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Synthesis and Properties of Analogs of Adenosine Diphosphate, Adenosine Triphosphate, and Nicotinamide-Adenine Dinucleotide Derived from 3-β-D-Ribofuranosyladenine (3-Isoadenosine)*

Nelson J. Leonard and Richard A. Laursen†

ABSTRACT: Coenzyme analogs ([3-isoadenosine]-5′-di- and triphosphates [3-iso-ADP and 3-iso-ATP], and the oxidized form of the 3-isoadenosine analog of nicotinamide-adenine dinucleotide [NMN-3-iso-AMP]) derived from 3-isoadenosine were synthesized from P^1-diphenyl P^2-([3-β-D-ribofuranosyladenine]-5′)-pyrophosphate by the anion-exchange method and were examined for their ability to replace the corresponding natural coenzymes in enzymatic reactions. Mixtures of 3-iso-AMP, 3-iso-ADP, and 3-iso-ATP were equilibrated by myokinase. 3-Iso-ATP replaced ATP in the hexokinase-catalyzed phosphorylation of glucose and in the production of light in the luciferin-luciferase system. 3-Iso-ADP was polymerized (A. M. Michelson, personal communication) by polynucleotide phosphorylase. The nicotinamide-adenine dinucleotide (NAD) analog, NMN-3-iso-AMP, was examined in six dehydrogenase systems and was found to be reduced from 6 to 140% as rapidly as NAD, depending upon the dehydrogenase. The hypochromicity of both the oxidized and reduced forms of NMN-3-iso-AMP suggests an interaction between the purine and pyridine rings; however, no energy transfer from the dihydronicotinamide to the adenine moiety was observed by fluorescence methods. The results suggest that 3-isoadenosine possesses many of the structural features of adenosine and that 3-isoadenosine derivatives may be useful in studies of the binding sites of certain enzymes.

In the preceding article (Leonard and Laursen, 1965) we described the synthesis of an isomer of adenosine, 3-isoadenosine,[1] and of (3-isoadenosine)-5′-phosphate (compound I). The biological activity (Leonard and Laursen, 1963; Gerzon *et al.*, 1964) of 3-isoadenosine in certain cell cultures suggested that 3-isoadenosine may have been metabolized in place of adenosine. This was not unanticipated in view of the gross structural similarities that exist between adenosine and 3-isoadenosine. In order to investigate some of the possible modes of action of 3-isoadenosine in living organisms and to explore the possibilities for using 3-isoadenosine

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[1] Abbreviations used in this paper are: 3-isoadenosine, 3-β-D-ribofuranosyladenine; 3-iso-AMP, 3-iso-ADP, and 3-iso-ATP, (3-isoadenosine)-5′-mono-, di-, and triphosphates, respectively. Other compounds are abbreviated in accordance with the recommendations of the International Union of Pure and Applied Chemistry (1963; *cf.* Dixon, 1960). NMN-3-iso-AMP and $NMNH_2$-3-iso-AMP, the oxidized and reduced forms of the 3-isoadenosine analog of nicotinamide-adenine dinucleotide, respectively (*cf.* Walter and Kaplan, 1963).

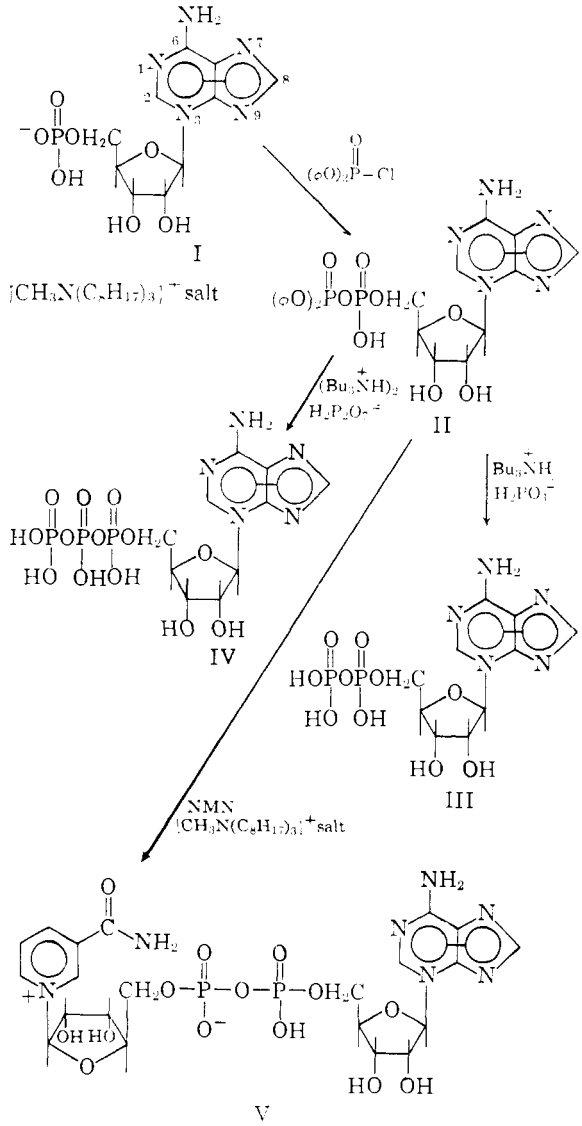
as an analog in studying the mechanisms of certain enzymes, we prepared some coenzyme analogs and examined them in several enzyme systems.

Synthesis of 3-Iso-ADP and 3-Iso-ATP. Coenzyme analogs derived from 3-isoadenosine were prepared by the anion-exchange method of Michelson (1958b, 1963). The methyltrioctylammonium salt of 3-iso-AMP (compound I) reacted with diphenylphosphoryl chloride to form the diphenyl pyrophosphate, P^1-diphenyl P^2-[(3-β-D-ribofuranosyladenine)-5′]-pyrophosphate (compound II). Compound II was then used immediately to prepare 3-iso-ADP (compound III) and 3-iso-ATP (compound IV) by reaction with the tributylammonium salts of phosphoric or pyrophosphoric acid, respectively. Yields were somewhat low (30–45%), chiefly because of losses encountered in purifying the products.

3-Iso-ADP and 3-iso-ATP were purified by elution from Dowex 1 (chloride) with lithium chloride–hydrochloric acid. 3-Iso-ADP had acid-labile phosphate–phosphate–adenine ratios of 1.00:2.03:1.09, the theoretical being 1.00:2.00:1.00, and contained no inorganic phoshate. 3-Iso-ATP contained a considerable amount of inorganic pyrophosphate which could not be removed by chromatography on Dowex 1 (chloride) or on DEAE-cellulose (bicarbonate) (Moffatt, 1964). A contributing cause was the high basicity of 3-isoadenosine (pK_a' 5.5), compared with adenosine (pK_a' 3.45), which resulted in the 3-isoadenosine derivatives being eluted much more rapidly than the corresponding adenosine compounds.

In the synthesis of 3-iso-ATP, the reaction time was found to be critical. Ordinarily in the synthesis of triphosphates by the anion-exchange method, the reaction time is about 2 hours; the reaction solvent is pyridine. On one occasion, when the reaction was allowed to proceed for 14 hours, 3-iso-ADP, small amounts of the tetraphosphate, and only small amounts of the triphosphate were isolated. The dismutation of ATP to ADP and higher phosphates in the presence of inorganic pyrophosphate in pyridine has recently been studied by Verheyden *et al.* (1964).

