5-fluoronicotinamide, 3-acetylpyridine, and thionicotinamide,

[1] The α-NAD employed contained approximately 4% β-NAD as determined by enzymatic assay. This amount of β-NAD could not have produced the slight noncompetitive inhibition observed employing α-NAD.

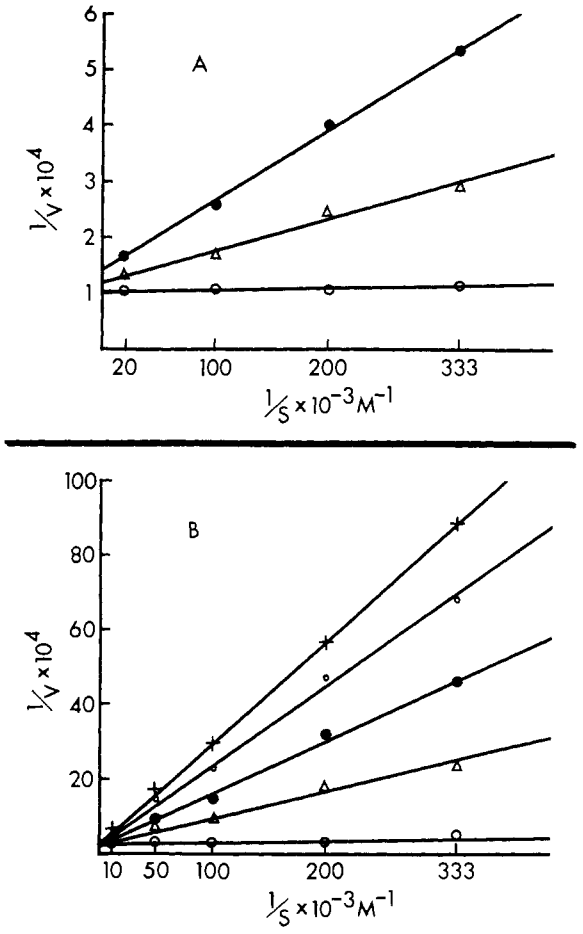

FIGURE 3: Double-reciprocal plots of nicotinamide phosphoribosyltransferase activity varying the concentrations of nicotinamide in the presence of various concentrations of α-NAD and nicotinamide riboside. See Figure 1 for experimental details. The concentrations of α-NAD shown in part A are: (○) none, (△) 5.0×10^{-4} M, and (●) 1.0×10^{-3} M. The concentrations of nicotinamide riboside shown in part B are: (○) none, (△) 3.6×10^{-5} M, (●) 7.2×10^{-5} M, (◦) 1.1×10^{-4} M, and (×) 1.4×10^{-4} M.

were found to be linear competitive inhibitors of nicotinamide phosphoribosyltransferase when nicotinamide was used as the variable substrate. The K_i slope and K_i intercept data are presented in Table I.

Discussion

The data demonstrate that inhibition of rat liver nicotinamide phosphoribosyltransferase by pyridine derivatives is in general either linear competitive or linear noncompetitive. In all cases studied, except nicotinate–adenine dinucleotide, the K_i slope values are smaller than the K_i intercept values. All of the free pyridine bases, nicotinamide nucleoside, $NMNH_2$ and 3-acetylpyridine–adenine dinucleotide, appear within the precision of the experiments to be competitive inhibitors, *i.e.*, they have K_i intercepts which approach infinity. All of the other pyridine derivatives studied appear to be linear noncompetitive with varying K_i slopes and K_i intercepts depending on structural modification.

If one postulates that the K_i slope results primarily from combination at the nicotinamide binding site ("nicotinamide site") and that the K_i intercept results primarily from combination at that portion of the active site not involved in nicotinamide binding ("PRPP site"), the overall result is the combination of all these separate effects.[2]

"Nicotinamide Site" Binding. Free pyridine bases combine primarily with the nicotinamide site as probably do nicotinamide nucleoside, $NMNH_2$, and pyridine dinucleotides, where the amide nitrogen has been replaced by other groupings, *i.e.*, CH_3. The substitution of sulfur for the carbon-7 in the nicotinamide has no effect on binding nor does the addition of fluorine on the 5 position of the pyridine ring. Affinity for the nicotinamide site as measured by K_i slope is significantly decreased when the configuration of the ribose attached to the pyridine is altered, as in the case of NADH or NADPH. The addition of a phosphate grouping on the 2 position of the ribose of the adenine nucleotide moiety has no effect on the affinity of the dinculeotide for the nicotinamide site.

Non-nicotinamide Active-Site Binding (PRPP Site). β-NAD, β-NADP, NADH, thionicotinamide–adenine dinucleotide, and β-NMN bind to the PRPP site as indicated by K_i intercept values (Table I). The oxidized forms of 3-acetylpyridine–adenine dinucleotide bind significantly less than, *e.g.*, β-NAD, exhibiting a K_i intercept that approaches infinity. α-NAD and nicotinate AD have significantly less affinity to the PRPP site than does β-NAD. Reduction of β-NAD, β-NADP, and β-NMN produced a marked decrease in the affinity for the PRPP site. In the case of $NMNH_2$, the K_i intercept (Table I) approached infinity, indicating that there is virtually no binding of $NMNH_2$ at the PRPP site.

That pyridine dinucleotide exists in solution in more than one form has been demonstrated by fluorescence studies (Weber, 1957; Velick, 1958), nuclear magnetic resonance (nmr) spectra (Majer *et al.*, 1962; Jardetzky and Wade-Jardetzky, 1966; Sarma *et al.*, 1968; Sarma and Kaplan, 1969a,b, 1970a,b), optical rotatory dispersion (ORD) (Bender and Grisolia, 1968), or chemical relaxation as demonstrated by the temperature-jump method (Czerlinski and Hommes, 1964).

