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Nicotinamide Adenine Dinucleotide as Substrate of the Nucleotide Phosphotransferase from *Escherichia coli*†

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ABSTRACT: The phosphate transfer to NAD catalyzed by the nucleotide phosphotransferase of *Escherichia coli* W has been investigated. Nucleotides, such as adenosine 2′-phosphate and thymidine 3′,5′-diphosphate, but not ATP, can serve as phosphate donors for the enzymic synthesis of NADP. The K_m for NAD is 2.2 mM. The NADP produced is composed of about 40% of the 2′ isomer and 60% of the 3′ isomer. The composition was demonstrated by the separation of the NADP isomers by liquid chromatography, by the proportions of the 2′,5′- and 3′,5′-adenosine diphosphates released by treatment with pyrophosphatase, and by assay with NADP-specific isocitrate dehydrogenase. The 3′ isomer is dephosphorylated by 3′-nucleotidase. The findings were also verified by comparison with the mixture of 2′ and 3′ isomers obtainable by acid isomerization of NADP. Both isomers of NADP can serve as phosphate donors for the nucleotide phosphotransferase, transferring their 2′- or 3′-phosphate residues to suitable acceptors, including NAD.

The formation of 2′-NADP[1] from NAD under the influence of NAD-kinase (EC 2.7.1.23), with ATP functioning as the donor of the phosphoryl group, is a well-established biological fact. Under these conditions, the transferred phosphate residue is inserted into the 2′ position of the adenosine moiety of NAD (Kornberg and Pricer, 1950). The discovery in *Escherichia coli* of a phosphotransferase capable of transferring a phosphate residue from a suitable phosphate donor, such as a mononucleotide, to the 2′- or 3′-hydroxyl of another nucleotide or nucleoside (Brunngraber and Chargaff, 1970, 1973) prompted the question whether NAD could act as acceptor in such a transfer reaction.

This proved to be the case. The enzymic phosphorylation by means of the *E. coli* phosphotransferase gave, in fact, rise to a mixture of two NADP isomers, *viz.*, 2′-NADP and 3′-NADP. The latter compound, which does not function as coenzyme in enzymic reactions requiring NADP, already has been described as the product of the acid isomerization of 2′-NADP (Shuster and Kaplan, 1955). The present paper examines the enzymic phosphate transfer to NAD in some detail.

Materials and Methods

Compounds. The nucleosides and nucleotides employed, as well as NAD^+, NADH, $NADP^+$, and NADPH, were commercial preparations, with the exception of the 3′-phosphate and the 3′,5′-diphosphate of thymidine and of the 2′,5′- and 3′,5′-diphosphates of adenosine which were synthesized as reported in the preceding paper (Brunngraber and Chargaff, 1973). Reagents used were of reagent or analytical grade.

Enzymes. Nucleoside phosphotransferase from carrot (Brunngraber and Chargaff, 1967) and nucleotide phosphotransferase from *E. coli* W (Brunngraber and Chargaff, 1973) were prepared as described previously. The purity of the latter enzyme corresponded to step 1-4 of the preceding paper. NAD-kinase (EC 2.7.1.23), pig heart isocitrate dehydrogenase (EC 1.1.1.42), and rye grass 3′-nucleotidase (EC 3.1.3.6) came from Sigma. A preparation of phosphodiesterase from *Crotalus adamanteus* venom (Sigma) served as source of nucleotide pyrophosphatase.

Analytical. For the chromatographic separation of nucleotides on filter paper two solvent systems were employed: (1) 1-propanol-concentrated ammonia–water (11:7:2, v/v); (2) ethanol-1 M ammonium acetate (7:3, v/v). Liquid chromatography was performed in the Varian aerograph LCS 1000 when the NADP isomers or the mixture of adenosine diphosphates to which they gave rise were to be characterized. The conditions for the NADP isomers were: flow rate, column 20 ml/hr, gradient 35 ml/hr; starting eluent, 0.1 M KH_2PO_4, pH 6.3; gradient eluent, 1 M KH_2PO_4, pH 4.3; gradient delay, 5 min; initial volume, 35 ml. The same conditions served for the adenosine diphosphates, except that the starting eluent was 0.3 M KH_2PO_4 (pH 4.3). The relative proportions of the separated components were estimated by planimetry.

Enzymic Phosphorylation Experiments. The normal NADP isomer, 2′-NADP, was prepared from NAD and ATP with NAD-kinase (Wang *et al.*, 1954). The product was partially purified on paper with solvent 2. In this system NADP and ADP are not completely separated, but this does not interfere with the subsequent analysis of the newly formed NADP by liquid chromatography since, with the conditions specified before, ADP is eluted after NADP.