Synthesis of NMN-3-iso-AMP. A large number of analogs of NAD, having modifications in the purine (Fawcett and Kaplan, 1962; Kaplan *et al.*, 1952; Windmueller and Kaplan, 1961; Honjo *et al.*, 1963; Pfleiderer *et al.*, 1963a; Atkinson *et al.*, 1961, 1962) and nicotinamide (Atkinson *et al.*, 1961; Walter and Kaplan, 1963; Kaplan and Ciotti, 1956) portions of the molecule, have been synthesized. The first chemical synthesis of NAD, in which the coenzyme was actually isolated, was carried out by Hughes *et al.* (1957), who condensed adenosine monophosphate with nicotinamide mononucleotide (NMN) in the presence of dicyclohexylcarbodiimide. This has since been the method of choice for preparing analogs from the corresponding nucleotides (Fawcett and Kaplan, 1962; Honjo *et al.*, 1963; Pfleiderer *et al.*, 1963a,b; Atkinson *et al.*, 1962), although the yields are usually low (10–30%), even when an excess of nucleotide (AMP, and the like) is used. In cases where the nucleotide (e.g., 3-iso-AMP) is the limiting reagent (and not NMN, as is usually the case), this method is less attractive. And indeed, when equimolar amounts of NMN and 3-iso-AMP were condensed, the desired product, NMN-3-iso-AMP, was obtained in only 1% yield.



In view of the successful synthesis of 3-iso-ADP and 3-iso-ATP via the anion-exchange method, we felt this method might be useful for preparing NMN-3-iso-AMP. When compound II was allowed to react with the methyltrioctylammonium salt of NMN, NMN-3-iso-AMP (compound V) was formed in about 30% yield. After chromatography on Dowex 1, the product appeared to be homogeneous, as judged by its behavior on paper chromatography and electrophoresis. NMN-3-iso-AMP was cleaved by venom pyrophosphatase to NMN and 3-iso-AMP. The ratio of adenine to nicotinamide to phosphate was 1.00:1.00:1.91, the theoretical ratio being 1.00:1.00:2.00. In this case, too, the relatively high basicity of 3-isoadenosine made separation of NMN-3-iso-AMP from the other components [NMN, 3-iso-AMP, and (3-iso-AMP)$_2$] of the reaction mixture more difficult than in the case of NAD. However, by chromatographing the product on Dowex

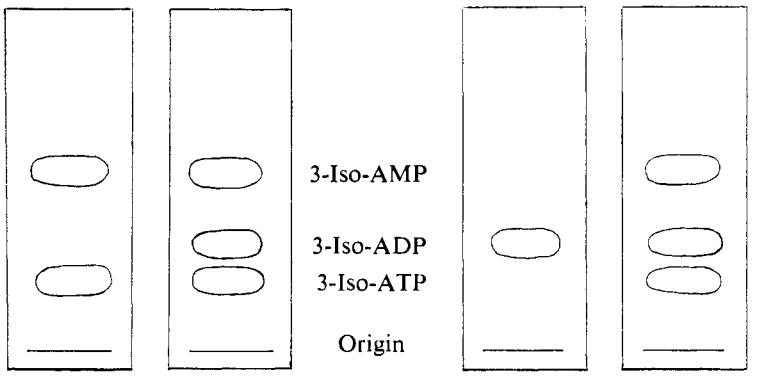


FIGURE 1: Reactions of (3-isoadenosine)-5′-phosphates with myokinase. Chromatography of reaction mixtures.

1 (chloride and formate) columns, we were able to obtain a reasonably pure sample.

Nicotinamide mononucleotide was prepared by hydrolysis of commercial NAD with snake venom pyrophosphatase. The procedure was essentially that of Kaplan and Stolzenbach (1957) combined with the work-up procedure of Haynes *et al.* (1957). The product obtained in this manner was chromatographically homogeneous and free from inorganic ions.

Venom pyrophosphatase for the preparation of NMN and for other experiments was freed from 5′-nucleotidase by chromatography on the DEAE-cellulose column used by Boman and Kaletta (1957) for the separation of venom phosphodiesterases. Using the same chromatographic procedures as these authors, it was possible for us to obtain a preparation of pyrophosphatase containing only about 2% of 5′-nucleotidase activity.

Reaction of 3-Iso-AMP, 3-Iso-ADP, and 3-Iso-ATP with Myokinase. In view of its reported specificity (Noda and Kuby, 1957; Callaghan and Weber, 1959) for adenosine nucleotides in the reaction

$$ATP + AMP \rightleftharpoons 2\ ADP$$

it seemed that myokinase (adenylate kinase) would be a good enzyme with which to compare the ability of 3-isoadenosine to replace adenosine.

A mixture of 3-iso-ATP and 3-iso-AMP was incubated with myokinase for 1 hour, and the reaction mixture was chromatographed on paper according to Oliver and Peel (1956). As can be seen in Figure 1, the mixture of 3-iso-ATP and 3-iso-AMP was equilibrated to a mixture of the mono-, di-, and triphosphates. In a test of the reverse reaction, 3-iso-ADP also disproportionated to an equilibrium mixture of phosphates. Since there are other enzymes which catalyze similar exchanges but which are less specific for the nucleoside than myokinase (Callaghan and Weber, 1959; Dixon, 1960; Dixon and Webb, 1964), the possibility that the observed activity with the 3-isoadenosine phosphates was owing to a contaminating enzyme was considered. As controls, inosine diphosphate (IDP) and cytidine diphosphate (CDP) were incubated with myokinase under the same conditions. With these nucleotides, however, nodismutation was observed.

Reactions with Myokinase and Hexokinase. Hexokinase catalyzes the phosphorylation of glucose and other hexoses with ATP, for example, the reaction:

$$\text{glucose} + ATP \rightarrow \text{glucose-6-phosphate} + ADP$$

It has been reported that ATP can be replaced by deoxy-ATP (Siebert and Beyer, 1961) and by ITP (Kleinzeller, 1942; Kaplan *et al.*, 1952), although in the latter case it has been suggested (Berg and Joklik, 1954) that the reactivity is owing to impurities—ADP and a transphosphorylase that catalyzes the reaction:

$$ADP + ITP \rightleftharpoons ATP + IDP$$

In order to confirm the results with myokinase and to test the ability of 3-iso-ATP to react with hexokinase, 3-iso-ADP was incubated with myokinase and hexokinase (Figure 2). Myokinase catalyzes the dismutation of ADP to ATP and AMP, and the ATP formed phosphorylates glucose (equation 1).

$$2\ ADP \overset{\text{myokinase}}{\rightleftharpoons} ATP + AMP$$

$$\frac{ATP + \text{glucose} \overset{\text{hexokinase}}{\longrightarrow} \text{glucose-6-phosphate} + ADP}{ADP + \text{glucose} \rightarrow \text{glucose-6-phosphate} + AMP} \qquad (1)$$

The reaction was followed by measuring the amount of acid-labile phosphate remaining in the reaction mixture. It can be seen in Figure 2 that the 3-isoadenosine analogs replaced the adenosine phosphates in both cases, although the over-all reaction was slower with 3-iso-ADP than with ADP. Inosine diphosphate was not effective

