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Studies on the Mechanism of Rat Liver Nicotinamide Mononucleotide Pyrophosphorylase*

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ABSTRACT: Preparations of rat liver have been shown to possess a nicotinamide mononucleotide: pyrophosphate phosphoribosyl transferase (EC 2.4.2.12) which specifically requiresadenosine triphosphate, as well as 5-phosphoribosyl 1-pyrophosphate. These same preparations will synthesize nicotinamide mononucleotide in the absence of adenosine triphosphate if the concentration of both 5-phosphoribosyl 1-pyrophosphate and magnesium is greatly increased. Similar heat labilities, identical gel electrophoretic patterns, and molecular weights indicate that nicotinamide mononucleotide synthesis stimulated by adenosine triphosphate or high levels of 5-phosphoribosyl 1-pyrophosphate and magnesium is probably catalyzed by the same enzyme. These data, together with an analysis of the kinetics of cotinamide mononucleotide formation in the presence and in the absence of adenosine triphosphate, lead to the suggestion that adenosine triphosphate is a positive allosteric effector of nicotinamide mononucleotide pyrophosphorylase. Analysis of the product inhibition data for nicotinamide mononucleotide synthesis in the presence of adenosine triphosphate and initial velocity patterns of the reaction in the absence of adenosine triphosphate indicate that the mechanism for the enzymatic formation of nicotinamide mononucleotide is either an ordered sequence beginning with the binding of 5-phosphoribosyl 1-pyrophosphate and culminating in the release of nicotinamide mononucleotide, or an Iso–Theorell–Chance mechanism, in which case the order of substrate binding cannot be predicted from the data.

Rat liver NMN pyrophosphorylase requires ATP as well as nicotinamide and PRPP (Dietrich *et al.*, 1966), and is markedly inhibited by physiological levels of NAD (Dietrich and Muniz, 1966). The enzyme fraction which synthesizes NMN in the presence of ATP will also form NMN in the absence of ATP if the levels of both PRPP and magnesium are greatly increased (Powanda *et al.*, 1968). In addition, the activity of this enzyme is decreased some 40% by adrenalectomy or hypophysectomy (Dietrich *et al.*, 1967). These data suggested that nicotinamide mononucleotide pyrophosphorylase is a likely point of control in pyridine nucleotide metabolism and that ATP may be a positive agent of control. The following presents a probable mechanism for enzymatic NMN formation together with the evidence that ATP does act as a positive allosteric effector.

Methods and Materials

Enzymatic Material. The enzyme was purified as previously reported (Dietrich *et al.*, 1966). This preparation (fraction A), or a more purified fraction (fraction B), was used throughout as indicated. The latter preparation, which has a specific activity of *ca.* 200,000 cpm/mg of protein and is free of inorganic pyrophosphatase, was prepared by heating fraction A at 55° for 5 min and centrifuging at 20,000*g* for 15 min. The resultant supernatant material was used and represents a 300-fold purification over the homogenate. Neither fraction A nor B contains any detectable NAD glycohydrolase, nicotinate phosphoribosyl transferase, NAD pyrophosphorylase, nicotinamide deamidase, or NAD kinase. Enzymatic destruction of NMN and PRPP cannot be observed. ATP degradation measured by luciferin–luciferinase assay (Strehler and McElroy, 1957) is, under the assay conditions, less than 4%.

Enzymatic Assay. This is the same as previously de-

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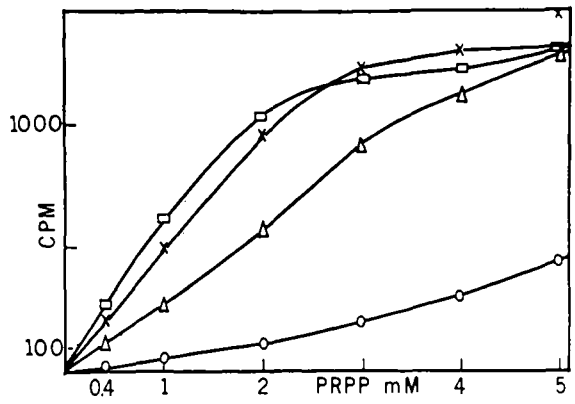

FIGURE 1: [^{14}C]NMN synthesized at various ratios of magnesium to PRPP. (○) 2:1, (△) 5:1, (×) 10:1, and (□) 15:1. Nicotinamide was constant at 1×10^{-4} M and had a specific activity of 10 μCi/μmole. The reaction was incubated for 1 hr. At this time the reaction was stopped by boiling and 100-μl aliquots were assayed for radioactivity. Duplicates were run and their values were averaged. Reaction mixtures void of PRPP were run at the same time and used for background corrections. The range of the duplicates for the ratios of magnesium to PRPP, 10:1 and 15:1, at the 5×10^{-3} M PRPP level overlap. Fraction A was the enzyme source.

