Structural Determination of a Cyclic Metabolite of NAD\(^+\) with Intracellular Ca\(^{2+}\)-mobilizing Activity*

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Incubation of NAD\(^+\) with extracts from sea urchin eggs resulted in production of a metabolite which could immobilize intracellular Ca\(^{2+}\) stores of the eggs. In this study we present structural evidence indicating that the metabolite is a cyclized ADP-ribose having an N-glycosyl linkage between the anomic carbon of the terminal ribose unit and the N\(^6\)-amino group of the adenine moiety. In view of this structure we propose cyclic ADP-ribose as the common name for the metabolite. The purification procedure for the metabolite consisted of deproteinizing the incubated egg extracts and sequentially chromatographing the extracts through three different high pressure liquid chromatography (HPLC) columns. The homogeneity of the purified metabolite was further verified by HPLC on a Partisil 5 SAX column. Using radioactive precursor NAD\(^+\) with label at various positions it was demonstrated that the metabolite was indeed derived from NAD\(^+\) and that the adenine ring as well as the adenylyl alpha-phosphate were retained in the metabolite whereas the nicotinamide group was removed. This was confirmed by \(^1\)H NMR and two-dimensional COSY experiments, which also allowed the identification of all 12 protons on the two ribosyl units as well as the two protons on the adenyl ring. From the chemical shifts of the two anomeric protons it was concluded that the C-1 carbons of both ribosyl units were still bonded to nitrogen. The positive and negative ion fast atom bombardment mass spectra showed (M + Na)\(^+\), (M + H + 2Na)\(^+\), (M - H)\(^+\), and (M + 2H + Na)\(^-\) peaks at m/z 564, 586, 540, and 562, respectively. Exact mass measurements indicated a molecular weight of 540.0526 for (M - H)\(^+\). This together with the constraints imposed by the results from NMR, radioactive labeling, and total phosphate determination uniquely specified a molecular composition of C\(_{16}\)H\(_{21}\)N\(_{5}\)O\(_{14}\)P\(_2\). Analysis by \(^1\)H NMR and mass spectroscopy of the only major breakdown product of the metabolite after prolonged incubation at room temperature established that it was ADP-ribose, thus providing strong support for the cyclic structure.

Mobilization of intracellular Ca\(^{2+}\) is a common response to activation of a variety of surface receptors. In many cases the second messenger involved in this signal transduction process is IP\(_3\). However, IP\(_3\) may not be the only messenger molecule responsible for mobilizing intracellular Ca\(^{2+}\). For example, arachidonic acid has also been found to be as potent as IP\(_3\) in releasing Ca\(^{2+}\) from the endoplasmic reticulum in pancreatic islets (1). Similarly, cyclic IP\(_3\) was shown to be even more active than IP\(_3\) in evoking a light response when microinjected into the Limulus photoreceptor (2). Both cyclic IP\(_3\) and arachidonic acid, on the other hand, are metabolites related to the polyphosphoinositide pathway and therefore could be considered as part of the same metabolic system which generates IP\(_3\). In a previous report we showed that a completely independent system involving pyridine nucleotide metabolites may also be involved in mobilization of intracellular Ca\(^{2+}\) in sea urchin eggs (3). We have developed a highly stable cell-free system using egg homogenates as a biological assay for Ca\(^{2+}\)-releasing activators (4). With this screening assay we found a Ca\(^{2+}\)-releasing metabolite of NAD\(^+\) which was produced by incubation with high speed supernatants of the egg. The metabolite which we originally called "enzyme-activated NAD" (E-NAD) was purified by HPLC. Microinjection of E-NAD into intact eggs elicited transient Ca\(^{2+}\) increases as well as cortical reactions showing that it was active both in vitro and in vivo. Structural evidence is presented in this article which indicates that the metabolite is a cyclized ADP-ribose having an N-glycosyl linkage between the anomic carbon of the terminal ribose unit and the amino group at the 6-position of the adenine ring. In view of this novel structure we propose cyclic ADP-ribose as a more descriptive common name for the metabolite instead of E-NAD. For the sake of consistency we will retain the use of the name E-NAD in this article.

**EXPERIMENTAL PROCEDURES
Preparation of Egg Homogenates and the Ca\(^{2+}\) Release Assay—Eggs of Lytechinus pictus were dejellied, washed once with artificial seawater, once with Ca\(^{2+}\)-free seawater containing 1 mM EGTA, twice with Ca\(^{2+}\)-free seawater without EGTA, and then once with an intracellular medium containing gluconate. The compositions of various seawater formulations were as described previously (4), and the intracellular medium contained 250 mM N-methylglucamine, 250 mM

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The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; E-NAD, enzyme-activated NAD\(^+\); HPLC, high pressure liquid chromatography; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; CPR, Corey-Pauling-Koultun; MS, mass spectrometry.