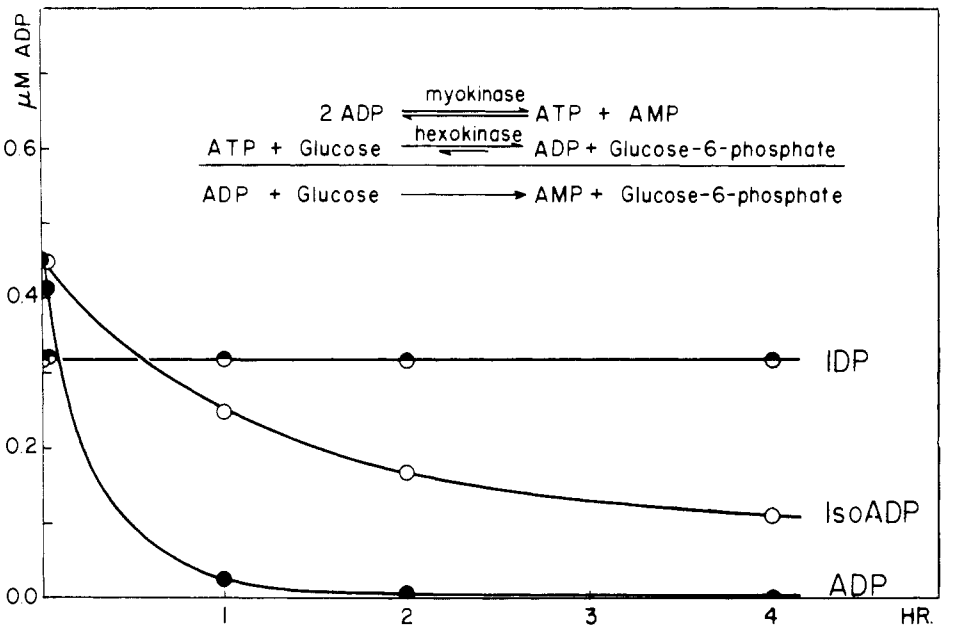


FIGURE 2: Reactions of nucleoside diphosphates with myokinase and hexokinase.

in the reaction, because although ITP reacts with hexokinase, IDP does not react with myokinase (Kaplan *et al.*, 1952).

Enzymatic Reduction of NMN-3-iso-AMP. The use of analogs to study the binding sites and mechanisms of action of dehydrogenases and for demonstrating the species heterogeneity of dehydrogenases catalyzing the same reaction is well established (Anderson and Kaplan, 1959; Kaplan *et al.*, 1956; Pfleiderer *et al.*, 1963b; Fawcett, 1962; Walter and Kaplan, 1963). With a large body of data available for comparative purposes, we felt it would be of interest to compare the rates of enzymatic reduction of NMN-3-iso-AMP with those of NAD and other NAD analogs.

The dehydrogenases used for the reduction of NAD and NMN-3-iso-AMP were chosen on the basis of their being readily available in a reasonably pure state. They were also enzymes which Kaplan and co-workers (Fawcett and Kaplan, 1962; Windmueller and Kaplan, 1961; Anderson and Kaplan, 1959) have used in their analog studies.

Table I shows the relative rates of reduction of NAD and NMN-3-iso-AMP in several dehydrogenase-catalyzed reactions. Relative rates were calculated by measuring the slope of the initial reaction curve. The rates were reproducible to within 5%, with the exception of that of glyceraldehyde-3-phosphate dehydrogenase which varied by as much as 10%, apparently because of instability of the enzyme. The NAD used for comparison in these studies was a commercial sample and was not purified before use. Since it has been shown by Dalziel (1963) that commercial NAD contains small amounts of inhibitory impurities, and since the NMN-3-iso-AMP may have contained small amounts of undetected impurities, an even larger margin for error must be allowed for the values in Table I. However, the range of relative rates obtained shows that NMN-3-iso-AMP varies widely in its ability to react with dehydrogenases.

Spectral Properties of NMN-3-iso-AMP. The hypochromism exhibited by nucleic acids and P^1,P^2-dinucleoside-5′-pyrophosphates has been interpreted as resulting from the interaction of the π-electron systems of the purine or pyrimidine rings (Michelson, 1958a; Weber, 1950; Laland *et al.*, 1954). This phenomenon has also been observed in reduced NAD (Weber, 1958), so it was of interest to see whether hypochromism could be observed with NMN-3-iso-AMP.

Ultraviolet spectra of the oxidized and reduced forms of NAD and NMN-3-iso-AMP were obtained before and after hydrolysis with venom pyrophosphatase. Figures 3 and 4 show that in all cases a hyperchromic shift occurred when the molecules were cleaved with pyrophosphatase, and that in this respect NMN-3-iso-AMP behaves identically with NAD.

TABLE I: Relative Rates of Enzymatic Reduction of NMN-3-iso-AMP.

Dehydrogenase	Source	$\frac{k_{\text{NMN-3-iso-AMP}}}{k_{\text{NAD}}} \times 100$
Alcohol	Horse liver	140
Glutamic	Beef liver	121
Lactic	Rabbit muscle	60
Glyceraldehyde-3-phosphate	Rabbit muscle	55
Malic	Pig heart	19
Alcohol	Yeast	6

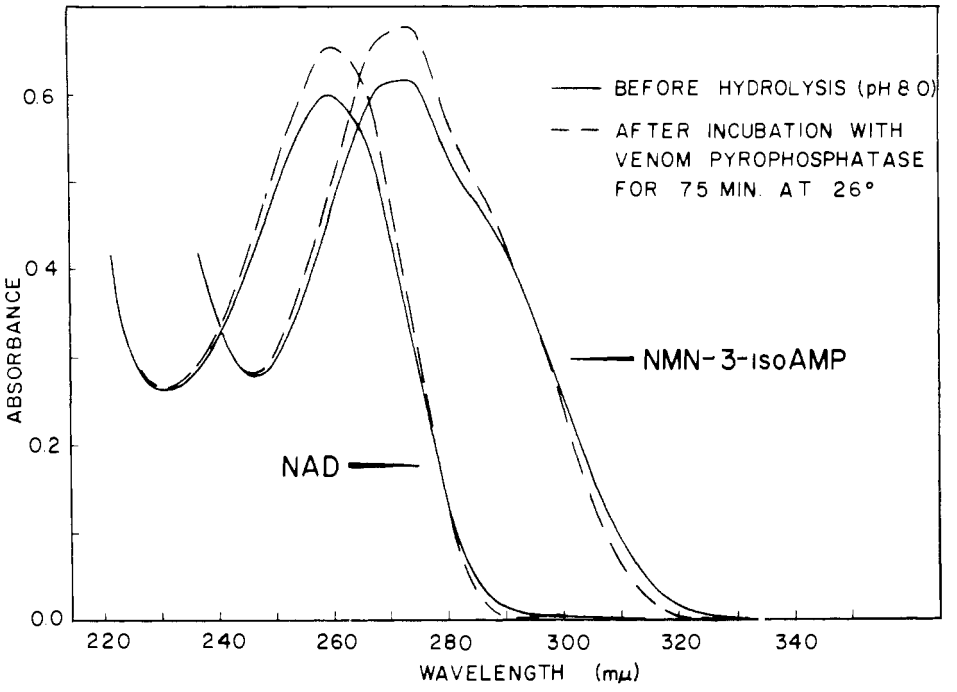


FIGURE 3: Hypochromicity of NAD and NMN-3-iso-AMP.