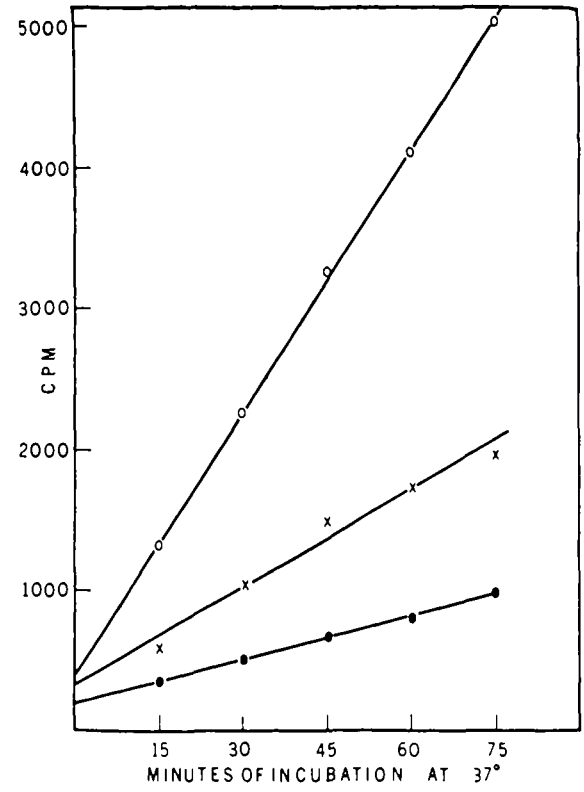

FIGURE 2: NMN synthesis with time. Nicotinamide (10 μCi/μmole) was constant at 1×10^{-4} M. The ratio of magnesium to PRPP was 12:1 in all cases. (●) 6×10^{-4} M PRPP, (×) 1.2×10^{-3} M PRPP, and (○) 4×10^{-3} M PRPP. The assay was run in duplicate and the results were averaged. Fraction A was the enzyme source. Data are uncorrected for background or efficiency.

scribed (Dietrich *et al.*, 1966), except that the buffer used in the present studies is 0.05 M Tris-Cl (pH 8).

Determination of Radioactive Background. Measurement of the background consists of the counting of areas of a paper chromatogram corresponding to the expected location of NMN after chromatographing boiled reaction mixtures containing only enzyme and the levels of [^{14}C]nicotinamide used in each experiment. This procedure eliminates the possibility that observed activity was due merely to the "tailing" of the labeled substrate.

Analysis of Kinetic Data. The kinetic data were analyzed using an IBM 7040 computer and FORTRAN program, supplied by Dr. W. W. Cleland of the University of Wisconsin. The use of these programs is described in detail (Cleland, 1963a).

Disc Gel Electrophoresis Studies. A Canalco 5% separating gel system was used with a Buchler polyanalytic electrophoresis apparatus. After preparation of the gels, 0.1-ml samples of the enzyme diluted 1:2 in 40% sucrose were placed atop the spacer gel. The amperage was maintained at 1.25 mA/tube until the sample entered the separating gel (length 2.25 in.), at which time the current was raised to 2.5 mA. When the tracking dye reached a point 4–5 mm from the bottom of the gel, the run was considered complete.

Reagents. [7-^{14}C]Nicotinamide (10–11 mCi/mmole) was purchased from the New England Nuclear Corp. ATP was obtained as the sodium salt from Sigma Chemical Co., while NMN was obtained from P & L Biochemicals. The concentrations of the reagents were checked either spectrophotometrically or, in the case of nicotinamide, by measuring the radioactivity. PRPP was purchased as the magnesium salt from P & L Biochemicals. The concentration of solution of PRPP was assumed to be that which results from dissolving a known weight in a given volume. Chromatographic assay using labeled orotate and the orotidine 5′-phosphate pyrophosphorylase isolated from yeast (Flaks, 1963) supported this assumption. A 1×10^{-2} M solution of Mg_2PRPP exhibited an ultraviolet spectrum similar to that of sodium pyrophosphate, there being no defined peak. If the difference in absorbance were due to ATP, the level of contamination would be 0.1%. This is $^1/_{50}$ the amount of ATP which would be needed to produce enzymatic activity equivalent to that seen at high levels of PRPP. Thus, it was assumed that the stimulation produced by PRPP is not due to ATP contamination.