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potassium gluconate, 20 mM HEPES, and 1 mM MgCl₂. The pH was adjusted to 7.2 by the addition of acetic acid. The eggs were then resuspended in 10 volumes (10% egg suspension) of intracellular medium. Aprotinin (10 μg/ml), soybean trypsin inhibitor (25 μg/ml), leupeptin (10 μg/ml), benzamidine (2.5 mM), ATP (0.5 mM), phosphocreatine (4 mM), creatine kinase (2 units/ml), EGTA (10 μM), and 10% homogenate were added, and the suspension was agitated for 5–10 min before homogenizing with a Dounce-type homogenizer with a size A pestle. The homogenates were centrifuged for 20–40 s (13,000 × g, 4 °C) in a microcentrifuge (model 235A, Fisher). The supernatant, referred to as 10% homogenate, was collected and was either fresh or frozen at −70 °C for storage.

Calcium release from microsomes in the homogenates (0.8–1 ml) was measured using the Ca²⁺ indicator, fura-2, as described previously (3). The 10% homogenate was first thawed and incubated at 17 °C for about 1 h before it was diluted 2–4 times (2.5–5% homogenate) with intracellular medium containing 1 μM fura-2; all of the protease inhibitors, and the ATP-regenerating system described above. The diluted homogenate was further incubated at 17 °C for an additional 1–2 h before use. The incubation periods allowed the microsomes to sequester the excess Ca²⁺ in the medium.

Preparation of Egg Extracts—Eggs of Strongylocentrotus purpuratus were harvested and washed as described above except that the last wash was with a medium containing 0.72 M glucose, 1 mM MgCl₂, and 20 mM HEPES, pH 7.2. Eggs were finally resuspended in 3 volumes of the same medium, and aprotinin (10 μg/ml), leupeptin (10 μg/ml), soybean trypsin inhibitor (50 μg/ml), benzamidine (2.5 mM), EGTA (0.1 mM), and 10% homogenate were added. The egg suspension (25%) was then homogenized with a Dounce homogenizer and centrifuged for 30 min at 18,000 rpm (0–4 °C) in a J-21B centrifuge with a JA-20 rotor (Beckman Instruments). The supernatant was collected and was either used fresh or stored frozen at −70 °C. Egg extracts from both L. pictus and S. purpuratus were active in producing the metabolite. However, much larger amounts of eggs could be obtained from the latter species and were used in this study for the purpose of making egg extracts.

Purification of the NAD⁺ Metabolite—Egg extracts (25%) were incubated with 2 mM NAD⁺ for 8–12 h at 17 °C. The increase in Ca²⁺ release activity was monitored by taking small aliquots (1–5 μl) of the mixture to egg homogenates (2.5–5%, 0.8 ml), and the resultant Ca²⁺ release was measured fluorometrically using the indicator fura-2 as described previously (3). The reaction was terminated at the end of the incubation period by the addition of an equal volume of acetone. The precipitated protein was removed by centrifugation, and the acetone was evaporated from the supernatant by a stream of N₂ gas. The pH of the supernatant was then adjusted to 7.6 by the addition of NaOH or Tris base. The first step of the purification procedure employed an HPLC anion exchange column. The resin, AG MP-1 (Bio-Rad), was slurry-packed into a stainless steel column (10 cm) under pressure using an HPLC pump (model II0B, Beckman) operating at a flow rate of 8 ml/min. The flow was 4 ml/min. The UV (254 nm)-absorbing peaks of the eluent were collected by a fraction collector operating at a peak-detecting mode and tested for Ca²⁺ release activity using the egg homogenate assay. The E-NAD eluted in a peak between 13 and 16 min. The column was cleaned afterward as described above except that the last wash was with a medium containing 0.72 M glucose, 1 mM MgCl₂, and 20 mM HEPES, pH 7.2. Eggs were finally resuspended in 3 volumes of the same medium, and aprotinin (10 μg/ml), leupeptin (10 μg/ml), soybean trypsin inhibitor (50 μg/ml), benzamidine (2.5 mM), EGTA (0.1 mM), and 10% homogenate were added. The egg suspension (25%) was then homogenized with a Dounce homogenizer and centrifuged for 30 min at 18,000 rpm (0–4 °C) in a J-21B centrifuge with a JA-20 rotor (Beckman Instruments). The supernatant was collected and was either used fresh or stored frozen at −70 °C. Egg extracts from both L. pictus and S. purpuratus were active in producing the metabolite. However, much larger amounts of eggs could be obtained from the latter species and were used in this study for the purpose of making egg extracts.