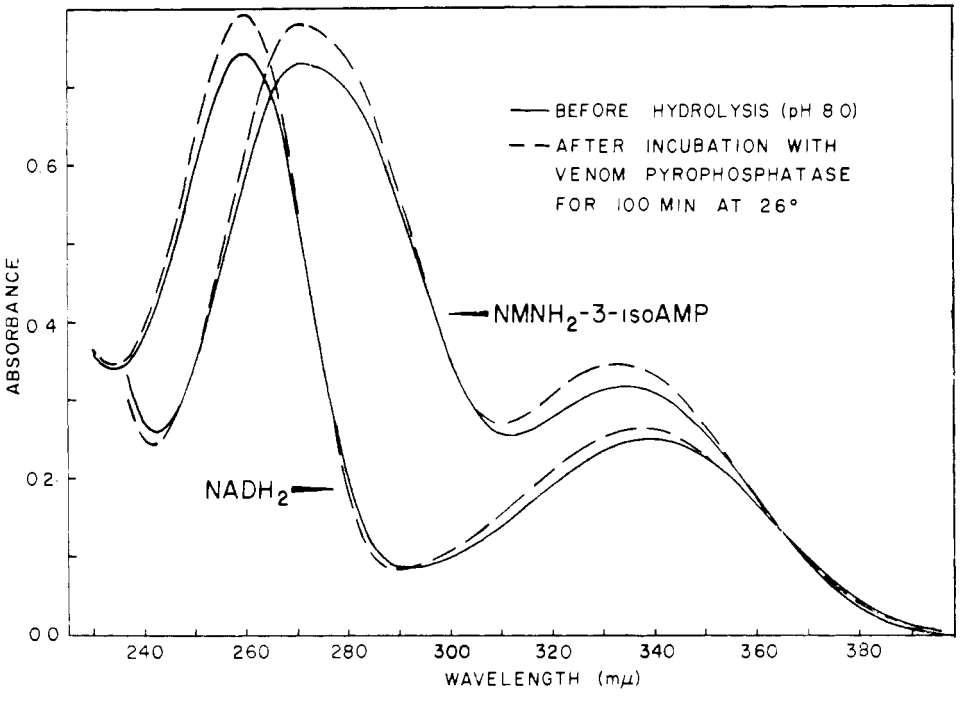


FIGURE 4: Hypochromicity of $NADH_2$ and $NMNH_2$-3-iso-AMP.

The transfer of energy from the adenine to the dihydronicotinamide chromophore noted by Weber (1958) in reduced NAD with fluorescence methods has also been observed with the NAD analogs $NADH_2$-P, $NMNH_2$-dAMP (Fawcett and Kaplan, 1962), and the analog derived from 6-(2-hydroxyethylamino)purine (Windmueller and Kaplan, 1961). The fluorescence spectra shown in Figure 5 reveal that whereas $NADH_2$ has a peak at 260 mμ, indicating energy transfer from adenine to dihydronicotinamide, $NMNH_2$-3-iso-AMP does not have a corresponding peak at 275 mμ (the absorption maximum of 3-iso-AMP) and that there is no energy transfer.

The emission spectrum (Figure 6) of $NMNH_2$-3-iso-

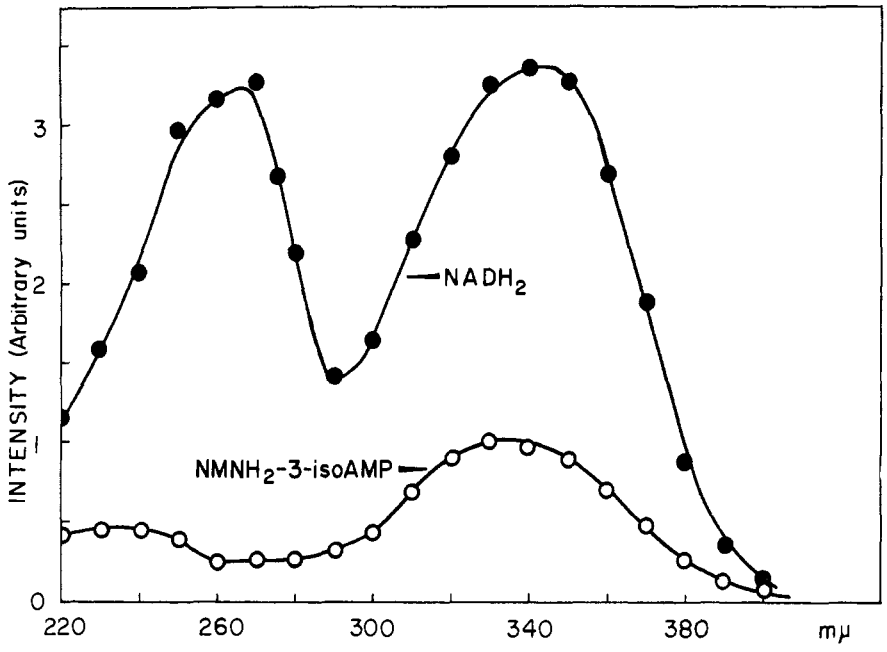


FIGURE 5: Fluorescence excitation spectra of chemically reduced NAD and NMN-3-iso-AMP.

AMP is similar to that of $NADH_2$, having a maximum at 450 mμ. However, the fluorescence yield for $NMNH_2$-3-iso-AMP is only about 40% that for $NADH_2$.

Reaction of 3-Iso-ATP with Firefly Luciferase. Firefly luciferase is reportedly (McElroy and Green, 1956; McElroy and Seliger, 1963) specific for ATP in the reaction:

$$LH_2 + ATP + E \overset{Mg^{2+}}{\rightleftharpoons} E \cdot LH_2\text{-}AMP + PP_i$$
$$\downarrow O_2$$
$$E \cdot L\text{-}AMP + \text{light} + H_2O$$

where luciferin (LH_2) and luciferase (E) combine with ATP to form a complex which emits light on oxidation (McElroy and Seliger, 1961, 1962, 1963; Rhodes and McElroy, 1955). Experiments with crude firefly extracts have shown that 3-iso-ATP is also able to stimulate light production, although at a lower intensity. These results have been confirmed by W. D. McElroy (personal communication) with purified luciferase and will be described in detail in a forth coming joint publication.

Reaction of 3-Iso-ADP with Polynucleotide Phosphorylase. Preliminary results of A. M. Michelson (personal communication) indicate that 3-iso-ADP acts as a substrate for *Azotobacter vinelandii* polynucleotide phosphorylase (Grunberg-Manago *et al.*, 1956) in the exchange reaction:

$$\text{3-iso-A-P-P} + {}^{32}P_i \rightleftharpoons \text{3-iso-A-P-}{}^{32}P + P_i$$

and the polymerization reaction:

$$n\,(\text{3-iso-ADP}) \rightarrow (\text{3-iso-AMP})_n + n\,P_i$$

Details of the polymerization of 3-iso-ADP, the physical and *in vitro* (non-) coding properties of poly-3-iso-adenylic acid, and the properties of complexes of poly-3-isoadenylic acid with other polynucleotides will be described in a subsequent communication.

