ENZYMATIC CLEAVAGE OF DIPHOSPHOPYRIDINE NUCLEOTIDE WITH RADIOACTIVE PYROPHOSPHATE

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The reaction of nicotinamide mononucleotide (nicotinamide ribose phosphate) with adenosinetriphosphate (ATP), catalyzed by a specific enzyme in liver and yeast, has been shown to result in the production of diphosphopyridine nucleotide (DPN) and inorganic pyrophosphate (PP) (1). Similarly, the reaction of riboflavin phosphate with ATP, catalyzed by another yeast enzyme, produces flavin-adenine dinucleotide and PP (2). These reactions are freely reversible; cleavage of the dinucleotides by PP results in the formation of ATP and the respective mononucleotides. No information is available, however, as to the exact way in which the phosphate is distributed in these molecular rearrangements. A hypothetical mechanism is one resembling the reversible splitting of polysaccharides, disaccharides, and nucleosides by inorganic orthophosphate. Evidence to support such a "pyrophosphorylase" action has now been obtained by studies with radioactive PP. The reaction may be formulated as

(1) $P^{32}P^{32}$ + nicotinamide-ribose-P-P-adenosine (DPN) $\rightleftarrows$

nicotinamide-ribose-P + $P^{32}P^{32}$-P-adenosine (ATP)

Methods

Materials—$P^{32}P^{32}$ was prepared by dehydration of $Na_2HP^{32}O_4$. $H_3P^{32}O_4$ in acid solution was obtained from the Oak Ridge National Laboratory and had a stated specific activity of 0.025 mg. per mc. and a stated radiochemical purity of 99.9 per cent. 0.2 ml. (0.77 mc.) was adjusted with NaOH to about pH 9, added to 0.40 ml. of 0.1 M Na_2HPO_4 in a platinum crucible, dried at 110°, and then heated at 225° for about 24 hours to constant weight. The product contained 0.02 mole of orthophosphate per mole of phosphate; 99 per cent was hydrolyzable by 1 N HCl in 10 minutes at 100° and by purified yeast inorganic pyrophosphatase. Chromatographic analysis on Dowex-1 (chloride form) with 0.05 M NaCl in 0.01 N HCl as the eluant indicated that 1.7 per cent of the counts were in the orthophosphate region of the elution curve and 86.8 per cent in the PP region. Some preparations, perhaps as a result of overheating or incorrect pH adjustment, contained polyphosphate, determined as acid-labile phos-

phate, which resisted hydrolysis by inorganic pyrophosphatase. This polyphosphate fraction remained adsorbed to Dowex-1 with 0.05 M NaCl in 0.01 N HCl as the eluant, but was promptly and quantitatively eluted by 1 N HCl.

The enzyme carrying out the pyrophosphorolytic cleavage of DPN was purified from hog liver acetone powder as previously described (1). The final ammonium sulfate precipitate was washed with cold 0.80 saturated ammonium sulfate solution to remove adherent orthophosphate. With the purified preparation, 8 μM of DPN were synthesized from ATP and nicotinamide mononucleotide per hour per mg. of protein. Slight ATPase activity remaining in the final preparation was suppressed by the use of fluoride.

Inorganic pyrophosphatase purified from bakers' yeast was kindly furnished by Dr. L. A. Heppel. Only PP, among all organic and inorganic phosphate compounds tested, was acted upon. Hexokinase (Fraction 3) was prepared by the method of Berger *et al.* (3) and *Zwischenferment* as previously described (4).

Myokinase was prepared essentially according to Colowick and Kalckar (5). Rabbit muscle extract was heated at pH 3, neutralized, and filtered. Ammonium sulfate (252 gm.) was added to the filtrate (600 ml.), and the precipitate was removed by centrifugation. To the supernatant fluid were added 100 gm. of ammonium sulfate, and the precipitate collected by centrifugation was dissolved in water to a volume of 90 ml.

Triphosphopyridine nucleotide (TPN) (purity 0.86) was obtained by ion exchange chromatography of a crude liver fraction.[1] DPN (1) and nicotinamide mononucleotide (6) were prepared as previously described. ATP and adenosinediphosphate were purified from commerical products by ion exchange chromatography (7).