Results

The synthesis of NMN, in the absence of ATP, as a function of PRPP concentration is shown in Figure 1. The magnitude of response is markedly affected by the amount of magnesium present. Double-reciprocal plots of these data are not linear. The upward curvature at lower levels of substrate observed in the double-reciprocal plots is apparently not an artifact occasioned by a differential breakdown of PRPP as a function of concentration. This is demonstrated in Figure 2 which shows that at a magnesium to PRPP ratio of 12:1, NMN synthesis is linear with time for concentrations of PRPP which span the range used in the experiment depicted in

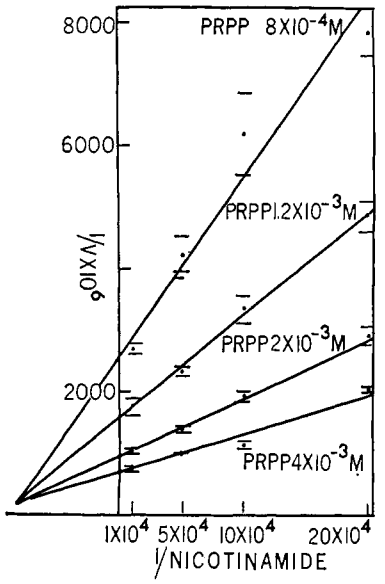

FIGURE 3: Double-reciprocal plots of activity *vs.* nicotinamide concentration at various fixed levels of PRPP. The magnesium to PRPP ratio was constant at 10:1. The incubation time was 1 hr at 37°. Duplicates were run and 100-μl aliquots assayed for radioactivity. A background of enzyme plus nicotinamide was incubated for each level of the latter. The ranges of the reciprocal values are shown by the horizontal bars. The nicotinamide employed had a specific activity of 10 μCi/μmole. Fraction A was the enzyme source.

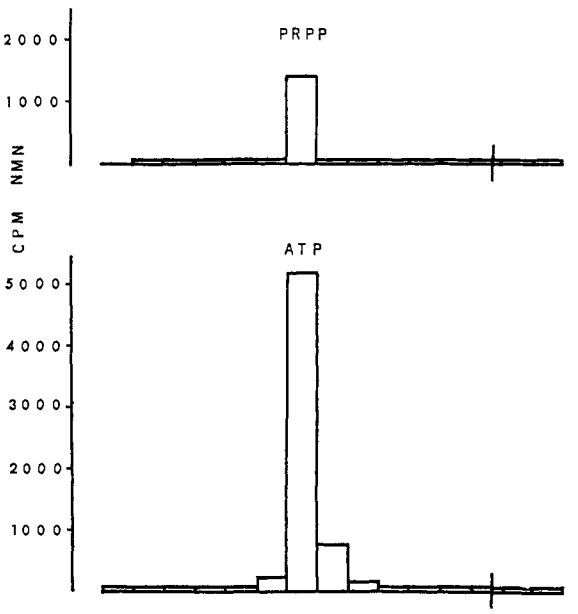

FIGURE 4: Disc gel electrophoresis of NMN pyrophosphorylase activity stimulated by ATP or high levels of magnesium and PRPP. After completion of a run employing a 5% cross-linked gel (see text for details), the gel was longitudinally divided into two equal parts and each longitudinal half was cut into 15 0.4-cm pieces, as depicted in the chart, and minced with 1 ml of reaction mixture. One set of longitudinal segments was incubated with PRPP (5 μmoles), $MgCl_2$ (50 μmoles), [^{14}C]nicotinamide (0.4 μmole, specific activity 10 μCi/μmole), and Tris (50 μmoles, pH 8.0). The second set of longitudinal segments was incubated in the presence of ATP (0.5 μmole), $MgCl_2$ (0.5 μmole), PRPP (0.2 μmole), [^{14}C]-nicotinamide (10 μCi/μmole, 0.1 μmole), and Tris (50 μmoles, pH 8.0). Incubation was carried out for 4 hr in a total volume of 1 ml. Duplicate 100-μl aliquots were employed to determine the amount of NMN formed. Position of tracking dye is indicated.