Radioactive Labeling of E-NAD—E-NAD was double labeled by incubating egg extracts (5 ml) with 1.9 mM NAD⁺ and a tracer amount of [3H]NAD⁺ (5 μCi) together with 2.5 μCi of either [14C]NAD⁺ or [15N]NAD⁺. The position of the label on the radioactive precursor used were adenosine-2',8'-H, adenosine-1',3'-H, carboxyl-14C, adenosyl-14C, and nicotinamide-4'-H. The last two radioactively labeled forms of NAD⁺ were first purified by passage through an AG MP-1 HPLC column, and the others were used without further purification. The incubation was carried out at 17 °C for 4–8 h, and the labeled E-NAD was purified afterward as described above.

Determination of the Extinction Coefficient—The amount of radioactively labeled E-NAD was determined by dividing the counts in the purified E-NAD by the specific activity of the precursor NAD⁺, which was determined from the radioactivity and the amount of NAD⁺ in an aliquot of the reaction mixture. The extinction coefficient was then calculated from the absorbance at 254 nm and the concentration of the labeled E-NAD.

Analytical HPLC Procedures—The purity of the E-NAD was further verified by chromatographing it on a Whatman Partisil 5 SAX (Whatman International, Maidstone, UK) analytical column (0.46 × 25 cm) packed with 30–40 pm of silica, and the solvent program used was a linear gradient of 0–0.4 M formic acid (pH 4.0 with NH₄OH) in 30 min at a flow rate of 1 ml/min. The column was re-equilibrated with formic acid for another 8 min before the next injection. The Vydas column consisted of a low capacity ion exchange material on a high efficiency protected silica substrate. It is specifically designed for nucleotides and can completely resolve the 12 major ribonucleotides in 10 min. Solvent A was 0.045 M NH₄COOH, pH 4.6, with H₂PO₄, solvent B was 0.5 M NaH₂PO₄, pH 2.7, with HCOOH. The solvent program used was a linear gradient of 0–0.6 M formic acid (pH 4.0 with NH₄OH) in 30 min at a flow rate of 1 ml/min. Other analytical separations employed a 0.46 × 15-cm AG MP-1 column at a flow rate of 1 ml/min. Solvent A was water, and solvent B was 150 mM trifluoroacetic acid. The solvent program started with 1% B for 1 min and increased in steps to 2, 4, 8, 16, 32, 64, and 100% B in each step. The increase was linear to the next higher step in 5 min, except the last step which took 10 min.

¹H NMR Procedures—The one-dimensional ¹H NMR spectra were obtained using a Bruker Instruments, Inc. WM-250 superconducting spectrometer equipped with a variable temperature control, a noise-modulated broadband decoupler, and a 5-mm selectively tuned ¹H coil. Spectra were obtained at 26 °C, with a spectral width of 9000 Hz, with a quadrature pulse sequence at a frequency of 250.13 MHz with an acquisition time of 2.048 s and a relaxation delay of 10 s. Each spectrum was accumulated in 16,000 data points and transformed into 8,000 real data points. The number of transients was between 32 and 1000 depending on the concentration of the samples.

The two-dimensional COSY spectra were obtained with a Bruker AM-300 spectrometer. A 90°-r-90° pulse sequence was used with a magnitude calculation and contour plot to display the connectivities of juxtaposed ¹H-H. The basic F2 matrix was constructed from 16 transients and 512 t values using the eg value.

Mass Spectrometry Procedures—All mass spectrometry experiments were conducted at the Midwest Center for Mass Spectrometry (Lincoln, NE) with a Kratos Analytical Instruments MS-50 triple analyzer equipped with a fast atom bombardment source, a high resolution MS-1 of Nier-Johnson geometry followed by an electrostatic analyzer used as MS-II. Samples were dissolved in water at about 1 μg/ml, and 1-μl aliquots was added to a matrix of either dithiothreitol/dithioerythritol for positive ion spectra or triethanolamine for negative ion spectra. Fast atom bombardment by 7-keV argon atoms was used to desorb the preformed ions from the matrix using a 60° incident angle at a flow rate held at +8 kV (positive ion mode) or −8 kV (negative ion mode).