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[1] Abbreviations used are: 2′-NADP, nicotinamide adenine dinucleotide 2′-phosphate; 3′-NADP, nicotinamide adenine dinucleotide 3′-phosphate. The carbon atoms of the ribose component of the AMP moiety of NAD are designated 2′, 3′, etc. Other abbreviations as in *Biochemistry 5*, 1445 (1966).

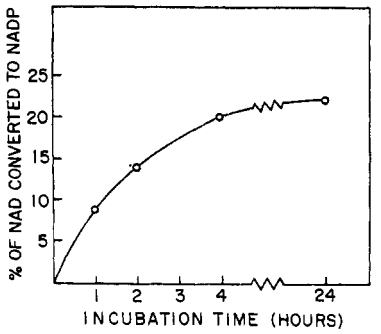

FIGURE 1: Enzymic phosphate transfer to NAD from thymidine 3′,5′-diphosphate. The incubation mixture (total volume, 1 ml) consisted of 0.1 M sodium acetate buffer (pH 5.2), which was 12.6 mM with respect to thymidine 3′,5′-diphosphate, 10 mM with respect to NAD, and contained 13 μg of purified *E. coli* phosphotransferase corresponding to step 2-5 in Table I of preceding paper (Brunngraber and Chargaff, 1973). Incubation at 37° for indicated periods when portions of 30 μl were removed and developed in ethanol–ammonium acetate (7:3, v/v) at pH 7.5. The molar absorption coefficient of NADP (at 260 nm) served for the estimation of the quantities of NADP produced enzymically.

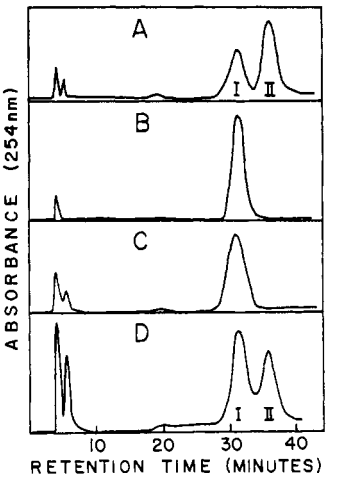

FIGURE 2: Liquid chromatography of (A) NADP from the phosphorylation of NAD by *E. coli* nucleotide phosphotransferase; (B) commercial NADP; (C) NADP from the phosphorylation of NAD by NAD kinase; (D) NADP isomerized with 0.5 N HCl.

A mixture of 2′- and 3′-NADP was prepared by treating 2′-NADP with 0.5 N HCl at room temp for 40 hr (Shuster and Kaplan, 1955). After neutralization with NaOH the NADP was separated on paper (solvent 2).

The conditions for the action of nucleotide phosphotransferase of *E. coli* on NAD were as follows; total volume, 1 ml; 0.1 M sodium acetate buffer of pH 5.2; 20 mM NAD; 4 μg of enzyme. Three different phosphate donors were used: 100 mM *p*-nitrophenyl phosphate, 25 mM adenosine 2′-phosphate, 25 mM thymidine 3′,5′-diphosphate. Incubation was at 37° for intervals ranging from 1 to 24 hr. The NADP formed was, prior to analysis, separated on paper with solvent 2.

Adenosine 2′,5′-diphosphate was synthesized (Tunis and Chargaff, 1960) from adenosine 2′-phosphate with the use of carrot nucleoside phosphotransferase of a purity corresponding to step IV (Brunngraber and Chargaff, 1967). The incubation of 20 mM adenosine 2′-phosphate and 100 mM *p*-nitrophenyl phosphate in 0.1 M sodium acetate (pH 5.2) was carried out at 37° for 2 hr. The newly formed adenosine diphosphate was separated on paper in solvent 1.

Cleavage of Pyrophosphate Link. The various NADP preparations were cleaved enzymically with the nucleotide pyrophosphatase of snake venom (Wang, 1957) in Tris-HCl buffer (0.1 M, pH 7.5) at 37° for 3 hr. Solvent 2 served for the paper chromatography of the products. The fractions of adenosine diphosphate obtained were identified by their 250:260 and 280:260 absorbance ratios, the ratios of adenosine to phosphate, and the R_F values recorded by paper and liquid chromatography.

Assays. 2′-NADP was assayed through the formation of NADPH catalyzed by pig heart isocitrate dehydrogenase with the use of the procedure described in the TPN information booklet of Boehringer/Mannheim. In addition, assays were performed by means of the cyanide addition reaction (Ciotti and Kaplan, 1957).

Results

Enzymic Phosphate Transfer to NAD. When NAD is incubated with the nucleotide phosphotransferase of *E. coli* W in the presence of a nucleotide (adenosine 2′-phosphate or thymidine 3′,5′-diphosphate) a new ultraviolet-absorbing spot is observed on the chromatograms irrigated with either solvent 2 or buffered ammonium isobutyrate. This component has the same R_F value as NADP in these solvents. It also has the same absorbance ratio 0.83 (250:260) and 0.23 (280:260). A typical experiment, in which more than 20% of NAD was converted to NADP by phosphate transfer from pTp, is shown in Figure 1.

