The NAD glycohydrolase (NADase) (EC 3.2.2.5) from *Bungarus fasciatus* (banded krait) venom was purified (1000-fold) to electrophoretic homogeneity through a 3-step purification procedure, the last step being affinity chromatography on Cibacron blue agarose. The purified NADase is a glycoprotein containing two subunits of Mr = 62,000 each. Nicotinamide and adenosine diphosphoribose were produced in a 1:1 stoichiometry and were the only products formed when the purified NADase was incubated with NAD. These results were confirmed by high performance liquid chromatography. The enzyme exhibited a broad pH profile with optimum pH for hydrolysis at 7.5 with very little change in $K_m$ from pH 6.6 to pH 8.5. The NADase is only slightly affected by changes in ionic strength. The enzyme studied titrimetrically at pH 7.5 and 38 °C exhibited a $K_m$ of 14 μM and a $V_{max}$ of 1380 μmol of NAD cleaved/min/mg of protein. The activation energy for the enzyme-catalyzed hydrolysis of NAD was 15.7 kcal/mol.

In addition to NAD and NADP, a number of NAD analogs were shown to function as substrates for the enzyme. Product inhibition studies demonstrated nicotinamide to be a noncompetitive inhibitor with a $K_i$ of 1.6 mM and adenosine diphosphoribose to be a competitive inhibitor with a $K_i$ of 0.36 mM. Procion blue HB (Cibacron blue F3GA) was shown to be a competitive inhibitor with a $K_i$ of 33 nmol. The purified NADase catalyzed the pyridine base exchange reaction between 3-acetylpyridine and the nicotinamide moiety of NAD.

In recent years, the developing importance of the participation of NAD in nonoxidation-reduction reactions such as ADP-ribosylation and the synthesis of polyADP-ribose has promoted a renewed interest in the properties of NAD glycohydrolases (NADases), especially with respect to the catalysis of transglycosidation reactions. All NADases are characterized as catalyzing the hydrolysis of the nicotinamide-ribosidic bond of NAD to produce nicotinamide and ADP-Rib\(^1\) in equimolar amounts. NADases have been demonstrated in various microorganisms and in a variety of animal tissues (1-11). Several mammalian NADases have been known to catalyze, in addition to the hydrolytic reaction, a transglycosidation reaction, frequently referred to as the pyridine base exchange reaction. This property of mammalian NADases has been used successfully to synthesize pyridine nucleotide analogs containing pyridine bases other than nicotinamide. More than 70 NAD analogs have been prepared in this fashion, many of which have become effective reagents of importance in biochemical research. The applications of these compounds in studies of the mechanism of action of dehydrogenases, transhydrogenases, and reductases, in the development of analytical techniques for clinical diagnosis and in *in vivo* studies related to chemotherapy, document well the importance of these derivatives and the reactions through which they are synthesized.

Enzyme-catalyzed pyridine base exchange and ADP-ribosylation share the common property of being transglycosidation reactions involving NAD as substrate. Mechanistic studies of the pyridine base exchange reaction have been hampered somewhat by the lability of solubilized membranous NADases known to catalyze this reaction. Soluble NADases such as those purified from *Neurospora crassa* and bovine seminal plasma are sufficiently stable but do not catalyze the transglycosidation reaction. The recent studies (12-17) reporting the presence of soluble NADases in various snake venoms, some of which were demonstrated to catalyze the pyridine base exchange reaction, were of obvious interest with respect to the possibility of purifying one of these enzymes to provide the experimental system for studying transglycosidation reactions. In the present study, the NADase from *B. fasciatus* (banded krait) venom, first reported by Bhattacharya (13), was purified 1000-fold to electrophoretic homogeneity and various properties of the enzyme were characterized. Of special interest was the demonstration of a base exchange reaction catalyzed by this enzyme from the genera *Bungarus*.