The MS-MS experiments were performed using MS-I to select the sample ion, and the collisionally activated decomposition spectra were obtained by activating the ion in the third field-free region by a 90° collision with helium gas in a cell at a pressure which reduced the resolution to 25% of its maximum value. The mass spectrometer was held at 200 °C, and the MS-MS spectra were obtained by scanning the electrostatic analyzer (MS-II). Detection was with a...
postacceleration detector at 15 kV with respect to ground. 10–30 scans were signal-averaged for each spectrum by using software developed at the Midwest Center for Mass Spectrometry.

Materials—Sea urchins were purchased from Marinus, Inc. (Long Beach, CA). Radioactive NAD + with labels on various positions was from either Du Pont-New England Nuclear or Amersham Corp. NAD + was from either Sigma or Boehringer Mannheim. Fura-2 was from Molecular Probes.

RESULTS

Purification of the NAD + Metabolite—Incubation of sea urchin egg extracts with NAD + produced a multitude of metabolites. The Ca 2+-releasing metabolite, E-NAD, was previously purified from the mixture by anion exchange and ion pair reverse phase HPLC (3). This procedure, however, was not suitable for large scale purification of E-NAD, and the use of ion pair reagents in the reverse phase also complicated the subsequent structural characterization of the purified product. To overcome these disadvantages we introduced several modifications of the previous procedure. A semipreparative anion exchange (AG MP-1) column was used in the first step which allowed routine processing of 200–300 ml of deproteinized egg extracts. The large volume of eluent (0.5–1 liter) was then vacuum-dried. The active materials were redissolved in small volumes and applied to an analytical reverse phase column. The chromatography was performed isocratically with formic acid which is a volatile acid. The resolution of this reverse phase system was not as good as the ion pair reverse phase HPLC (3). This procedure, however, was not suitable for large scale purification of E-NAD, and the use of ion pair reagents in the reverse phase also complicated the subsequent structural characterization of the purified product. To overcome these disadvantages we introduced several modifications of the previous procedure. A semipreparative anion exchange (AG MP-1) column was used in the first step which allowed routine processing of 200–300 ml of deproteinized egg extracts. The large volume of eluent (0.5–1 liter) was then vacuum-dried. The active materials were redissolved in small volumes and applied to an analytical reverse phase column. The chromatography was performed isocratically with formic acid which is a volatile acid. The resolution of this reverse phase system was not as good as the ion pair system used earlier, and therefore an additional HPLC procedure employing the mixed mode column was introduced as the final step of purification.

As in the previous study, the induction of Ca 2+-release from egg homogenates was used as an assay to monitor the metabolite through all of the purification procedures. In order to quantify the purification in each step, the total and specific Ca 2+ release activities were defined and determined as follows. A unit of Ca 2+ release activity was defined as the amount sufficient to produce half-maximal Ca 2+ release from 0.8 ml of a 2.5% egg homogenate. Therefore, each fraction containing activity was serially diluted, and 2–10 µl was added to 0.8-ml aliquots of homogenate to determine the dilution and volume required to produce half-maximal Ca 2+ release. From the volume (µl) added to the homogenate, the serial dilution required, and the initial volume of the fraction, the total units of activity in the initial fraction were computed. The specific Ca 2+ release activity was determined by dividing the total activity by the amount of UV (254 nm)-absorbing material in the fraction, which was estimated by the absorbance and the extinction coefficient measured for E-NAD (14.3 \( \times 10^5 \) M⁻¹ cm⁻¹) as described below. The Ca 2+ release activity was found to elute as a single peak with UV 254 absorbance from all three HPLC columns.

Typical results of the purification procedure are shown in Table I. Passage through the AG MP-1 column increased the specific Ca 2+ release activity by 15-fold with a yield of about 28%. The loss was likely due to the combination of reduced resolution of the semipreparative column and the requirement for large volume reduction of the eluent by rotary evaporation and vacuum drying before the next step. After the reverse phase and the mixed mode columns, the specific Ca 2+ release activity increased by another 19-fold, and there was essentially no loss in activity. The overall purification was 284-fold with approximately a 25% yield. Typically, 4–8 nmol of E-NAD can be purified from each milliliter of egg extract incubated with 2 mM NAD + , so the percent conversion of NAD + to E-NAD is about 0.8–1.6% after correcting for the loss during purification.

The homogeneity of the purified E-NAD was further verified by chromatographing it on a Whatman Partisil 5 SAX HPLC column as shown in Fig. 1. Essentially a single UV 254 absorbing peak was observed with a recovery of 91%. The first 30 fractions were tested for Ca 2+ release activity, and only the two fractions coinciding with the UV peak contained activity. Indicated also on Fig. 1 are the elution times of 11 standard metabolites of NAD + , and none of them are found to coelute with E-NAD, suggesting its uniqueness.