Figure 1. Straight lines, however, do result at magnesium to PRPP ratios of 10:1 and 15:1 if one graphs the data as $1/v$ *vs.* $1/(\text{PRPP})^2$ (unpublished data). Plotting the data in Figure 1 according to the Hill empirical equation, $\log v/V_{max} - v = n \log S - \log K$, using linear regression analysis, the values of n obtained are 1.7, 1.9, 2.3, and 1.7 when the magnesium to PRPP ratios are 15:1, 10:1, 5:1, and 2:1, respectively (unpublished data). This is taken to mean that more than one molecule of PRPP is involved in NMN synthesis in the absence of ATP.

Double-reciprocal plots of initial reaction studies were carried out in the absence of ATP, employing nicotinamide (Figure 3).

Evidence that the enzymatic activity catalyzed by high levels of PRPP and magnesium or low levels of PRPP plus ATP is carried out by the same protein is presented in Figures 4 and 5. When the enzyme preparations are subjected to disc gel electrophoresis in a loosely cross-linked system (5%) and the gel segments are subsequently assayed for activity, the sites of NMN formation coincide (Figure 4) and the extent of synthesis is directly proportional to the amount of protein placed on the column (unpublished data). The two activities are also identical when compared with heat lability (Figure 5). The enzymatic activity comes off a calibrated gel column (Andrews, 1964) as a single peak, having a molecular weight of *ca.* 64,000. Both ATP and high levels of PRPP and magnesium activate enzymatic activity obtained from the gel column (unpublished data).

Double-reciprocal plots of product inhibition studies employing nicotinamide as the variable substrate against NMN and PP_i, and PRPP as the variable substrate against NMN and PP_i, are shown in Figures 6–9. Employing the same data used to plot these graphs, values for $1/V$, the intercept; and K/V, the slope; and their standard errors were obtained by computer analysis. These values were replotted against the various inhibitor concentrations. K_i's, the points of interception with the horizontal, were read from these replots (Cleland, 1963b). These K_i's are listed in Table I.

Discussion

On the basis of the data so far collected, there seems to be little doubt that the same protein catalyzes the synthesis of NMN from PRPP and nicotinamide in the presence and absence of ATP. NMN formation in both instances follows the same course of heat lability (Figure 5) and exhibits the same electrophoretic mobility (Figure 4). Furthermore, both activities are simultaneously eluated from a Sephadex G-100 column. Assuming that there is but one NMN pyrophosphorylase in the preparations studied, analysis of the kinetics of NMN production in the presence and absence of ATP leads to the conclusion that ATP functions as an allosteric effector.

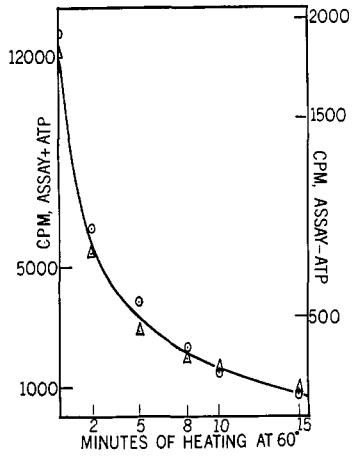

FIGURE 5: Heat lability of NMN pyrophosphorylase activity assayed in the presence of ATP or high levels of magnesium and PRPP. The enzyme used was fraction B diluted 1:4 with 0.05 M Tris-Cl buffer (pH 7.3). Aliquots of this were heated at 60° for the time noted in the graph. The supernatant material obtained after centrifugation was assayed for NMN pyrophosphorylase. The concentrations of the reactants in the presence of ATP (represented by ○) were: ATP (8×10^{-3} M), $MgCl_2$ (8×10^{-3} M), nicotinamide (1×10^{-4} M; specific activity 10 μCi/μmole), and PRPP (2×10^{-4} M). In the case of high levels of magnesium and PRPP (represented by △), the concentrations of the reactants were: PRPP (5×10^{-3} M), $MgCl_2$ (5×10^{-2} M), and nicotinamide (10 μCi/μmole, 1×10^{-4} M). The counts per minute represent a 100-μl aliquot taken after 1-hr incubation. Duplicate assays were run and the average values minus background plotted.

NMN synthesis at high levels of magnesium and PRPP is characterized by parabolic plots of $1/v$ *vs.* 1/PRPP with n, the coefficient of the Hill equation, approximating 2. NMN synthesis in the presence of ATP occurs at lower levels of magnesium and PRPP and yields $1/v$ *vs.* 1/PRPP plots which are linear (Dietrich *et al.*, 1966). This conversion of double-reciprocal plots from parabolic into linear by the addition of a low molecular weight substance is cited as evidence of allosteric activation (Worcel *et al.*, 1965; Okazaki and Kornberg, 1964).