**EXPERIMENTAL PROCEDURES**

**Materials**

All mono- and dinucleotides were purchased from Sigma, except c-NAD and the thiocinotinamide, 3-aminopyridine, 3-aminomethylpyridine, 3-pyridinedaldehyde, pyridine, 3-methylpyridine, 3-pyridylcarbinol, 4-aminopyridine, isonicotinic acid hydrazide, and isonicotinamide analogs of NAD which were prepared by published procedures (18-20). Molecular weight standards and *B. fasciatus* snake venom were purchased from Sigma. 3-Acetylpyridine was purchased from Aldrich. Matrex gel blue A was purchased from Amicon. Phosphocellulose P-11 was purchased from Whatman. Bovine seminal plasma NADase was purified by the method of Yuan and Anderson (8). Procion blue HB was purchased from Aldrich and further purified through preparative paper chromatography. All other chemicals used were reagent grade.

**Methods**

*Activation of Phosphocellulose—Whatman phosphocellulose P-11 was stirred in a 3-fold volume of 0.5 N NaOH for 15 min; after settling,
the fines were removed by decanting. The suspension was then washed with 10 liters of water through a fritted glass funnel; the pH after this step was 8.0 to 8.5. The resin was then stirred in a 3-fold volume of 0.5 M HCl for 15 min and brought to pH 6.0 by washing with water as before. Phosphocellulose was stirred in a 5-fold volume of 0.5 M HCl for 12 h. The resin was titrated to pH 8.2 with 6 N NaOH. Before use, phosphocellulose columns were equilibrated with 0.05 M Tris HCl, pH 8.2.

Assays of NADase activity.—When crude samples of NADase were to be analyzed, the potassium cyanide assay was used. The method was a modification of the assay described by Yian and Anderson (8). The reaction mixtures contained 1 mM β-NAD, 50 mM potassium phosphate buffer, pH 7.5, and NADase, to a final volume of 3 ml. All assays were performed at 38°C. Initial velocities were measured by taking 0.2-ml aliquots from the reaction mixture and immediately adding to 2.8 ml of 1 M potassium cyanide. Remaining absorbance at 237 nm was read against a blank of 0.2 ml of 30 mM potassium phosphate buffer, pH 7.5, plus 2.8 ml of 1 M potassium cyanide. A unit of NADase was defined as the amount of enzyme required to hydrolyze 1 μmol of β-NAD/min.

A fluorometric assay employing β-NAD as substrate was used to monitor NADase activity in column fractions during purification. The assay was 1000 times more sensitive than the cyanide assay and was a modification of the method described by Pekala and Anderson (10). Reaction mixtures contained 0.05 mM β-NAD, 50 mM potassium phosphate buffer and NADase, in a final volume of 1.0 ml. All fluorimetric assays were carried out at room temperature. Fluorescence emission was measured at 560 nm and Coomassie blue stained gels at 550 nm. NADase activity was expressed as relative fluorescence increase/min. All fluorimetric measurements were performed on a Perkin-Elmer 650-40 spectrophotofluorometer.

All kinetic experiments were done titrimetrically in 4 ml of reaction mixtures using a Radiometer type TTTIII titrator, type SBR 2C microtitration assembly equipped with a GK 2320C combination electrode. Reaction rates were determined by the consumption of 1 μl NaOH titrant needed to maintain a constant pH of 7.5. The pH and temperature were 7.5 and 38°C, respectively, unless otherwise stated. All water solutions were prepared as microequivalents H+ released/min, which equaled micromoles of NAD cleaved/min.

Sucrose Gradients.—Linear sucrose gradients (20 to 50%) were made with an ISCO model 570 gradient former. For all gradients used, 20% (w/v) of ice-cold sucrose in 0.05 M sodium phosphate buffer (pH 7.4) and 5% (w/v) of ice-cold sucrose in 0.05 M sodium phosphate buffer (pH 7.4) were used. The volume of each solution used was 18.5 ml.

SDS Gel Electrophoresis.—Purity and molecular weights were determined by SDS gel electrophoresis by the method of Laemmli (21). Separating gels were 8% acrylamide while stacking gels were 3% acrylamide. Slab gel electrophoresis was carried out in a Bio-Rad model 220 slab gel electrophoresis unit at 25 mA/slab. Molecular weight of NADase was determined by measuring its relative mobility in relation to other standard proteins. Slabs were stained for 4 h with 0.25% Coomassie blue R, 25% isopropyl alcohol, and 10% acetic acid. Slabs were destained with 10% acetic acid.