The studies of Sarma and Kaplan utilizing nmr data to determine the geometric disposition of oxidized and reduced pyridine nucleotides are of particular interest. These studies indicate that the ribose conformation attached to the pyridine is distinctly different in oxidized and reduced pyridine nucleotides. The conformation of the D-ribose attached to the adenine moiety of oxidized and reduced nicotinamide–adenine dinucleotide, nicotinamide–adenine dinucleotide phosphate, and the corresponding hypoxanthine analog is indicated by Sarma and Kaplan (1970a,b) to be $C_{3'}$-exo,$C_{4'}$-endo.[3] The conformation of the D-ribose attached to the dihydropyridine ring of reduced pyridine dinucleotides is $C_{2'}$-endo, $C_{3'}$-exo. These changes may be very conspicuous and it is thought that the geometric alterations resulting in the reduction of the pyridine nucleotides are continued to the

[2] It is realized that both slope and intercept will be affected by any inhibitor capable of combining with free enzyme provided the ordered mechanism of Powanda *et al.* (1969) is correct. Such an inhibitor could conceivably combine at (or near) either the PRPP or nicotinamide site to cause noncompetitive inhibition. However, for the sake of simplicity throughout this paper, changes in K_i slope and K_i intercept will be attributed primarily to changes at the "nicotinamide site" and "PRPP site," respectively.

[3] X-Ray and neutron diffraction data (Sundaralingam, 1965, 1969) indicated that the puckering in the furanose ring involves the $C_{2'}$ or $C_{3'}$ atom, resulting in four conformeric possibilities: $C_{2'}$-endo, $C_{3'}$-exo, $C_{3'}$-endo, and $C_{2'}$-exo. The $C_{3'}$-exo,$C_{4'}$-endo configuration has not been observed to date in β-nucleotides employing this method. Regardless of the exact changes in configuration, these studies indicate a change in configuration of the furanose ring during the reduction of NAD.

diphosphate backbone. Furthermore, the geometric disposition between the pyridine base and the D-ribofuranose ring is syn in α-NAD, β-NAD, β-NADH, and 3-acetylpyridine–ADH, and anti in the acetylpyridine analogs of β-NAD and NADP.

Utilizing these interpretations of the nmr data, the kinetic studies presented in Table I may be employed to speculate on the spacial configuration of the nicotinamide and PRPP binding sites of rat liver nicotinamide phosphoribosyltransferase. The nicotinamide and PRPP binding sites presumably lie adjacent to each other since β-NMN, the product of the reaction, appears to occupy both sites simultaneously. It would appear that binding of the 5′-phosphate of the second substrate, PRPP, or the product, NMN, is essential since loss of this binding group, as in the case of nicotinamide riboside, results in loss of PRPP site affinity but retention of nicotinamide site affinity. The $C_{3'}$-exo,$C_{4'}$-endo configuration of the ribose of the pyridine nucleotide appears also to be critical since conversion of the ribose configuration into the $C_{2'}$-endo,$C_{3'}$-exo form results in significantly weaker binding of either pyridine mono- or dinucleotide to the PRPP site. This can be seen in Table I by comparing the K_i intercepts obtained employing β-NAD, β-NADP, and β-NMN with those observed with β-NADH, β-NADPH, and β-$NMNH_2$. The geometric disposition between the pyridine base and the D-ribofuranose ring appears also to be critical since when 3-acetylpyridine-AD, which exists in the anti form, is substituted for the β-NAD (syn form), marked changes in the affinity for the PRPP site, as indicated by an increase in K_i intercept, are observed (Table I). This is not due to an effect on the 3-acetylpyridine portion of the molecule since 3-acetylpyridine alone has a high affinity for the nicotinamide binding site (Table I).[4]

A scheme of enzymatic reactions presented in Figure 4 is compatible with the kinetic data and the interpretations of the nmr data as presented by Sarma and Kaplan. The credibility of such a scheme must await more detailed studies on the enzymatic structure.

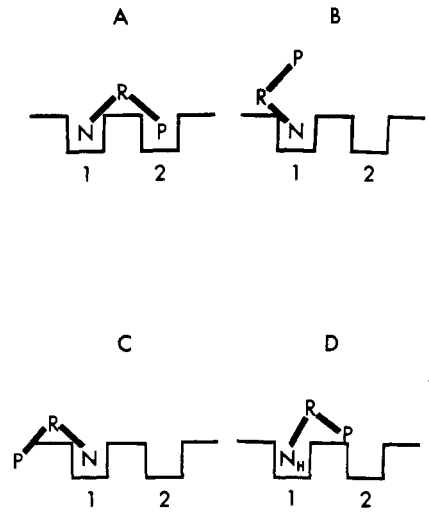

FIGURE 4: Schematic representation of the active site of nicotinamide phosphoribosyltransferase. N–R–P = nicotamide–ribose–phosphate, 1 and 2 are the nicotinamide and 5′-phosphate binding sites, respectively. (A) β-cis NMN, (B) α-cis NMN, (C) β-anti NMN, and (D) β-$NMNH_2$.

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[4] Unpublished data from this laboratory indicate that of the pyridine bases studied, two of them (3-acetylpyridine and thionicotinamide) can serve effectively as substrates for nicotinamide phosphoribosyltransferase. 6-Aminonicotinamide and 5-fluoronicotinamide do not serve as substrates for this system. The K_i slopes obtained employing these compounds are not statistically different from those observed with NAD.