Discussion

Adenosine and 3-isoadenosine resemble each other in that both are ribonucleosides of roughly equivalent bulk having three potential hydrogen-bonding sites (N-1, N-7, and 6-NH_2) in common on the purine ring and a π-electron system which may be capable of forming complexes with portions of enzymes. The nucleosides differ somewhat in the spatial relationships of the amino group, purine ring, and ribose moieties and in the electronic configuration of the purine ring. Evidence of the latter is seen in the difference in basicities of adenosine (pK_a' 3.45) and 3-isoadenosine (pK_a' 5.5) and in the differences in nucleophilicity of the nitrogen atoms in 9- and 3-substituted adenines; e.g., 9-substituted adenines are alkylated at N-1 (Leonard and Fujii, 1964) whereas 3-substituted adenines are alkylated at N-7 (Leonard and Fujii, 1963). These factors may also affect the manner in which adenosine and 3-isoadenosine participate in hydrogen bonding with other nucleosides. Since alkylation and hydrogen bonding (in DNA) (Watson and Crick, 1953) appear to be favored at N-1 with adenosine (Figure 7A) it would not be surprising, in view of the alkylation data, to find preferential hydrogen bonding at N-7 with 3-isoadenosine (Figure 7B). However, hydrogen bonding at N-7 of adenosine or 9-alkyladenines has been observed in certain crystalline complexes (Haschemeyer and Sobell, 1963; Hoogsteen, 1959), and thus it is

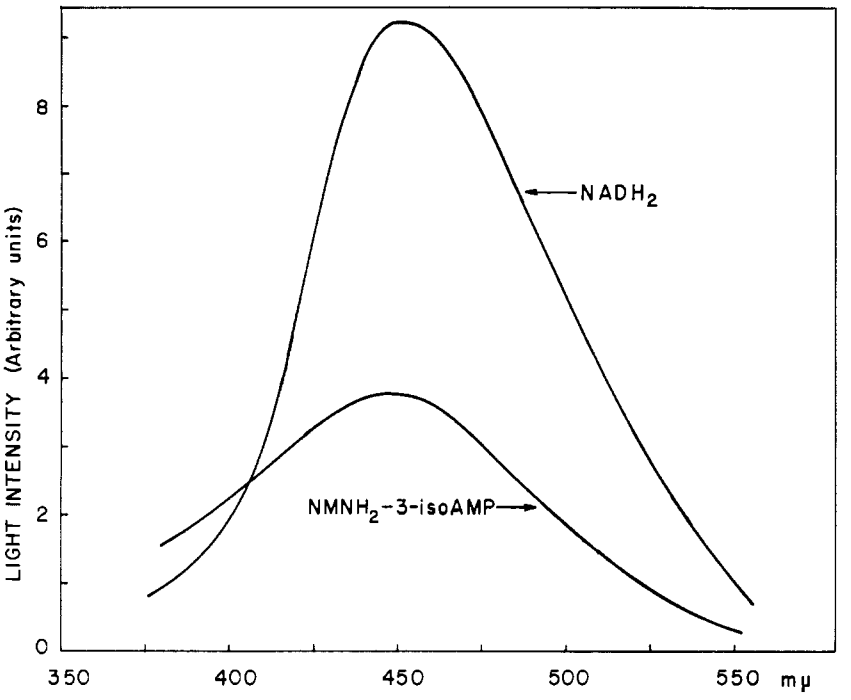


FIGURE 6: Emission spectra of chemically reduced NAD and NMN-3-iso-AMP.

apparent that factors (crystal packing, helix formation) other than the relative nucleophilicities of the nitrogen atoms may also be important.

With the dehydrogenases examined, we have observed roughly the same pattern noted by Kaplan and coworkers (Fawcett and Kaplan, 1962; Windmueller and Kaplan, 1961), namely, a relatively low specificity for beef liver glutamic and horse liver alcohol dehydrogenases and a high specificity for yeast alcohol dehydrogenase. It is interesting that NMN-3-iso-AMP is actually a better cofactor than NAD for two of the enzymes.

Evidence for the interaction of purine and nicotinamide rings in NMN-3-iso-AMP is provided by the hypochromicity observed in both the oxidized and reduced forms of NMN-3-iso-AMP. This behavior, noted previously with NAD (Weber, 1958; Fawcett and Kaplan, 1962), suggests that NMN-3-iso-AMP possesses a folded configuration. The lack of energy transfer from adenine to dihydronicotinamide in $NMNH_2$-3-iso-AMP does not necessarily mean that the molecule does not possess the folded configuration, since the electronic structures may simply be unfavorable for transfer by the coupled oscillator mechanism of Weber (1958). It is perhaps significant that of the NAD analogs examined so far, only $NADH_2$, $NADH_2$-P, $NMNH_2$-dAMP (Fawcett and Kaplan, 1962), and the analog derived from 6-(2-hydroxyethylamino)purine (Windmueller and Kaplan, 1961), compounds in which the pyrimidine ring is still "intact," show energy transfer; the analogs derived from 1-(2-hydroxyethyl)adenine (Windmueller and Kaplan, 1961), thymidine and uridine (Fawcett and Kaplan, 1962), inosine (Shifrin and Kaplan, 1959), and 3-isoadenosine do not. One of the requirements for energy transfer is a sufficiently long-lived excited state of the purine moiety. Börresen (1963a,b) and Walaas (1963) have observed that adenosine and its phosphate derivatives have a small but measurable fluorescence at 380–400 μ, whereas the values for ITP and IMP are only one-tenth that of adenosine. A preliminary experiment has indicated that 3-isoadenosine has no measurable fluorescence at *p*H 3.3 or 7.2.

In conclusion, the ability of 3-isoadenosine to replace adenosine in reactions with a number of enzymes, particularly specific enzymes such as myokinase and

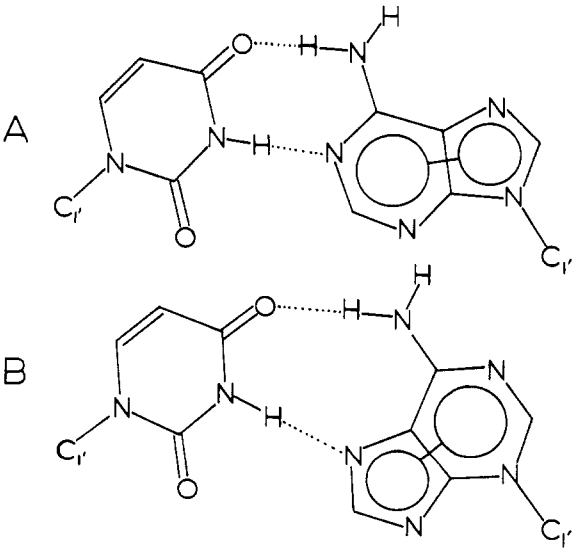


FIGURE 7: Hydrogen bonding at N-1 of adenosine (A) and N-7 of 3-isoadenosine (B).

luciferase, suggests that 3-isoadenosine has many of the structural features of adenosine, and that coenzyme analogs derived from 3-isoadenosine, in conjunction with other analogs, may be useful for studying the mechanisms of action of a number of enzymes.

Experimental

Purification and Manipulation of Solvents. Pyridine was distilled from calcium hydride and was stored over calcium hydride or Linde molecular sieves (Type 4A). Dimethylformamide was distilled from phosphorus pentoxide and stored over molecular sieves. Dioxane was distilled from lithium aluminum hydride and stored over sodium or molecular sieves. Small quantities of solvents were stored over molecular sieves in bottles fitted with serum caps and were transferred by means of a syringe.

Evaporation of Solvents. Because the nucleotides and nucleotide anhydrides described here were very unstable, an effort was made to prevent decomposition by overheating. Unless otherwise noted, solvents were removed using a Büchi "Rotavapor" thin-film rotary evaporator and a vacuum pump at a bath temperature of less than 40°.