Enzyme purity was determined by using the same method of SDS electrophoresis except electrophoresis was carried out in 9-cm tubes, run at 3 mA/gel. Proteins were stained with Coomassie blue or periodic acid-Schiff reagent for glycoproteins (22). Gels were scanned using a Gilford 250 spectrophotometer. Periodic acid-Schiff gels were scanned at 560 nm and Coomassie blue stained gels at 550 nm.

High performance liquid chromatography of products formed in the NADase-catalyzed reactions was performed on a Spectra-Physics SP 8000, using SP model 770 spectrophotometric detector. The column (5 μm) was an Alttech RSIL AN. Solvent system used was 5 mM sodium phosphate, pH 4.0, and 500 mM sodium phosphate, pH 4.0, 75 and 25%, respectively.

Spectrophotometric measurements were performed on a Beckman Acta MVI recording spectrophotometer. Protein concentrations were measured by the method of Bradford (23) with crystalline bovine serum albumin as a standard. Both the normal and microprotein determinations were used. Ultrafiltration was carried out using Amicon YM-10 membranes.

RESULTS

Purification of Enzyme

Phosphocellulose Chromatography.—Two hundred milligrams of lyophilized B. fasciatus venom were dissolved in 10 ml of 0.05 M Tris-HCl, pH 8.2 (4°C) (Fraction 1) and applied to a phosphocellulose column (1.5 × 40 cm) previously equilibrated with 0.05 M Tris-HCl, pH 8.2. The column was washed with this buffer until absorbance at 280 nm was essentially zero. A linear gradient of 0.05 M Tris-HCl, pH 8.2, and 0.05 M Tris-HCl, pH 8.2, 0.2 M potassium chloride (600 ml each) was then applied to the phosphocellulose column. Fractions of 5 ml were collected. A typical elution profile is shown in Fig. 1. Fractions containing NADase activity were pooled and concentrated by ultrafiltration (Fraction 2).

Gel Filtration.—A column was packed with Sephadex G-100 (4.0 × 100 cm) and equilibrated with 0.05 M sodium pyrophosphate buffer, pH 7.0. Fraction 2 was applied to the column and eluted with the same buffer at a flow rate of 0.6 ml/min. Fractions of 5 ml were collected. The elution profile is shown in Fig. 2. Fractions containing NADase activity were pooled and concentrated by ultrafiltration (Fraction 3).

Affinity Chromatography.—A column was packed with Amicon Matrex gel blue A (0.9 × 5 cm) and equilibrated with...
0.05 M sodium pyrophosphate, pH 7.0. Fraction 3 was applied and eluted with the same buffer. After all the 280 nm of absorbing material had been removed, the column was eluted with 0.05 M sodium pyrophosphate, pH 7.0, 0.5 M KCl. After 20 to 30 column volumes of this buffer wash, 0.005 M potassium phosphate, pH 8.0, 2 M KCl was applied to the column. Fractions containing 1.0 ml were collected. The elution profile is shown in Fig. 5. Fractions containing NADase were pooled, concentrated, and washed free of salt using ultrafiltration. A summary of the purification procedure is shown in Table I. Since the enzyme exhibited a very tight binding to Amicon Matrex gel blue A, inhibition by the free ligand, Procion blue HB, was investigated. Inhibition by this dye was studied by varying the substrate concentration at five constant inhibitor concentrations. The results plotted according to Lineweaver and Burk (24) are shown in Fig. 4. Replots of the slopes and apparent $K_m$ values are shown in the inset of Fig. 4. Procion blue HB was observed to be a linear competitive inhibitor with a $K_I$ of 0.033 μM.

Properties of Enzyme

Estimation of Purity—When 40 μg of NADase from Amicon Matrex gel blue A was analyzed by SDS-polyacrylamide electrophoresis and stained with Coomassie blue, only one protein band was observed. When gels run simultaneously under identical conditions were stained with periodic acid-Schiff reagent for the detection of carbohydrates, a distinctly positive red band was observed which migrated exactly the same distance as the Coomassie blue stained band.

