A Method for the Purification of Nicotinamide Mononucleotide[1]

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Introduction

Since nicotinamide mononucleotide (NMN) prepared by the method of Kornberg and Pricer (1) contains a fairly large quantity of salts, a modification of the procedure of the previous authors was developed. The essential steps include incubation of diphosphopyridine nucleotide (DPN) with purified pyrophosphatase (1) in a nonphosphate buffer and in the presence of fluoride [under these conditions it was not found necessary to fractionate the pyrophosphatase to as high a degree of purity as that employed by Kornberg and Pricer, inasmuch as fluoride in the absence of inorganic phosphate inhibits phosphatases but not the pyrophosphatase (1)]. The NMN is next adsorbed on charcoal followed by elution with aqueous amyl alcohol (2). The eluates are then passed through a bed of Dowex 1 chloride anion exchanger. NMN is washed out of the ion-exchange resin with water while DPN and 5-adenylate are retained. NMN is precipitated from a slightly acidic solution by a large volume of acetone.

Procedure

DPN,[3] 0.75 g., is dissolved in 20 ml. of 0.1 *M* glycine buffer (pH 7.7) and adjusted to pH 7.6 with 2 *N* KOH. Three milliliters of 1 *M* sodium fluoride and enough potato pyrophosphatase [step II-2c (1)] to guarantee complete destruction of DPN in less than 10 min. are added. The final volume is made up to 30 ml. with 0.1 *M* glycine buffer (pH 7.7). The mixture is incubated at 37°. The progress

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[3] DPN of 50–90% purity has been used.

of the reaction is followed by measurement of the disappearance of DPN by the alcohol dehydrogenase assay (3). When more than 90% of the DPN has disappeared,[4] the mixture is chilled to 5° in an ice bath, and 3 ml. of 50% perchloric acid is added. Precipitated proteins are removed by centrifugation in the cold. The clear supernatant is adjusted to about pH 5.5 with 2 *N* potassium hydroxide and left to stand at 0° for about 10 hr. The precipitated potassium perchlorate is then centrifuged off in the cold. The supernatant solution is diluted with 9 vol. of cold (5°) water. Five grams of an activated carbon (Nuchar C 190N[5]) is added, and the mixture is stirred mechanically for 10 min. in an ice bath. The suspension is filtered with suction through No. 3 Whatman paper until almost all the fluid has passed through. The moist cake is then washed with 30 ml. ice-cold distilled water on the filter. The cake is sucked dry and suspended in 50 ml. cold 10% (*v/v*)

TABLE I

Protocol of a Typical Preparation of NMN

Fraction	DPN	NMN[a]
	total μmoles	*total μmoles*
0.74 g. 69% DPN	775	—
After pyrophosphatase treatment	27	756
Charcoal treatment:		
Raffinate	—	9
Eluate	—	407
Eluate from Dowex	2	380
Acetone precipitate (redissolved)	1	352

[a] It was assumed that the molecular extinction values for reduced DPN and NMN were equal at 340 mμ. The NMN value was calculated as the difference between the hydrosulfite and alcohol dehydrogenase assay values.

isoamyl alcohol and stirred for 30 min. in an ice bath followed by vacuum filtration through No. 3 Whatman paper. This elution procedure is repeated three times. The separate eluates are assayed for the nicotinamide ribose moiety by the hydrosulfite procedure (4) (50–70% recovery on the basis of the original DPN). The eluate fractions containing the bulk of the compound are combined and carefully concentrated to about 50 ml. *in vacuo*. This solution is then passed through a well-washed Dowex[6] 1 chloride column (5) (diameter 6.5 cm., height 3.5 cm.)

[4] The capacity of the glycine buffer is not quite adequate to hold the pH at the optimum for the enzyme. If an inadequate amount of enzyme is used the reaction will be slowed down considerably before complete hydrolysis is obtained. In such a case readjustment of the pH to 7.7 with dilute potassium hydroxide solution during the course of the reaction is helpful.

[5] Obtained from the Industrial Chemical Sales Division, West Virginia Pulp and Paper Company, Tyrone, Pennsylvania.

[6] Dow Chemical Company, Midland, Michigan; 200–400 mesh. The resin is sus-

followed by 300–600 ml. of distilled water (rate of flow 4–5 ml./min.). The progress of the compound through the column can be followed by measurement of absorption at 260 mμ. The tubes containing the bulk of the NMN are combined, adjusted to pH 5.5–6, and carefully concentrated *in vacuo* to about 10 ml. (recovery from column 70–90%). This solution is adjusted to pH 3 with dilute nitric acid in an ice bath and then treated with 20 vol. of acetone at 0–5°. The heavy white precipitate is centrifuged at 0°. (The residue can be dried *in vacuo* at this stage; however, the compound was found more stable when kept in solution frozen.) The gummy residue is dissolved in water and adjusted to pH 6 with dilute KOH. Analysis indicates that the molar ratio of nicotinamide ribose (4) to ribose (6) to phosphate (7) is very close to theory in this preparation. On a weight-hydrosulfite assay basis, NMN of 70–90% purity was obtained, the final purity being somewhat dependent on the quality of DPN used initially. Data obtained in a typical preparation are summarized in Tables I and II.

TABLE II

Chemical Analysis of NMN[a]

Nicotinamide-ribose:pentose:phosphate	= 1:0.95:1.07
Purity (hydrosulfite assay *vs.* weight)	= 77%
Inorganic phosphate	= 0

[a] The preparation analyzed was the acetone precipitate described in Table I.

Summary

Purified nicotinamide mononucleotide (NMN) has been prepared by incubation of diphosphopyridine nucleotide (DPN) with potato pyrophosphatase in a nonphosphate buffer and in the presence of fluoride. The NMN formed is adsorbed on charcoal followed by elution with aqueous isoamyl alcohol. This eluate is passed through Dowex 1 chloride, and NMN is washed out of the anion exchanger with water, DPN and adenylic acid being retained by the resin. NMN is precipitated from a slightly acidic solution with acetone.

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pended in distilled water and centrifuged at 150–200 × *g* for 5 min. The fines are decanted. This process is repeated till the exchanger is free of fines. The resin is then treated on a suction funnel with 4% NH_4OH, and washed with water followed by 1 *N* HCl. This process is repeated 6–7 times. After the last water wash the resin is placed in a column, and 1 *N* HCl is passed through until there is no absorption at 260 mμ. Distilled water is then passed through the column till the titratable acidity is negligible. The thoroughness of the final water wash is of extreme importance here since it has been observed that DPN can be eluted from these columns with 0.001 *N* HCl. The resin can be stored in distilled water for an indefinite period of time. It is advisable to wash stored resin with distilled water in the column prior to use.

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