The product made enzymically was analyzed by liquid chromatography and the elution pattern compared with the profiles obtained with commercial NADP, with NADP synthesized by NAD-kinase, and with NADP isomerized by treatment with acid. These patterns are shown in Figure 2. Commercial NADP (curve B) and kinase-produced NADP (curve C) revealed a single peak with identical retention times. The products made by the action of phosphotransferase on NAD (curve A) and by the treatment with acid of normal NADP (curve D) both showed two peaks of which the faster one, component I, which is coincident with normal NADP, must be assigned to 2′-NADP and the slower, component II, to 3′-NADP. This assignment is supported by the observation that the treatment of the several NADP preparations with the 3′-nucleotidase of rye grass eliminated component II with the appearance of a new faster peak belonging to NAD which was also independently identified by paper chromatography (Figure 3). 2′-NADP is not affected by this nucleotidase.

The relative proportions of the two isomers of NADP can be estimated from the areas under the chromatographic peaks. In several experiments, the NADP synthesized by the *E. coli* phosphotransferase was found to contain 60–70% 3′-NADP, that produced by acid isomerization of 2′-NADP contained 40–50% of 3′-NADP. In the transfer reaction, no influence by the nature of the phosphate donor or by the length of incubation on the proportion of 2′ and 3′ isomers was observed. It may be of interest to recall that the proportion of 3′-NADP produced by the enzyme is similar to that of 3′-phosphates formed by phosphate transfer to the nucleoside or nucleotide of adenine. Thus, adenosine gave rise to 59% of 3′-adenylic acid and 5′-adenylic acid formed 58% of adenosine 3′,5′-diphosphate (Brunngraber and Chargaff, 1970).

Adenosine Diphosphates Released from Enzymically Made NADP. When the enzymically made NADP is split at the pyrophosphate bridge, the expected proportions of the 2′,5′- and of the 3′,5′-diphosphates of adenosine are released.

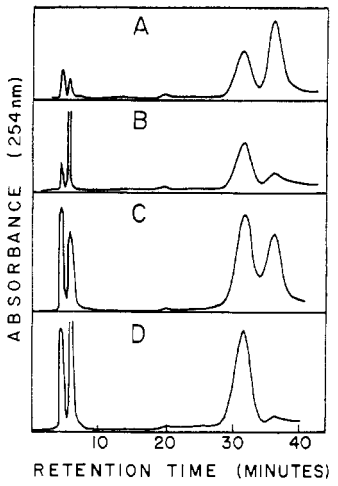

FIGURE 3: Liquid chromatography of (A) NADP synthesized by *E. coli* nucleotide phosphotransferase; (B) the same after treatment with 3′-nucleotidase; (C) NADP isomerized with 0.5 N HCl; (D) the same after treatment with 3′-nucleotidase.

Figure 4 shows the elution patterns of the adenosine diphosphates liberated from commercial NADP (A), NADP synthesized by NAD-kinase (B), NADP produced through phosphate transfer by the *E. coli* phosphotransferase (D), acid-isomerized NADP (E). For comparison the separation of the mixture of adenosine 2′,5′- and 3′,5′-diphosphates synthesized by the action of the phosphotransferase on 5′-adenylic acid is shown in curve C. The fast component (5 min) seen in curves A and B is due to some NADP that was not hydrolyzed by pyrophosphatase. The principal peak (37 min) liberated by the cleavage of 2′-NADP is adenosine 2′,5′-diphosphate which proved identical chromatographically with the authentic diphosphate synthesized by the action of nucleoside phosphotransferase of carrot on 2′-adenylic acid. The slower

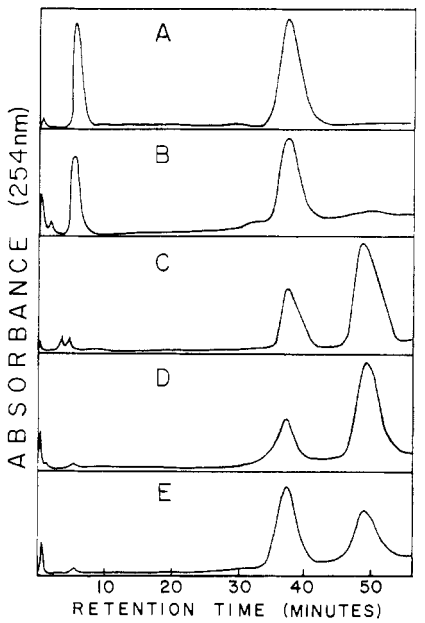

FIGURE 4: Liquid chromatography of adenosine diphosphates obtained from the hydrolysis by pyrophosphatase of (A) commercial NADP; (B) NADP synthesized by NAD-kinase; (D) NADP synthesized by *E. coli* nucleotide phosphotransferase; (E) NADP isomerized with 0.5 N HCl. Panel C shows the pattern of a mixture of authentic adenosine 2′,5′- and 3′,5′-diphosphate.