Molecular Weight—Molecular weight determinations of B. fasciatus venom NADase were done by gel filtration on a Sephacryl S-200 column (1.5 × 85 cm). The column was equilibrated with 0.05 M potassium phosphate buffer (pH 7.5), 0.1 M potassium chloride. Column standardization was accomplished with cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase, and catalase. Under these conditions, the snake venom enzyme exhibited an apparent $M_2$, 125,000. Results are shown in Fig. 5. Molecular weight was also determined by the zone sedimentation-sucrose density gradient centrifugation method of Martin and Ames (25). Catalase and yeast glutathione reductase were used as molecular weight standards. The molecular weight of the snake venom enzyme determined by this technique was 130,000.

When the molecular weight of the NADase was determined under the denaturing conditions of SDS-polyacrylamide gel electrophoresis, an apparent $M_2$, 62,000 was obtained (Fig. 5).

![Fig. 3. Affinity chromatography on Amicon Matrex Gel blue A.](image)

**TABLE I**

| Purification of NADase from B. fasciatus | Fraction | Total protein | Total activity | Specific activity | Yield | Purification |
|----------------------------------------|----------|---------------|----------------|------------------|-------|-------------|
|                                        | mg       | units         | units/mg       | %                |       | -fold       |
| Crude                                  | 200      | 260           | 1.3            | 100              |       | 1           |
| Phosphocellulose                       | 8.1      | 280           | 35             | 108              | 27    |             |
| Sephadex G-100                         | 0.65     | 260           | 400            | 100              |       | 398         |
| Amicon Matrex Gel blue A               | 0.19     | 250           | 1320           | 96               | 1030  |             |
5) Stability—The purified NADase at a concentration of 400-500 μg/ml in 0.005 M potassium phosphate buffer, pH 7.5, retained over 90% of its activity over 4 weeks when stored at 4 °C. However, the purified enzyme was stable at -15 °C for over a period of 4 months with no apparent loss in activity. It is of interest to note the enzyme did not lose any activity when incubated at 37 °C, pH 7.6, for 48 h.

Effect of pH on $V_{\text{max}}$ and $K_m$—The activity of the enzyme was measured at 8 pHs between pH 6.0 to pH 8.5. At each pH value, five concentrations of substrate were employed. The reactions were carried out at 38 °C, and initial velocities were measured by the titrimetric assay described above. $K_m$ and $V_{\text{max}}$ values were determined by the method of Cleland (26) and plotted according to Lineweaver and Burk (24) at each pH value. The hydrolysis of NAD catalyzed by snake venom NADase exhibited a broad pH profile, with a pH optimum for hydrolysis of 7.5. There was very little change in the measured $K_m$ value over the pH range studied.

Effect of Ionic Strength on Kinetic Parameters—The effect of potassium chloride on the kinetic parameters of hydrolysis was investigated. Initial rates of hydrolysis of NAD were determined as previously described. The ionic strength was varied by adding different amounts of KCl to the reaction mixtures. Increasing the ionic strength from 10 to 87 mM resulted in a slight increase in maximum velocity with no effect on the $K_m$ value. An ionic strength of 87 mM was therefore chosen for the following kinetic studies of the enzyme. At a higher ionic strength, 500 mM, an increase in $K_m$ and a decrease in $V_{\text{max}}$ was observed.

$K_m$ and $V_{\text{max}}$ under Optimal Assay Conditions—Using the optimal assay conditions found for ionic strength and pH, kinetic constants for the enzyme reaction were determined. The concentration of NAD was varied from 10 to 400 μM and the initial velocities were determined titrimetrically at two different enzyme concentrations. $K_m$ and $V_{\text{max}}$ were determined by the method of Cleland (26) and plotted according to Lineweaver and Burk (24) in Fig. 6. From these data, a $K_m$ for NAD of 14 μM and a $V_{\text{max}}$ of 1300 and 1380 μmol of NAD cleaved/min/mg of protein were found when 34 and 68 ng of NADase were used, respectively. NAD hydrolysis was directly proportional to the amount of NADase added at saturating substrate concentrations.

Effect of Temperature on NADase Activity—Rates of hydrolysis of NAD were determined titrimetrically at 15 temperatures in the range 13-45 °C. The Arrhenius plot of these data was linear throughout the temperature range studied. An activation energy of 15.7 kcal/mol was determined from these data. Thermal denaturation of the B. fasciatus NADase was observed at 50 °C as indicated by a loss of activity with a half-life of 8 min and $k_{\text{diss}}$ of 0.078 min$^{-1}$.