A similar degree of homogeneity of the purified E-NAD was indicated when chromatographing it on yet another HPLC column, a Vydac 303NT405, which was capable of completely resolving all 12 major ribonucleotides (mono-, di-, and triribonucleotides of adenine, cytosine, guanine, and uracil). Only a single UV 254 peak with all of the Ca 2+ release activity was eluted (data not shown). The purified E-NAD was also eluted as a single peak when chromatographed on each of the three columns used in the purification procedure; therefore, it was judged homogeneous by five different HPLC columns.

Radioactive Labeling of E-NAD—To demonstrate that E-NAD was indeed derived from NAD + and to determine the structural changes brought about through conversion of NAD + to E-NAD, radioactive NAD + labeled at various positions was used as a precursor. In each experiment, two precursor NAD + s with label on different positions were used.
The ratio of the two labeled NAD\(^{+}\) precursors (\(R_{\text{NAD}}\)) was determined by dual isotope counting of aliquots of the reaction mixture. A similar ratio of the purified E-NAD (\(R_{\text{E-NAD}}\)) was also determined. If both labels were conserved in E-NAD then the two ratios should be the same. That is, the isotopic ratio of E-NAD divided by the isotopic ratio of NAD\(^{+}\) (\(R_{\text{E-NAD}}/R_{\text{NAD}}\)) should equal unity. On the other hand, if one label was removed during the reaction, the quantity (\(R_{\text{E-NAD}}/R_{\text{NAD}}\)) should be 0 (or infinite). Therefore, by measuring \(R_{\text{E-NAD}}/R_{\text{NAD}}\) with various combinations of radioactive NAD\(^{+}\) precursors, it was possible to determine which part of the NAD\(^{+}\) molecule was modified by the enzymatic reaction.

Table II summarizes the results of a series of such labeling experiments. By using NAD\(^{+}\) precursors with \(^{3}\)H labels on the 2- and 8-positions of the adenine ring and also uniform \(^{14}\)C labels on all of the carbons, the measured \(^{14}\)C/\(^{3}\)H ratio in E-NAD was found to be the same as in the precursors, giving an \(R_{\text{E-NAD}}/R_{\text{NAD}}\) value of 0.99. Similarly, for the paired precursors with adenylate-\(\alpha\)-\(^{31}\)P and adenine-2,8-\(^{3}\)H labels the \(R_{\text{E-NAD}}/R_{\text{NAD}}\) value (0.94) was also very close to unit. On the other hand, if the precursors were labeled on the nicotinamide group, either nicotinamide-4-\(^{14}\)C or carboxyl-\(^{14}\)C, the labels were lost from the E-NAD. This is shown in Table II for the last three precursor pairs, all of which gave \(R_{\text{E-NAD}}/R_{\text{NAD}}\) values of essentially 0 (0.01-0.04). These results indicate that both the adenine ring and the adenylate \(\alpha\)-phosphate were conserved in E-NAD while the nicotinamide group was removed.

The radioactive labeling of E-NAD provided a means for measuring the extinction coefficient of the molecule as follows. First, the concentration of the radioactively labeled E-NAD (either on the adenine ring or on the adenylate \(\alpha\)-phosphate) was determined from its radioactivity and the known specific activity of the precursor NAD\(^{+}\). Then dividing the absorbance of E-NAD at 254 nm by the concentration gave a value of (14.5 ± 1.5) \times 10\(^{5}\) M\(^{-1}\) cm\(^{-1}\) for the extinction coefficient of E-NAD. This value was about 85% of the extinction coefficient of NAD\(^{+}\) at the same wavelength. The lower extinction coefficient of E-NAD is consistent with the absence of the UV-absorbing nicotinamide group in E-NAD. The UV-absorption property of E-NAD was therefore determined only by the adenine group, and indeed we have previously shown that the UV spectrum of E-NAD was indistinguishable from that of ADP-ribose (3). The value for the extinction coefficient of E-NAD determined here was used in the rest of the study for the determination of E-NAD concentration.

\(^{1}\)H NMR of E-NAD—Fig. 2 compares the \(^{1}\)H NMR spectrum of E-NAD with those of ADP-ribose and NAD\(^{+}\). The partial assignments of the peaks in the ADP-ribose and the NAD\(^{+}\) spectra were based on published spectra (5, 6). Comparison between the E-NAD and the NAD\(^{+}\) spectra reveals an immediately noticeable difference, namely all of the proton peaks associated with the nicotinamide group, \(H_{a2}, -4, -5, -6\), were absent from the E-NAD spectrum. The two adenine protons, \(H_{a2}\) and -8, were still present, although the \(H_{a8}\) was shifted quite a bit downfield. These interpretations are consistent with the radioactive labeling results indicating the presence of the adenine protons and the removal of the nicotinamide group.