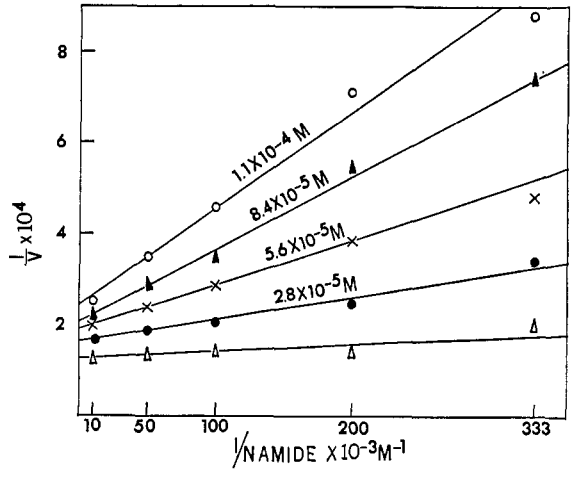

FIGURE 6: Double-reciprocal plots of activity *vs.* NMN at various levels of nicotinamide. The specific activity of the nicotinamide was 8 μCi/μmole. Each reaction flask contained: 0.2 μmole of PRPP, 2.0 μmoles of ATP, 10 μmoles of $MgCl_2$, 50 μmoles of Tris (pH 8.0), and enzyme in a total volume of 1 ml. Incubation was carried out at 37° for 1 hr. Enzyme B was employed in these studies.

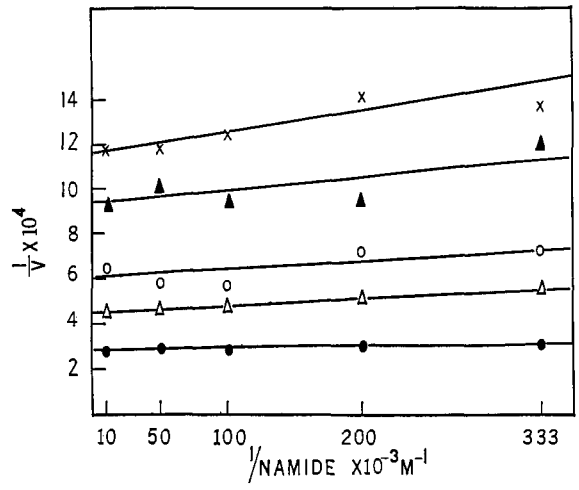

FIGURE 7: Double-reciprocal plots of activity *vs.* inorganic pyrophosphate at various levels of nicotinamide. (●) None, (△) 6×10^{-4} M, (○) 1×10^{-3} M, (▲) 2×10^{-3} M, and (×) 4×10^{-3} M. Each reaction flask contained: 0.2 μmole of PRPP, 2.0 μmoles of ATP, 10 μmoles of $MgCl_2$, 50 μmoles of Tris (pH 8.0), and enzyme in a total volume of 1 ml. Incubation was carried out at 37° for 1 hr. Enzyme B was employed in these studies.

The designation of ATP as an allosteric activator, however, reveals little as to how it enhances the rate of NMN formation. In this regard the data presented in Figure 1 may offer a clue. It would appear that magnesium, at an unphysiological level well in excess of that needed to convert all the free PRPP into Mg–PRPP, is serving as a modifier of NMN pyrophosphorylase activity. It may be that ATP is utilized as a highly stereospecific molecular tool for inserting magnesium into the proper molecular environment for the most efficient catalytic action. Such a postulate does not preclude the possibility that ATP may be utilized during the reaction. In an analogous reaction, the synthesis of nicotinate ribonucleotide from nicotinate and PRPP, stoichio-

TABLE I: Product Inhibitor Constants.[a]

Inhibitor	Variable Substrate	K_i Slope (μM)	K_i Intercept (μM)	Type of Inhibn
NMN	Nicotinamide	8	43	Noncompetitive
PP_i	Nicotinamide	300	940	Noncompetitive
NMN	PRPP	10	$\rightarrow \infty$	Competitive
PP_i	PRPP	160	840	Noncompetitive

[a] See Figures 6–9 and text for experimental details.