Determinations—PP was determined as orthophosphate released by the action of inorganic pyrophosphatase. Radioactivity was determined in solution in a metal dish with a mica end window Geiger-Müller counter; the background was 20 to 30 c.p.m. Glucose-6-phosphate was estimated spectrophotometrically by TPN reduction with *Zwischenferment.* The nicotinamide-ribose moiety was estimated according to Huff and Perlzweig (8).

Orthophosphate was determined by the method of Fiske and Subbarow (9); acid-labile phosphate was the phosphate liberated after 10 minutes hydrolysis in 1 N HCl at 100°. DPN and ATP were determined as previously described (1).

Ion exchange chromatography was carried out according to Cohn (10) in a cold room at 2°. Dowex-1 anion exchange resin (200-400 mesh) was

[1] Kornberg, A., and Horecker, B. L., unpublished.

prepared by washing the resin first with 3 N HCl until the washings were free of material absorbing at 260 mμ, and then with water until the chloride ion test was negative. The resin was converted to the formate form by repeated washing of the chloride form with 2 M sodium formate until the washings gave no test for chloride. Then the resin was washed with water. The incubation mixtures were adsorbed on Dowex-1 columns, washed with an equal volume of water, and eluted with the indicated eluants under a head of hydrostatic pressure adjusted to permit a flow rate of 0.5 to 1.0 ml. per minute.

Results

$P^{32}P^{32}$ was incubated with DPN in two experiments which differed only in the initial concentrations of these reactants; the reaction was allowed to proceed to or near the equilibrium point. After heat inactivation of the enzyme, an aliquot of the reaction mixture was treated with barium and ethanol which precipitated from 99.4 to 99.9 per cent of the radioactivity; another aliquot was analyzed by anion exchange chromatography. The soluble barium salt fraction, essentially free of radioactivity, contained 96 per cent or more of the DPN and nicotinamide mononucleotide (Table I). The ion exchange chromatogram (Fig. 1) indicates three radioactive areas which, from prior trial experiments, are known to correspond exactly to the orthophosphate, PP, and ATP regions. Phosphate analyses and enzymatic assays of samples in these respective areas in Experiment I confirmed these deductions; in the ATP area these analyses were in close agreement with the optical density values. (The ultraviolet light-absorbing material eluted initially consists of the pyridine nucleotides.) The recovery of ATP from the chromatogram, as judged by ultraviolet absorption, was over 90 per cent and, as determined by radioactivity (Table II), it was near theoretical. The specific activities of ATP and PP were identical within experimental error, as indicated in Fig. 1.

It was of interest to collect the formed radioactive ATP to establish that the location of P^{32} was exclusively in the two terminal positions. To the eluate, between 365 and 785 ml. (Experiment I, Fig. 1), were added 39.6 μM of carrier ATP. The pH was adjusted to 8.7 and 2.0 ml. of saturated barium acetate and 100 ml. of ethanol were added. The precipitate was dissolved in dilute HCl, freed of barium by passage through a column of Dowex-50 cation exchange resin (sodium form), 0.6 cm. × 1 sq. cm. The elutate was neutralized; final volume, 5.5 ml. (The supernatant which was discarded contained 12 per cent of the radioactivity and 12 per cent of the 260 mμ optical density.) Analysis of the final product, recovered in an over-all yield of 85 per cent, was as follows: ATP, total micromoles by optical density 40.7, by enzyme assay 40.3, total counts per

minute 2.8×10^6. The same procedure, applied to the ATP fraction of Experiment II, yielded comparable results.

TABLE I

Balance Data of DPN Pyrophosphorolysis

In Experiment I the incubation mixture contained 1.4 ml. of PP (0.02 M, pH 7.4), 1.2 ml. of radioactive PP (approximately 0.01 M), 1.0 ml. of DPN (0.1 M), 2.0 ml. of glycylglycine buffer (0.25 M, pH 7.4), 0.2 ml. of magnesium acetate (0.3 M), 2.0 ml. of potassium fluoride (1 M), 5.0 ml. of pyrophosphorolytic enzyme (0.8 mg. of protein per ml.), and water to a volume of 20 ml. After 70 minutes at 40°, the mixture was placed in a boiling water bath for 1.5 minutes, cooled, and centrifuged. An aliquot of 7.0 ml. was adjusted to pH 8.5 with NaOH and glycine buffer, and to it were added 1.0 ml. of 0.1 saturated barium acetate and 2.0 ml. of ethanol. The precipitate was removed by centrifugation and the supernatant was saved as the soluble barium salt fraction. Experiment II was similar to Experiment I and differed only in that the incubation mixture contained 1.0 ml. of DPN (0.04 M) and 1.0 ml. of potassium fluoride (1 M).