The positions of the two anomeric protons of the ribose units of E-NAD, \(H_{1}\)' and \(H_{1}'\), were in the same spectral region as in NAD\(^{+}\), indicating that both C-1 carbons still had nitrogen atoms bonded to them as in NAD\(^{+}\). This is in contrast to ADP-ribose which has an \(-\text{OH}\) group bonded to one of the anomeric carbons instead. As a result the position of that anomeric proton was shifted upfield to 5.3 and 5.2 ppm, respectively, for the \(\alpha\)- and \(\beta\)-anomers (\(H_{1}'\), \(H_{1}\')).

In order to be able to identify the resonances of the rest of the ribose protons two-dimensional COSY experiments were performed. The results are summarized in a contour plot shown in Fig. 3, which graphically displays the connectivities of the juxtaposed protons. Using the cross-correlation peaks and starting from the two anomeric protons, the rest of the six protons from each of the ribose units were sequentially traced and their positions assigned as shown.

Results presented so far indicate that except for the removal of the nicotinamide group, the rest of the NAD\(^{+}\) molecule including the adenine ring, the adenylate \(\alpha\)-phosphate, and the two ribosyl units remained intact in E-NAD. Furthermore, from the NMR results we can conclude that both anomic carbons of the two ribosyl units are bonded to nitrogen atoms.

Mass Spectrometry of E-NAD—The negative ion fast atom bombardment mass spectrum of E-NAD is shown in Fig. 4. The peaks at \(m/z\) 540 and 562 were from the molecular ions of E-NAD, \((M - H)^{-}\) and \((M - 2H + Na)^{-}\), respectively. The two low \(m/z\) peaks were due to matrix ions. The positive ion fast atom bombardment mass spectrum (data not shown) showed also two peaks at \(m/z\) 564 and 586 corresponding to molecular ions of \((M + Na)^{+}\) and \((M - H + 2Na)^{+}\).
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Fig. 3. Two-dimensional COSY spectrum of E-NAD. The spectrum was obtained from approximately 2 µmol of E-NAD dissolved in D2O (99.996%). The labels H1', -2', -3', -4', and -5' refer to protons on the ribose unit closer to the adenine moiety. The labels H1', -2', -3', -4', and -5' refer to protons on the terminal ribose unit.

These results together indicate a molecular weight of 541 for E-NAD. The inset in Fig. 4 shows the collisional-activated decomposition spectrum of the (M – H)− ion of E-NAD. The two major fragments at m/z 426 and 408 correspond to [M – H – (HPO4 + H2O)]− and [M – H – (HPO4 + 2H2O)]−, respectively. The facile loss of base typical of nucleotides (7) was not observed. The exact mass of the m/z 540 ion was determined by peak matching using CsI as a reference and was measured to be 540.0526 with an accuracy of better than 2 ppm. Examination of all possible combinations of C, H, N, O, and P (atoms present in E-NAD) which yield masses within 3 ppm of the observed m/z gave 23 possibilities. Evidence presented above established the presence of the adenine ring and the two ribosyl units in E-NAD. Therefore it must contain at least 15 carbons and 4 nitrogens. It should also contain at least one phosphate, the adenylyl α-phosphate. Imposing these constraints reduces the possible combinations to seven which are listed in Table III together with the deviations from the observed mass of 540.0526. To distinguish among these seven possibilities the total phosphate content of E-NAD was measured according to (8). A plot of phosphate content versus amount of E-NAD was constructed with 15 data points, and the slope of the linear regression line (correlation coefficient was 0.99) was found to be 2.2. This value indicates 2 mol of phosphate for each mole of E-NAD, and from Table III the molecular composition of E-NAD was uniquely determined to be C15H21N5O13P2.

Structure of E-NAD—The only structure that was found to be consistent with all of the data presented above is a cyclized ADP-ribose having an N-glycosyl linkage between the anomic carbon of the terminal ribose unit and the N6-amino group of the adenine moiety. The structural formula as well as the CPK model are shown in Fig. 5. This structure gives the exact molecular composition as determined above. It also accounts for the NMR findings that both anomic carbons of the two ribose units must have nitrogen atoms attached to them. The fact that the CPK model could be constructed indicates that there is no intrinsic steric hindrance prohibiting the cyclization. Both the structural formula and the CPK model in Fig. 5 show the conformation of the newly formed N-glycosyl bond in the α-configuration. However, a CPK model in the β-configuration can be constructed as easily, and we do not have definitive evidence for the actual conformation at the present time.