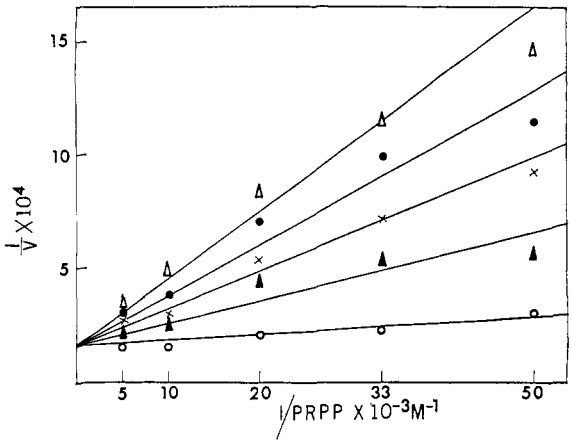

FIGURE 8: Double-reciprocal plots of activity *vs.* NMN at various levels of PRPP. (○) None, (▲) 2.8 × 10^{-5} M, (×) 5.6 × 10^{-5} M, (●) 8.4 × 10^{-5} M, and (△) 1.1 × 10^{-4} M. Each reaction flask contained: 2.0 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.1 μmole of [^{14}C]nicotinamide (8.0 μCi/μmole), 50 μmoles of Tris (pH 8.0), and enzyme in a total volume of 1 ml. Incubation was carried out at 37° for 1 hr. Enzyme B was employed in these studies.

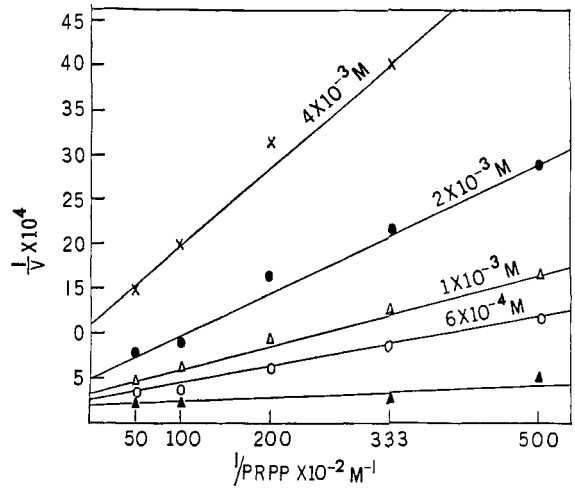

FIGURE 9: Double-reciprocal plots of activity *vs.* inorganic pyrophosphate at various levels of PRPP. Each reaction flask contained: 2.0 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.1 μmole of [^{14}C]nicotinamide (8.0 μCi/μmole), 50 μmoles of Tris (pH 8.0), and enzyme in a total volume of 1 ml. Incubation was carried out at 37° for 1 hr. Enzyme B was employed in these studies.

metric breakdown of ATP to ADP has been demonstrated (Honjo *et al.*, 1966; Smith and Gholson, 1969). Whether ATP is utilized in a similar manner in the case of NMN pyrophosphorylase cannot be assessed at the present stage of enzymic purity.

Investigation of the mechanism of NMN formation entailed obtaining initial velocities for the reaction in the absence of ATP and product inhibition patterns in the presence of ATP. These data were then interpreted assuming NMN synthesis is bireactant for both substrate and product.

The double-reciprocal plots of the series of initial reaction rates obtained in the absence of ATP are parabolic when $1/S$ equals 1/PRPP, but linear and intersecting when $1/S$ equals 1/nicotinamide (Figure 3). Such behavior is to be expected of data gathered from an allosteric reaction. That the $1/v$, 1/nicotinamide plots intersect indicates the mechanism of the reaction is sequential, that is, both substrates add before either product is released. Such data, however, do not differentiate between a random or an ordered sequential mechanism.

The study of the mechanism of inhibition produced by products of the reaction was carried out in the presence of ATP to minimize the complications which could arise from one of the precursors acting as both substrate and activator in the absence of ATP. The data so obtained gave linear double-reciprocal plots (Figures 6–9). The replots of the slopes and intercepts *vs.* inhibitor concentration (Table I) indicate that one product–substrate pair shows competitive inhibition[1] while the other pairs are noncompetitive in nature. There are two sequential BiBi mechanisms which fit these data (Cleland, 1963c): the ordered BiBi, in which PRPP would be the first substrate bound and PP_i the first product released; and the Iso–Theorell–Chance mechanism. In the latter case it would be impossible to determine from the available data which substrate was initially bound. Binding studies or isotopic exchange are essential to distinguish between these two mechanisms.

[1] The intercept values obtained by computer analysis of the data employed in plotting Figure 8 are not significantly different from one other at four concentrations of inhibitor employed.

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