	Experiment I				Experiment II			
	DPN	PP	ATP	N-R	DPN	PP	ATP	N-R
Reaction mixture								
Initial amount, μM*	97.8	28.1	0.0		39.2	37.4	0.0	
Final " "	70.6	5.6	22.4	100.0	17.3	10.1	19.9	50.4
Δ	−27.2	−22.5	+22.4		−21.9	−27.3	+19.9	
Soluble barium salt fraction								
Amount, μM	68.6			98.3	16.6			51.5
Recovery, *per cent*†	97.3			98.3	95.7			102

* Initial values were obtained by removing an aliquot into trichloroacetic acid immediately after mixing. The low initial values for PP may be related to a slight turbidity which formed instantly in the incubation mixture. Initial nicotinamide-ribose (N-R) determinations were not made and the high final value in Experiment II is unexplained.

† The recovery of DPN and of total N-R moiety (nicotinamide mononucleotide + DPN) in the soluble barium salt fraction is compared with the final amount in the reaction mixture.

The ATP was degraded according to the following equation.

$$\text{(2) Adenosine-P-P}^{32}\text{P}^{32} + 2 \text{ glucose} \xrightarrow[\text{myokinase}]{\text{hexokinase}} \text{adenosine-P} + 2 \text{ glucose-P}^{32}$$

Upon completion of the reaction in the presence of excess glucose, the incubation mixture was analyzed by ion exchange chromatography. The adenosine-5-phosphate fraction was recovered quantitatively in the pre-

dicted area in the elution diagram and was free of any trace of radioactivity (Table III). The glucose-6-phosphate fraction was then eluted; it contained 98.5 per cent of the radioactivity of the ATP from which it originated.

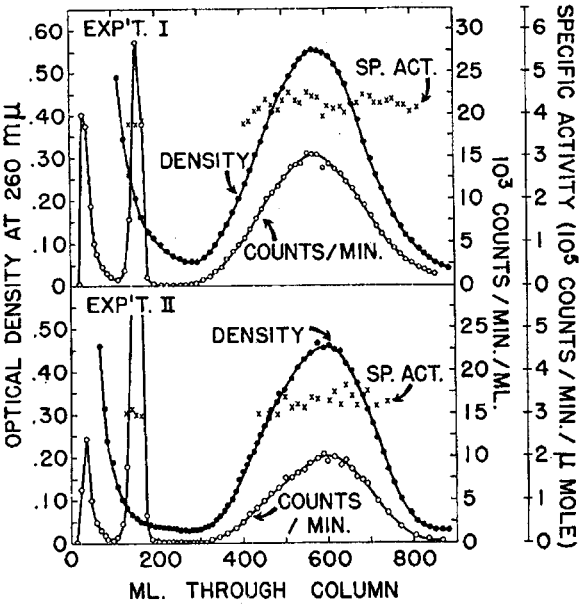

FIG. 1. Radioactivity of PP and ATP separated by ion exchange chromatography. A 10 ml. aliquot of the heated incubation mixture (see Table I) was adsorbed on a Dowex-1 (chloride form) column, 6 cm. × 1 sq. cm., and eluted with 0.01 N HCl in 0.05 M NaCl. The three areas of radioactivity are, in their order of elution, orthophosphate, PP, and ATP.

TABLE II

Recovery of Radioactivity

Experiments I and II are described in Table I and Fig. 1. In Experiment III the incubation mixture contained 0.5 ml. of ATP (0.04 M), 0.9 ml. of PP (0.02 M, pH 7.4), 0.2 ml. of radioactive PP (approximately 0.01 M), 1.0 ml. of glycylglycine buffer (0.25 M, pH 7.4), 0.1 ml. of magnesium acetate (0.3 M), 0.5 ml. of potassium fluoride (1 M), 2.0 ml. of pyrophosphorolytic enzyme (1.3 mg. of protein per ml.), and water to a volume of 10 ml. Experiment III-a differed only in that 1.0 ml. of nicotinamide mononucleotide (0.02 M) was included. After 90 minutes at 40°, the mixture was placed in a boiling water bath for 1.5 minutes, cooled, and centrifuged. An aliquot of 9.0 ml. was analyzed chromatographically as in Fig. 1.