The strongest support for the cyclic structure comes from the fulfillment of an obvious prediction of the structure, namely that the hydrolysis of the N-glycosyl linkage should produce ADP-ribose as diagramed in Fig. 5. This was indeed proven to be the case by the analysis of the breakdown product of E-NAD as described in the following section.

Analysis of the Breakdown Product of E-NAD—Hydrolysis of E-NAD was induced by prolonged incubation in aqueous medium at room temperature. About 2 µmol of E-NAD was purified as described under "Experimental Procedures." The purity was confirmed by 1H NMR which gave a spectrum identical to that shown in Fig. 2. The sample was allowed to incubate in the unbuffered D2O in the NMR tube for about 40 h at room temperature and then rechromatographed on an
FIG. 4. Mass spectrometry of E-NAD. Purified E-NAD was analyzed by fast atom bombardment mass spectrometry of the (M–H)⁻ ions. The inset shows the collisional activated decomposition spectrum of the (M–H)⁻ ion of E-NAD.

TABLE III
Molecular compositions with mass similar to E-NAD

| C | H | N | O | P | Deviation* ppm |
|---|---|---|---|---|---------------|
| 17 | 15 | 7 | 12 | 1 | 1.8 |
| 19 | 17 | 4 | 13 | 1 | −0.7 |
| 15 | 20 | 5 | 13 | 2 | −1.3 |
| 20 | 18 | 9 | 2 | 4 | −1.3 |
| 19 | 22 | 5 | 6 | 4 | 1.0 |
| 16 | 21 | 10 | 2 | 5 | −2.1 |
| 15 | 25 | 6 | 6 | 5 | 0.4 |

*Deviation from the observed mass of 540.0526 of the molecular ion of E-NAD.

AG MP-1 HPLC column. Two major UV-absorbing peaks were eluted. The first major peak was E-NAD since it contained Ca²⁺ release activity, and its elution time of about 17 min was characteristic of authentic E-NAD. The total amount of E-NAD in this peak was 0.87 µmol which was about half of the original amount. The breakdown product was in the second major peak which contained the other half (0.94 µmol) of the original material and was eluted at about 23 min. This peak did not have any Ca²⁺ release activity, and the retention time was characteristic of ADP-ribose (about 23 min). The peak was collected, and the ¹H NMR spectrum was found to be essentially identical to ADP-ribose showing the characteristic α- and β-anomeric protons at 5.3 and 5.2 ppm (cf. the ADP-ribose spectrum in Fig. 2). Even the shape of the complex group of ribose protons at the spectral region between 4 and 4.7 ppm was essentially identical to that of authentic ADP-ribose.

The negative ion fast atom bombardment mass spectrum of the breakdown product of E-NAD showed (M–H)⁻ and (M–2H + Na)⁻ peaks at m/z 558 and 580, respectively, indicating a molecular weight of 559 for the breakdown product. Exact mass measurement gave the mass of 558.0643 for (M–H)⁻ which is within 0.72 ppm of the calculated mass of (M–H)⁻ for ADP-ribose. Finally, Fig. 6 compares the collisional activated decomposition spectrum of the (M–H)⁻ of the breakdown product with that of authentic ADP-ribose. The three major fragment ions of m/z 424, 406, and 345 in the ADP-ribose spectrum corresponded to (M–base H)⁻, (M–base H – H₂O)⁻, and (M–(ribose + HPO₄)⁻), respectively. The two fragmentation patterns were virtually superimposable. Therefore, by criteria of HPLC, NMR, and mass spectrometry, it can be concluded unequivocally that the break-
The breakdown product of E-NAD was ADP-ribose, a compound larger than E-NAD by one water molecule.

**DISCUSSION**

Four different approaches were used in this study to characterize structurally E-NAD, the Ca\(^{2+}\)-releasing metabolite of NAD\(^{+}\).

1) The first approach used radioactive precursors, and the results indicated that the adenine ring was conserved in E-NAD while the nicotinamide group was not. That the pyrophosphate linkage was also unchanged was suggested by the conservation of the adenyate-\(\alpha\)-\(\beta\)P label and by the total phosphate determination showing 2 mol of phosphate/mol of E-NAD.

2) The second approach was \(^1\)H NMR, and four conclusions could be drawn. First, there were two, and only two, protons on the adenine ring. This rules out structures such as those in which the \(N^\alpha\)-amino group has been replaced by a proton. Second, both ribose units were intact with all 12 protons identified. Third, the nicotinamide group was removed which confirmed the results from radioactive labeling. Fourth, both anomeric carbons of the ribose units were bonded to nitrogen.