P^1-Diphenyl P^2-[(3-β-D-Ribofuranosyladenine)-5′]-pyrophosphate (II). Monomethyltrioctylammonium (3-β-D-ribofuranosyladenine)-5′-phosphate (0.5 mmole) was dissolved in 2.0 ml of dry dimethylformamide and 2.0 ml of dry dioxane (Michelson, 1958b). To the solution was added 0.15 ml of diphenylphosphoryl chloride and 0.3 ml of tributylamine. The solution was shaken and was kept at room temperature for 2.75 hours. The solvent was removed under vacuum, and 50 ml of dry ether was added. The mixture was shaken vigorously and then was allowed to stand for 1 hour in the refrigerator. The ether was decanted and the gummy residue was washed twice more with 25-ml portions of ether. Traces of water and ether were removed by co-distillation under vacuum with 5 ml of dry dimethylformamide.

(3-β-D-Ribofuranosyladenine)-5′-diphosphate (3-Iso-ADP) (III). To 0.6 mmole of P^1-diphenyl P^2-[(3-β-D-ribofuranosyladenine)-5′]-pyrophosphate (compound II) was added 0.8 mmole of tributylammonium phosphate in 0.7 ml of dry pyridine. The mixture was shaken vigorously and was kept at room temperature for 1.5 hours. Solvent was removed under vacuum and 20 ml of water was added. The solution was extracted with three 25-ml portions of ether, basified to *p*H 8.5 with ammonium hydroxide, filtered, and applied to a column (2.0 × 14 cm) of Dowex 1-X8 (chloride, 200–400 mesh). The column was washed with water until the absorbance at 275 mμ fell to zero, and was eluted with 500 ml of 0.0075 N HCl, which removed 3-iso-AMP and $(3\text{-iso-AMP})_2$ (69 units); 300 ml of 0.005 N HCl–0.05 M LiCl, which removed inorganic phosphate and then 3-iso-ADP (251 units); and 0.005 N HCl–0.25 M LiCl, which removed 3-iso-ATP (31 units); 20-ml fractions were collected and the absorbance at 275 mμ was measured. Fractions 40–46 were combined, neutralized with lithium hydroxide, and evaporated to dryness. Methanol (10 ml), followed by 50 ml of acetone, was added. The solid was triturated, collected by centrifugation, and washed with acetone and ether, 109 mg (theory 268 mg). The product obtained in this manner sometimes contained inorganic phosphate which could be removed by readsorbing the material on the Dowex 1 column, eluting with 0.005 N HCl–0.025 M LiCl, and collecting only the center fractions of the peak.

Analysis of Purified 3-Iso-ADP. Purified 3-iso-ADP (4.4 mg) was dissolved in 1.0 ml of water; 0.050-ml aliquots were removed for analysis. Total phosphate, acid-labile (7-minute) phosphate, and inorganic phosphate were determined by standard methods (Flynn *et al.*, 1954; Cowgill and Pardee, 1957). Adenine was estimated by hydrolyzing a sample at 100° in 1.0 N HCl for 15 minutes and measuring the ultraviolet absorption at 262 mμ in 0.02 N HCl, assuming $\epsilon_{262} = 13{,}100$ (Beaven *et al.*, 1955). 3-Isoadenosine was estimated by measuring the ultraviolet absorption of 3-iso-ADP in 0.02 N HCl at 275 mμ, assuming $\epsilon_{275} = 16{,}600$ for 3-iso-AMP and that the spectral properties of 3-iso-AMP and 3-iso-ADP are the same. Enzymatically hydrolyzable phosphate was measured by incubating 3-iso-ADP in 0.1 M Tris-HCl buffer (*p*H 8.0), 0.04 M magnesium chloride containing 1 mg of crude *Crotalus adamanteus* venom per mg of 3-iso-ADP, and determining inorganic phosphate liberated (Table II). A purified sample showed a single spot (R_F 0.31) on chromatography in isobutyric acid–water–ammonium hydroxide (66:33:1) (Pabst, 1961). Some unpurified samples contained an unidentified compound (R_F 0.41) which was not 3-iso-AMP (R_F 0.50) or 3-iso-ATP (R_F 0.18).

(3-β-D-Ribofuranosyladenine)-5′-triphosphate (3-Iso-ATP) (IV). To 0.29 mmole of P^1-diphenyl P^2-[(3-β-D-ribofuranosyladenine)-5′]-pyrophosphate (compound II) was added 0.6 mmole of bistributylammonium pyrophosphate in 1.5 ml of dry pyridine. The solution was kept at room temperature for 100 min, and 25 ml of water was added. The solution was extracted with three 25-ml portions of ether and was adjusted to *p*H 7.5 with ammonium hydroxide. The solution was applied to a column (1.8 × 15 cm) of Dowex 1-X8 (chloride, 200–400 mesh). The column was washed with 100 ml of water

TABLE II: Analysis of 3-Iso-ADP.

Analysis	Ratio	
	Theory	Found
7-Minute phosphate	1.00	1.00 (defined)
Total phosphate	2.00	2.03
Phosphate cleaved by snake venom	2.00	2.00
Inorganic phosphate	0.00	<0.01
Adenine	1.00	1.09
3-Isoadenosine	1.00	1.04
3-Iso-ADP (based on phosphate analysis)	89%	

and was eluted with 500 ml of 0.005 N HCl, which eluted 3-iso-AMP (8.3 units); 400 ml of 0.005 N HCl–0.05 N LiCl, which removed 3-iso-ADP (25 units); and 0.005 N HCl–0.1 N LiCl, which removed 3-iso-ATP (126 units); 24-ml fractions were collected and the absorbance at 275 mμ was measured. Fractions 62–80 were combined, neutralized with lithium hydroxide, and evaporated to dryness. Methanol (20 ml), followed by 150 ml of acetone, was added. The solid was triturated and was collected by filtration. The product was rechromatographed on the Dowex 1 column with 0.005 N HCl–0.15 N LiCl as the eluent. The center fractions of the peak were combined and treated as above, yielding 117 (theory 157) mg of product (dried under vacuum). The product was analyzed by the procedures described for 3-iso-ADP, and was found to contain about 63% 3-iso-ATP and considerable amounts of inorganic pyrophosphate. Small quantities of 3-iso-ATP were purified by electrophoresis on paper in 0.05 M sodium acetate buffer (*p*H 6.0) at 275 V for 3.5 hours. Under these conditions 3-iso-ATP migrated 12 cm and inorganic pyrophosphate 16.6 cm, 3-iso-ATP was located under ultraviolet light, and the band was excised and eluted.

Myokinase Reactions. AMP, ADP, and ATP were the products of Pabst Laboratories. IDP and CDP were obtained from Sigma Chemical Co. Reaction mixtures contained about 4 mg (total) of nucleotide(s) in 0.5 ml of 0.1 M potassium chloride, 0.01 M magnesium chloride, 0.1 M Tris-HCl buffer (*p*H 7.6). To half of the solution was added 50 μg of myokinase (Sigma). The solutions were kept at room temperature for 100 minutes. Aliquots of the mixtures and blanks were chromatographed on Whatman No. 1 filter paper in isobutyric acid–water–ammonium hydroxide (66:33:1) (Pabst, 1961) (see Figure 1).

Myokinase-Hexokinase Reactions. Reaction mixtures contained about 2 mg of nucleoside diphosphate, 125 μg of myokinase (Sigma), and 75 μg of hexokinase (Sigma, Type IV) in 1.0 ml of 0.05 M glucose, 0.1 M potassium chloride, 0.01 M magnesium chloride, 0.1 M Tris-HCl buffer (*p*H 8.5). Aliquots (0.1 ml) were removed periodically and analyzed for 7-minute phosphate (Cowgill and Pardee, 1957) (see Figure 2).