Substrate Specificity—The structural requirements for substrate activity of the snake venom NADase were investigated. Rates of hydrolysis of 25 pyridine nucleotide derivatives were studied at 37 °C, pH 7.5, in reaction mixtures containing 87 mM KCl, 400 μM nucleotide, and 56 ng of purified B. fasciatus NADase. Those compounds that did not react with the enzyme at 15 °C were further studied at five concentrations between 8 and 400 μM and the $K_m$ and $V_{\text{max}}$ values were determined as previously described (Table II). Compounds not serving as substrates were 3-pyridinedehyde-adenine dinucleotide, 3-pyridinedehyde-hypoxanthine dinucleotide, α-nicotinamide-adenine dinucleotide, 3-aminopyridine-adenine dinucleotide, pyridine-adenine dinucleotide, 3-methylpyridine-adenine dinucleotide, 3-pyridylcarbinol-adenine dinucleotide, 4-aminopyridine-adenine dinucleotide, isonicotinic acid hydradizide-adenine dinucleotide, isonicotinamide-adenine dinucleotide, 3-acetylpyridine-adenine dinucleotide, nicotinic acid-adenine dinucleotide, 3-aminomethyl pyridine-adenine dinucleotide, nicotinamide mononucleotide, NADH, and NADPH.

![Fig. 6. Effect of NAD concentration on the rates of hydrolysis catalyzed by purified B. fasciatus NADase.](image)

![Fig. 7. Noncompetitive inhibition of NADase by nicotinamide.](image)

**Table II**

| Substrate                                      | $K_m$ (μM) | $V_{\text{max}}$ (μmol/min) |
|------------------------------------------------|------------|------------------------------|
| Nicotinamide-adenine dinucleotide              | 14         | 0.074                        |
| Nicotinamide-adenine dinucleotide phosphate    | 5          | 0.058                        |
| Nicotinamide-ethenoadenine dinucleotide        | 20         | 0.065                        |
| Nicotinamide-hypoxanthine dinucleotide         | 5          | 0.055                        |
| Thionicotinamide-adenine dinucleotide          | 11         | 0.098                        |
| 3-Acetylpyridine-adenine dinucleotide          | 27         | 0.037                        |
| 3-Acetylpyridine-hypoxanthine dinucleotide     | 7          | 0.022                        |
| Thionicotinamide-adenine dinucleotide phosphate| 10         | 0.021                        |
| Nicotinamide-guanine dinucleotide              | 32         | 0.064                        |
Product Inhibition of the Enzyme—To determine the minimum kinetic mechanism for the hydrolysis of NAD, product inhibition by nicotinamide and ADP-Rib was studied. Inhibition constants for each product were determined at a constant inhibitor concentration while varying substrate concentration. Four different inhibitor concentrations were used in each experiment. Kinetic parameters for each inhibitor concentration were determined by the method of Cleland (26) and plotted according to Lineweaver and Burk (24). Results for nicotinamide and ADP-Rib inhibition are shown in Figs. 7 and 8, respectively. Replots of slopes and apparent $K_I$ values (or intercept values) are shown in the insets of these figures. ADP-Rib was a linear competitive inhibitor with a $K_I$ of 0.36 mM. Nicotinamide, on the other hand, was a purely noncompetitive inhibitor with a $K_I$ of 1.5 mM.