3) The third approach was mass spectrometry. The exact mass measurement allowed unique specification of the molecular composition of E-NAD to be C\(_{16}\)H\(_{21}\)N\(_{2}\)O\(_{13}\)P\(_{2}\).

4) The strongest evidence was provided by the fourth approach which was to analyze the only major breakdown product of E-NAD. Using HPLC, NMR, and mass spectrometry, the product was unequivocally identified as ADP-ribose.

The very fact that E-NAD differs from ADP-ribose by one water molecule and that E-NAD could be converted to ADP-ribose by simple hydrolysis strongly indicated that E-NAD was a cyclic compound. Theoretically there are several ways ADP-ribose can be cyclized. For example, a phosphodiester linkage could be formed with either ribose unit in a manner similar to cAMP. However, these types of cyclic structures are contrary to the NMR results showing that both anemic carbons of the ribose units were bonded to nitrogen. In fact the NMR results dictated that the cyclization must occur at the terminal anomeric carbon and must be linked to a nitrogen on the adenine moiety. There are four nitrogen atoms in the ring itself and one free amino group. If the linkage were to any of the ring nitrogens it would put a positive charge on the ring and would be expected to change the UV-absorption spectrum substantially as compared with ADP-ribose. Instead, it was observed that the UV spectrum of E-NAD was virtually identical to that of ADP-ribose (3). Furthermore, linkage to a ring nitrogen would result in a structure having a molecular weight less than the observed value by one proton.

The only structure that was found to be consistent with all available data was the one shown in Fig. 5. Analysis of the collisional activated decomposition spectrum of E-NAD (inset of Fig. 4) provided further support of this cyclic structure. For nucleotides such as ADP-ribose the major fragmentation pathway is the breakage of the pyrophosphate bond. As shown in Fig. 6, this gave rise to the major peak at m/z 345 corresponding to the loss of (ribose-HPO\(_3\)) from the molecule. However, this fragmentation pathway is not possible in a cyclized molecule such as E-NAD since the ribose phosphate is still attached to the molecule through the N-glycosyl linkage even after the pyrophosphate bond is broken. Indeed, as shown in the inset of Fig. 4, the major fragmentation pathway for E-NAD was a simple loss of phosphate and water (HPO\(_3\) + H\(_2\)O) giving rise to the m/z 426 peak. This was followed by loss of another water resulting in the peak at m/z 408. This pattern is consistent with the pyrophosphate being broken in
a manner similar to that observed in ADP-ribose, but because of the N-glycosyl linkage the ribose unit remained attached, and only HPO$_4$~$^2-$ + H$_2$O was lost.

The secondary fragmentation pathway in ADP-ribose was the loss of the adenine base giving rise to the two smaller peaks at $m/z$ 424 and 406 which corresponded to (M - base H)$^+$ and (M - base H - H$_2$O)$^-$, respectively. This pathway is not possible in E-NAD because the base is linked to both ribose units. Indeed, this facile loss of base, characteristic of nucleotides (7), was not observed. The collisional activated decomposition spectrum of E-NAD is therefore consistent with it being cyclic.

The cyclic structure of E-NAD can also account for the anomalous chemical shifts of the protons H$_8$ and H$_{2}'$ seen in the NMR spectrum (cf. Fig. 2). The unusual chemical environment in which these two protons exist is apparent from the CPK model shown in Fig. 5. In order for the molecule to cyclize, the sugar-phosphate backbone has to fold backward. Consequently, the H$_8$ proton is surrounded by the pyrophosphate group while the H$_{2}'$ proton is brought into close proximity with the adenine ring. The unusual environment in which these two protons exist could account for the downfield shifts observed for these protons.

The bulk of the structural characterizations presented in this study strongly indicates the cyclic structure for E-NAD. We therefore propose a descriptive common name, cyclic ADP-ribose, for the metabolite. The novelty of the structure itself warrants detailed investigation into the biochemistry of this NAD$^+$ metabolite. Moreover, cyclic ADP-ribose appears to be a commonly occurring metabolite since a variety of mammalian tissue extracts can also produce it upon incubation with NAD$^+$. Taken together, these results indicate that the importance of this metabolite could go beyond novelty and suggest that it may be a general second messenger like IP$_3$ for mobilizing intracellular Ca$^{2+}$. If so, the cyclic ADP-ribose would represent an addition to the already well known second messenger family of cyclic nucleotides.

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