Purification of Snake Venom Pyrophosphatase. Lyophilized *Crotalus adamanteus* venom (200 mg, Sigma), was dissolved in 7.0 ml of 0.02 M Tris-HCl buffer (*p*H 8.9); the *p*H was readjusted to 8.9 by the addition of solid Tris. The milky solution was centrifuged, and the clear supernatant was applied to a column (1.8 × 50 cm) of DEAE-cellulose which previously had been equilibrated with 0.02 M Tris-HCl buffer (*p*H 8.9) (Boman and Kaletta, 1957). Elution was carried out with 100 ml of 0.33 M Tris-HCl (*p*H 8.9), 60 ml of 0.60 M Tris-HCl (*p*H 8.9), and finally with 0.33 M Tris-HCl (*p*H 7.3). The flow rate was 0.35 ml/min; 3.5-ml fractions were collected. The transmittance at 254 mμ was measured automatically on a GME UV-254 absorption meter. Pyrophosphatase activity of the fractions was measured by the method of Kaplan and Stolzenbach (1957); 5′-nucleotidase activity was assayed by measuring the amount of inorganic phosphate (Leloir and Cardini, 1957) liberated from AMP at *p*H 8.5 in 0.02 M Tris-HCl buffer. Fractions 16–37 were combined and after lyophilization yielded 1.1 g of a powder having a pyrophosphatase activity of about 4 μmoles of NAD/hr/mg and a 5′-nucleotidase activity of about 0.05 μmole of AMP/hr/mg. The crude enzyme was stable for several months when stored at −20°.

Nicotinamide Mononucleotide. β-Nicotinamide-adenine dinucleotide (2.1 g, 2.9 mmole, 98% β-NAD, obtained from Sigma Chemical Co., St. Louis, Mo.) was dissolved in 40 ml of water, and the solution was adjusted to *p*H 6.0 with 1 N sodium hydroxide. Ten ml of 0.1 M sodium bicarbonate solution, 2.0 g of magnesium chloride hexahydrate, and 200 mg of crude venom pyrophosphatase were added, and the solution was adjusted to *p*H 8.2. As the reaction proceeded the *p*H decreased, and solid Tris was added to maintain the *p*H at 8.2. Additional enzyme was added when the reaction appeared to slow down. The course of the reaction was followed by the yeast alcohol dehydrogenase method (Kaplan and Stolzenbach, 1957). The reaction was 98% complete within about 18 hours. AMP and NAD were removed by adjusting the solution to *p*H 6.0 with formic acid and passing the solution through a column (2.6 × 19.5 cm) of Dowex 1-X8 (formate, 200–400 mesh). The column was washed with water, and 25-ml fractions were collected. Nicotinamide and cations were removed by combining fractions 1–7 (200 ml, 14,300 OD units at 266 mμ) and passing the solution through a column (2.6 × 10 cm) of Dowex 50-X8 (H^+, 200–400 mesh). The column was washed with water, and 150-ml fractions were collected. Fractions 4–7 (600 ml, 12,700 OD units) were combined and concentrated to about 50 ml. The solution was basified to *p*H 8.1 with ammonium hydroxide and was applied to a column (2.6 × 12 cm) of Dowex 1-X8 (formate, 200–400 mesh). The column was washed with water and was eluted with 0.01 N formic acid; 25-ml fractions were collected and the absorbance at 266 mμ was measured. Fractions 17–29 (8970 OD units) were combined, concentrated, and lyophilized, and yielded 677 mg of a white, fluffy solid which contained about 71% of NMN, based on spectral measurements. The product migrated as a single spot on chromatography in pyridine-water (2:1) (Burton and San Pietro, 1954) and on electrophoresis in 0.05 M sodium phosphate buffer (*p*H 8.5). The dissymmetry of the NMN peak suggested that an impurity, very similar in properties to NMN, might be present. Repeated chromatography on the Dowex 1 (formate) column led to more symmetrical elution curves, but also to severe losses of material. No material other than NMN could be detected by paper chromatography, by electrophoresis, or by ultraviolet spectral examination of the chromatographic fractions.

Methyltrioctylammonium Nicotinamide Mononucleotide. Methyltrioctylammonium hydroxide (0.5 mmole) in 0.83 ml of 50% aqueous methanol was added to a suspension of 238 mg (0.5 mmole) of hydrated nicotinamide mononucleotide in 5 ml of dimethylformamide

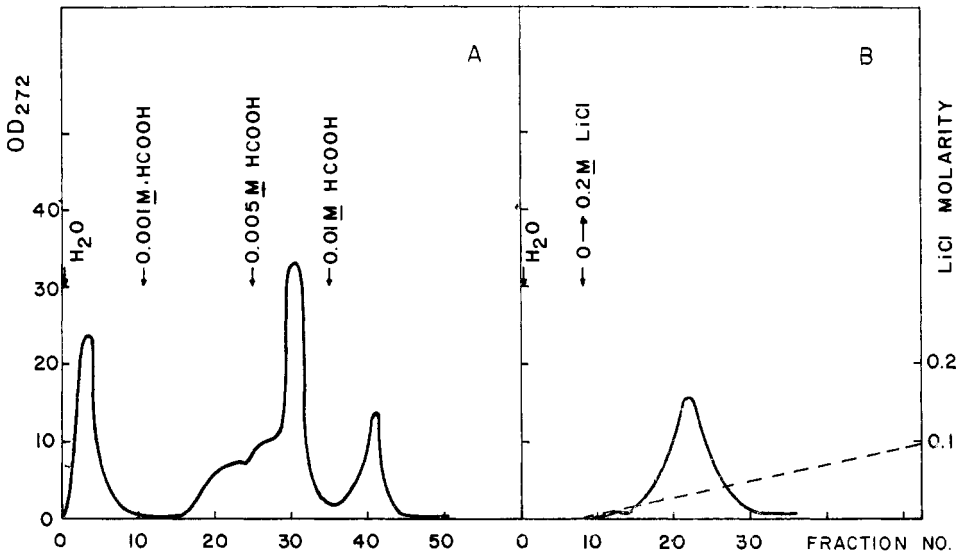


FIGURE 8: Purification of NMN-3-iso-AMP. (A) Chromatography on a column (1.8 × 14 cm) of Dowex 1-X8 (formate, 200–400 mesh). (B) Chromatography of fractions 24–35 from (A) on a column (1.8 × 12 cm) of Dowex 1-X8 (chloride, 200–400 mesh). Eluent as shown; 20-ml fractions.

and 0.5 ml of water. The mixture was shaken, and when solution was complete the solvent was evaporated under reduced pressure. Traces of water were removed by two evaporations with 5-ml aliquots of dry dimethylformamide.