Pyridine Base Exchange Reaction—The pyridine base exchange reaction was studied with both the purified $B. fasciatus$ venom NADase and the previously purified bovine seminal plasma NADase (8). Reaction mixtures contained 50 mM sodium phosphate buffer (pH 7.5), 1 unit of either NADase, 100 mM 3-acyetylpyridine, 3 mM NAD, in a total volume of 3 ml. At timed intervals, a 0.2-ml aliquot of reaction mixture was transferred to 0.2-ml of ice-cold 15% trichloroacetic acid. The concentration of dinucleotides present was determined by reduction of dinucleotides in reactions catalyzed by yeast alcohol dehydrogenase. The 3-acyetylpyridine dinucleotide on reduction exhibits an absorbance maximum of 365 nm, while reduced NAD has an absorbance maximum at 340 nm. Therefore, analog formation results in an increase in the 365:340 ratio. Yeast alcohol dehydrogenase reactions were assayed as follows; 0.2 ml of trichloroacetic acid reaction mixture filtrate was transferred into 2.8 ml of 0.2-ml aliquot of reaction mixture containing 0.5 M ethanol. Absorptions at 365 and 340 nm were recorded before and 2 min after the addition of 0.2 ml of a 10 mg/ml yeast alcohol dehydrogenase solution. Absorbance differences at each wavelength was then determined and plotted as $\Delta A_{365}/\Delta A_{340}$ versus time. In the reaction catalyzed by the snake venom NADase (Fig. 9, line 1) the $\Delta A_{365}/\Delta A_{340}$ increased with time indicating catalysis of the base exchange reaction by this enzyme. Purified bovine seminal plasma NADase, on the other hand, did not catalyze the pyridine base exchange reaction (Fig. 9, line 2). This latter result was reported previously by Yuan and Anderson (27). The concentration of 3-acyetylpyridine adenine dinucleotide formed in the reaction catalyzed by the snake venom enzyme was determined by the change in absorbance at 365 nm (Fig. 9, line 3). Maximum formation of analog occurred in 20 min beyond which a decrease in analog concentration was observed due presumably to the utilization of the analog as a substrate for the NADase.

**DISCUSSION**

The NADase from $B. fasciatus$ venom was purified to electrophoretic homogeneity through a 3-step procedure involving ion-exchange, gel filtration, and affinity chromatography. The purified enzyme migrated as a single band in SDS gel electrophoresis which stained positively for both protein and carbohydrate suggesting the enzyme to be a glycoprotein. The glycoprotein nature of the NADase from $B. fasciatus$ was further indicated in the present study by the difficulty encountered in eluting this enzyme from a concanavalin A-agarose column. When the purified enzyme was applied to such a column, no NADase activity could be eluted until the eluting buffer contained 2 M KCl plus 0.5 M $\alpha$-methylmannoside (data not shown). Under nondenaturing conditions, the enzyme was observed to have an apparent $M_r = 125,000$, determined by gel filtration on Sephadryl S-200 (Fig. 5). This apparent molecular weight for the native enzyme was further supported by zone sedimentation sucrose density gradient centrifugation, which indicated a $M_r = 130,000$. Under the denaturing conditions of SDS gel electrophoresis, the enzyme exhibited an apparent $M_r = 62,000$ (Fig. 5) which would be consistent with the enzyme existing as a dimer composed of two 62,000 molecular weight subunits.

The purified snake venom NADase contained no residual phosphodiesterase activity. All residual phosphodiesterase activity remaining after the Sephadex G-100 step during purifi-
cation was removed in the frontal elution of the Cibacron blue agarose with 50 mM sodium pyrophosphate buffer (data not shown). The disappearance of NAD in the presence of the purified snake venom NADase was attributed solely to the hydrolysis of the nicotinamide ribosidic bond, yielding only ADP-Rib and nicotinamide as products. The 1:1 stoichiometry of these products, along with the absence of any other ultraviolet absorbing products was verified by high performance liquid chromatography. Consistent with these results was the observation that identical rates of hydrolysis were obtained when enzyme activity was assayed by either the cyanide addition assay or the titrimetric assay.

Using the titrimetric assay, a $K_m$ for NAD of 14 $\mu$M and a $V_{max}$ of 1380 $\mu$mol of NAD cleaved/min/mg of protein were determined. In comparison, the membrane-associated bovine erythrocyte NADase (10) was reported to have a $K_m$ value of 16 $\mu$M, while other membrane-bound erythrocyte NADases exhibited $K_m$ values from 10 to 80 $\mu$M (11). The purified calf spleen NADase also exhibited a $K_m$ value of 56 $\mu$M for NAD (7). Yuan and Anderson (8) determined the soluble bovine seminal plasma NADase to exhibit a $K_m$ of 100 $\mu$M. The only other snake venom NADase studied, that from the partially purified Agkistrodon kalsys blomhoffii venom, exhibited a $K_m$ of 830 $\mu$M (14); this higher value might possibly be explained by differences in assays used.