Experiment No.	Initial counts	Soluble barium salts	Ortho-phosphate	PP	ATP	Total recovery
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
I	11.4×10^6	0.61	10.4	15.2	74.7	100.9
II	9.0×10^6	0.08	6.7	32.1	60.5	99.4
III	3.8×10^6				2.5	95.0
III-a	3.9×10^6				32.4	96.1

Doudoroff, Barker, and Hassid (11) proposed that, in the reversible condensation of glucose-1-phosphate with fructose (or other monosaccharides) by a *Pseudomonas* disaccharide phosphorylase, the intermediate formation of an enzyme-glucose complex took place. They observed a rapid incorporation of radioactive orthophosphate in the absence of any monosaccharide acceptor and an inhibition of this incorporation when fructose was present. By analogy with this transglucosidase concept, Equation 1 may be visualized as involving the formation of an intermediate enzyme-adenyl complex from which the adenyl group may be transferred either to PP or to nicotinamide mononucleotide. To test this hypothesis, ATP was incubated with enzyme and $P^{32}P^{32}$ in the complete absence of pyridine

TABLE III

Location of P^{32} in ATP^{32}

The incubation mixture contained 1.6 ml. of radioactive ATP (0.0073 M, see the text), 0.5 ml. of glucose (0.5 M), 2.0 ml. of glycylglycine buffer (0.25 M, pH 7.4), 0.3 ml. of magnesium acetate (0.3 M), 0.1 ml. of hexokinase, 0.5 ml. of myokinase (ammonium sulfate fraction), and water to a volume of 10 ml. After 30 minutes at 34°, the incubation mixture was adsorbed on a Dowex-1 (formate form) column, 10 cm. × 1 sq. cm., and eluted with 0.1 N formic acid. Adenosine-5-phosphate was eluted between 138 and 353 ml., 89 per cent appearing between 154 and 270 ml. After 552 ml. had passed through, the eluant was changed to 0.1 M sodium formate in 0.1 N formic acid. Glucose-6-phosphate was eluted between 13 and 39 ml.

	ATP	Adenosine-5-phosphate	Glucose-6-phosphate
Initial amount, μM	11.3	0.0	0.0
Final " "	0.0	10.6	23.1
Radioactivity, *c.p.m.*	622,000	0	613,000

nucleotide and in a control experiment in which nicotinamide mononucleotide was added (Experiments III and III-a, Table II). Only a small amount of radioactivity appeared in the ATP area in the absence of pyridine nucleotide (Experiment III) and there was no clear relation to the concentration of ATP. Furthermore, the incorporation of $P^{32}P^{32}$ in the presence of nicotinamide mononucleotide (Experiment III-a), rather than being inhibited, was more than 10 times as great. These data therefore provide no support for the occurrence of an enzyme-adenyl complex.

The specificity of PP in the cleavage of dinucleotides was demonstrated in previous work (1, 2) by the failure of orthophosphate or commercial metaphosphate to substitute for it. These observations have been extended to include highly polymerized metaphosphate prepared at 900° (12), an intermediate polyphosphate obtained at 225° (see "Methods"), and sodium tripolyphosphate (Monsanto or Blockson) which are also ineffective in replacing PP.

SUMMARY

1. The reversible enzymatic synthesis of DPN and inorganic pyrophosphate from nicotinamide mononucleotide and ATP was investigated with radioactive inorganic pyrophosphate.

2. Starting with DPN and inorganic pyrophosphate, the ATP formed was isolated and found to have the same specific activity as the inorganic pyrophosphate; the pyridine nucleotides were recovered quantitatively and found to be free of radioactivity. The location of P^{32} in ATP was shown to be exclusively in the two terminal groups.

3. The reaction mechanism may be regarded as the pyrophosphorolytic cleavage of a dinucleotide.

4. A convenient method is provided for isotopic labeling of ATP exclusively in the two terminal phosphate groups.

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