P^1-Ribosylnicotinamide-5' P^2-[(3-β-D-Ribofuranosyladenine)-5']-pyrophosphate (NMN-3-iso-AMP) (V). To 0.5 mmole of P^1-diphenyl P^2-[(3-β-D-ribofuranosyladenine)-5']-pyrophosphate (compound II) was added 0.5 mmole of methyltrioctylammonium nicotinamide mononucleotide in 1.0 ml of dry dimethylformamide and 0.5 ml of dry pyridine. The mixture was shaken until a homogeneous solution was obtained, and the solution was kept at 0° for 11 hours and at room temperature for 1.5 hours. The solvent was evaporated under vacuum, and 50 ml of ether and 2 ml of methanol containing 150 mg of lithium chloride was added. The solid which formed was triturated and removed by filtration, and was then dissolved in 50 ml of water. The solution was basified to *p*H 8.5 with lithium hydroxide and was extracted with 30 ml of ether to remove insoluble amines. The solution was applied to a column (1.8 × 14 cm) of Dowex 1-X8 (formate, 200–400 mesh). The column was washed with 200 ml of water and was eluted with formic acid (Figure 8A); 20-ml fractions were collected. NMN-3-iso-AMP was eluted in a peak having its maximum at tube 30. Tubes 24–35 were combined and evaporated to about 10 ml. The solution was diluted with 30 ml of water, was basified to *p*H 8.5 with lithium hydroxide, and was applied to a column (1.8 × 12 cm) of Dowex 1-X8 (chloride, 200–400 mesh). The column was washed with 100 ml of water and was eluted with a linear gradient of 1 liter of 0.2 M lithium chloride into 1 liter of water (Figure 8B); 20-ml fractions were collected. Tubes 18–28 were combined and evaporated to dryness, and traces of water were removed by azeotropic distillation with ethanol. Methanol (50 ml), followed by 100 ml of ether, was added. The precipitate was triturated and was collected by centrifugation. The residue was dissolved in 20 ml of water and the solution was evaporated to dryness. The solid was triturated in 10 ml of methanol and 30 ml of ether and was collected by centrifugation. The product, after being dried for several hours under high vacuum, weighed 96 mg (theory, 334 mg) and contained 79% NMN-3-iso-AMP, based on its phosphorus content.

Analysis of NMN-3-iso-AMP. NMN-3-iso-AMP (6.0 mg) was dissolved in 0.5 ml of 0.1 M Tris-HCl buffer (*p*H 8.0), 0.04 M magnesium chloride containing 0.4 mg of *Crotalus adamanteus* venom. The mixture was incubated at room temperature for 3 hours, and 0.2 ml of 1.0 N HCl was added. The solution was heated to 100° for 45 minutes and 0.05-ml aliquots were analyzed for phosphate (see analysis of 3-iso-ADP), adenine, and nicotinamide. For the latter analyses, 0.05-ml aliquots were spotted on Whatman No. 1 filter paper and the spots were neutralized with ammonia fumes. The chromatograms were developed in water-saturated 1-butanol, and the spots were eluted with 0.1 N HCl. Concentrations were estimated spectrophotometrically assuming ϵ_{262} = 13,100 for adenine (Beaven *et al.*, 1955), ϵ_{261} = 5300 for nicotinamide (Willi, 1954) and ϵ_{261} = 5500 for nicotinic acid (Hughes *et al.*, 1949) (formed in about 10% yield by hydrolysis of nicotinamide) (Table III).

Cleavage of NMN-3-iso-AMP with Venom Pyrophosphatase. NMN-3-iso-AMP (3.0 mg) and 10 mg of venom pyrophosphatase were incubated in 0.25 ml of 0.1 M Tris-HCl buffer (*p*H 8.0), 0.04 M magnesium

TABLE III: Analysis of NMN-3-iso-AMP.

Compound	R_F	Ratio Theory	Ratio Found
Adenine	0.39	1.00	1.00 (defined)
Nicotinamide	0.65	1.00	1.00
Nicotinic acid	0.18		
Phosphate		2.00	1.91

TABLE IV: Distances Migrated by NMN Derivatives.

Compound	Migration (cm)
NMN	1.6
3-Iso-AMP	3.5
NMN-3-iso-AMP	1.0
NAD	1.0

chloride for 3.75 hours at room temperature. Aliquots were then spotted on S and S Orange Label filter paper, and the strips were subjected to electrophoresis in 0.1 M sodium phosphate buffer (*p*H 8.5) for 5 hours at 275 V (see Table IV). Spots were detected under ultraviolet light; quaternary pyridinium salts could be detected as fluorescent spots after placing the strips in an ammonia–methyl ethyl ketone atmosphere (Kodicek and Reddi, 1951). Only 3-iso-AMP and NMN were detected. The spots were further identified by elution and determination of their ultraviolet spectra.

Enzymatic Reduction of NAD and NMN-3-iso-AMP. The procedures used were essentially those of Fawcett and Kaplan (1962). Enzymatic reductions were followed by measuring the increase in absorbance at 340 mμ with a Cary Model 15 recording spectrophotometer. Each cell (1.0 cm pathlength) contained 3.0 ml of 0.1 M Tris-HCl buffer (*p*H 9.0) and 0.6 μmole of NAD or NMN-3-iso-AMP. Substrate concentrations were: ethanol, 0.5 M; lithium DL-lactate, 0.1 M; sodium DL-malate, 0.5 M–sodium glutamate, 0.5 M. The glyceraldehyde-3-phosphate dehydrogenase reaction was carried out in 0.1 M Tris-HCl buffer (*p*H 8.5) containing sodium arsenate (0.0082 M) and 0.05 ml of triose phosphate ester solution (C. F. Boehringer and Soehne). Yeast and horse liver alcohol dehydrogenases, beef liver glutamic, pig heart malic, and rabbit muscle triosephosphate dehydrogenases were obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Rabbit muscle lactic dehydrogenase was purchased from Sigma Chemical Co., St. Louis. Reactions were initiated by adding 0.025 ml of a solution containing sufficient enzyme to give 10–20% reaction with NAD within 2 minutes. Relative rates were obtained by determining the slope of the rate curve between the first and third minute of reaction.

Chemical Reduction of NAD and NMN-3-iso-AMP. Reduction was carried out using a modification of the method of Lehninger (1957). NAD or NMN-3-iso-AMP (5 mg) and fresh sodium hydrosulfite (10 mg) were dissolved in 1.5 ml of 1.3% sodium bicarbonate. The initially yellow solution was kept at room temperature until the color faded (about 40 minutes) and oxygen was bubbled through the solution for a few minutes to oxidize excess hydrosulfite. The products from the oxidation of sodium hydrosulfite were not removed, controls having shown that they had no ultraviolet absorption in the region of interest. The reaction mixture was diluted with 0.1 M Tris-acetate (or hydrochloride) buffer (*p*H 8.0), and the solution was used within a few hours for spectral studies.

Fluorescence Excitation Spectra. Spectra were obtained on solutions having an absorbance of about 0.25 at 340 mμ. The spectrofluorometer consisted of an Osram xenon arc source, a monochrometer, a thermostated sample compartment, and an RCA IP 28 photomultiplier. Readings were made on a Keithley 414 microammeter. Emitted light was filtered to remove scattered light below 400 mμ. Corrections for variation in intensity of the arc source were made by measuring the intensity of the exciting beam with an RCA IP 28 photomultiplier in conjunction with the microammeter.

Emission Spectra. The apparatus used for determining emission spectra has been described by Weber and Young (1964).

Hypochromicity of Oxidized and Reduced NAD and NMN-3-iso-AMP. A solution of NAD or NMN-3-iso-AMP or the reduced analogs in 0.05 M Tris-HCl–0.04 M magnesium chloride buffer (*p*H 8.0) was placed in a 1.0-cm path length cell. Ultraviolet spectra were obtained on a Cary Model 15 recording spectrophotometer before and after the addition of snake venom pyrophosphatase.

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