The kinetic parameters of the B. fasciatus venom NADase exhibited little change over the pH range of 6.0 to 8.5. The optimum pH for hydrolysis of NAD as catalyzed by the enzyme was 7.5. Broad pH optima are characteristic of a number of NADases (7, 8, 19, 14). The kinetic parameters were only slightly affected by changes in ionic strength below 100 mM. The increase in $K_m$ at higher ionic strengths is similar to results obtained by Schuber et al. (7) with calf spleen NADase.

Studies of the substrate specificity of the snake venom NADase indicated that in addition to NAD and NADP, a number of pyridine nucleotide analogs served as substrates for the enzyme (Table II). The enzyme requires a complete oxidized dinucleotide as substrate and is specific for the B isomer of NAD. Although the adenyl portion of the dinucleotide was essential for substrate functioning, substitution of other purines for adenine did not greatly alter the functioning of the resulting dinucleotides as substrates. The enzyme was observed to be more sensitive to alterations in the pyridinium moiety of the substrate. Although thionicotinamide-adenine dinucleotide was essentially as effective as NAD as a substrate (Table II), another closely related analog, 3-acetylpyridine-adenine dinucleotide, exhibited a higher $K_m$ and lower $V_{max}$ value with the enzyme. This decreased effectiveness was further accentuated by the total inability of the 3-acetylpyridine analog of NADP to function as a substrate. Other analogs containing a variety of pyridine bases in place of nicotinamide were likewise inactive as substrates. The substrate specificity of the B. fasciatus venom NADase is more closely related to that reported for calf spleen NADase (28) than that observed for bovine seminal plasma NADase (27).

Product inhibition studies of the snake venom NADase demonstrated nicotinamide to be a noncompetitive inhibitor with respect to NAD and ADP-Rib to be a competitive inhibitor (Figs. 7 and 8). The same inhibitor patterns were observed in product inhibition studies of bovine seminal plasma NADase (27) and calf spleen NADase (29). These product inhibition patterns are consistent with an ordered Uni Bi mechanism in which nicotinamide is the first product released and ADP-Rib is the second. A number of erythrocyte NADases have been demonstrated to function through an opposite order of product release (10, 11).

Procion blue HB was shown to be an extremely effective linear competitive inhibitor of purified $B. fasciatus$ NADase and the most tightly bound inhibitor thus far observed with this enzyme. The inhibition constant of 0.033 $\mu$M represents a 10 to 100-fold better binding of this dye to the NADase than that observed with other NAD-requiring enzymes. Schuber and Pascal (30) have demonstrated competitive inhibition by this dye of calf spleen NADase with a $K_i$ of 0.4 $\mu$M, a value similar to that observed in the inhibition of several dehydrogenases (31).

It was of special interest in the present study that the highly purified form of $B. fasciatus$ venom NADase effectively catalyzed a pyridine base exchange reaction. This as previously stated is the first report of the pyridine base exchange reaction carried out by a NADase from snake venom of the genera, Bungarus. As shown in Fig. 9, the enzyme catalyzes the formation of 3-acetylpyridine-adenine dinucleotide from NAD and 3-acetylpyridine. The rate of synthesis of the analog was linear to a point of maximum accumulation followed by a second phase of the reaction where the concentration of analog decreases. This second phase of the reaction would be expected since 3-acetylpyridine-adenine dinucleotide also serves as a substrate for the enzyme. The pyridine base exchange reaction was also shown to occur with other pyridine bases and in the case of the formation of 3-aminopyridine-adenine dinucleotide, a higher yield of analog was obtained since this analog does not function as a substrate.

The demonstration of the transglycosidase activity of the purified snake venom enzyme has several important implications. Since other NAD-hydrolyzing enzymes such as phosphodiesterase and nucleotide pyrophosphatase have been removed during the purification procedure, greater yields of analog formation can be expected and the analysis of products should be less complicated. In addition to the obvious advantage for analog synthesis, the stable purified enzyme provides an ideal experimental system for studying the mechanism of pyridine base exchange. Such a study is currently in progress.

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