TABLE I: Composition of NADP Formed by Enzymic Phosphate Transfer to NAD and of NADP Produced by Acid Treatment of 2′-NADP.

Method of Determination	Composition of NADP (as % of Total)			
	Produced by Nucleotide Phosphotransferase		Produced by Acid Isomerization	
	2′-NADP	3′-NADP	2′-NADP	3′-NADP
Liquid chromatography of NADP	42	58	59	41
Liquid chromatography of adenosine 2′,5′- and 3′,5′-diphosphates released from NADP	41	59	58	42
Isocitrate dehydrogenase assay	43	57	55	45

component (48 min) appearing in curves C, D, and E is due to the isomeric adenosine 3′,5′-diphosphate. In several instances the components were analyzed and exhibited invariably a P:adenosine ratio around 2.0. The relative amounts of adenosine 2′,5′- and 3′,5′-diphosphates released from the NADP preparations consisting of a mixture of 2′- and 3′-NADP equaled the proportion of the isomers determined by direct chromatography or by enzyme assay (Table I).

Assay of NADP Produced by Phosphate Transfer. When NADP preparations produced through phosphate transfer by the nucleotide phosphotransferase were assayed by the cyanide addition reaction (Ciotti and Kaplan, 1957) the values obtained were 3–4% lower than those given by the NADP standard. This could be due to a slight contamination of the products with pAp formed by a trace of pyrophosphatase in our enzyme preparations; on paper chromatograms, NADP and pAp are not well separated.

The 2′-NADP assay by means of pig heart isocitrate dehydrogenase gave values that were entirely in line with the other determinations listed in Table I. For instance, a solution containing 1.4 mg of NADP/ml (made with the *E. coli* enzyme and estimated by its absorption at 260 nm and pH 7.0) showed a concentration of 0.61 mg/ml of 2′-NADP, corresponding to a content of 44% 2′-NADP in the total product. In this assay NAD was inactive.

In Table I, the composition of NADP produced from NAD by the *E. coli* phosphotransferase is compared with that of acid-isomerized NADP.

Donors in NAD Phosphorylation. Several organic phosphates were tested for their capacity to serve as donors for the synthesis of NADP by the nucleotide phosphotransferase. As is shown in Table II, *p*-nitrophenyl phosphate and several 2′- and 3′-nucleotides acted as phosphate donors, but the 3′-phosphate and the 3′,5′-diphosphate of thymidine proved more efficient. Phospho derivatives reported as inactive in the transferase reaction (Brunngraber and Chargaff, 1973), such as glucose 1- or 6-phosphate or ATP, could not promote the phosphorylation of NAD.

NADP as Phosphate Donor. Both NADP isomers can serve as phosphate donors in the enzymic reaction studied here by transferring the phosphate residue either to NAD or to

TABLE II: Phosphate Donors in the Synthesis of NADP by *E. coli* Nucleotide Phosphotransferase.

Expt[a]	Phosphate Donor	NADP Formed as % of Substrate NAD
1	*p*-Nitrophenyl phosphate	12
	2′-Adenylic acid	8
	3′-Adenylic acid	4
	2′- + 3′-Uridylic acid	3
	2′- + 3′-Cytidylic acid	4
2	*p*-Nitrophenyl phosphate	2
	2′-Adenylic acid	2
	3′-Thymidylic acid	9
	Thymidine 3′,5′-diphosphate	21

[a] In both experiments, the total volume of the assay mixtures was 0.4 ml, the buffer 0.1 M sodium acetate (pH 5.2), the NAD concentration 10 mM, the incubation temperature 37°. Experiment 1: 100 mM phosphate donor, 65 μg of enzyme corresponding to purification step 1-3 (Brunngraber and Chargaff, 1973), incubation time 4 hr. Experiment 2: 25 mM phosphate donor, 22 μg of enzyme corresponding to purification step 1-4, incubation time 1 hr. The assay samples were chromatographed in solvent 2.

TABLE III: NADP Isomers as Substrate of Phosphotransferase in the Presence and Absence of Acceptor.[a]

Phosphate Donor	Acceptor	Reaction Products (μmol/ml) 2′-NADP	3′-NADP	NAD	3′-TMP
2′-NADP	H_2O	10.8	0.6	9.5	
	Thymidine	16.2	0	4.2	1.1
2′- + 3′-NADP	H_2O	6.2	1.8	10.0	
	Thymidine	9.2	5.0	5.0	1.6

[a] The assay mixtures contained, in a total of 1 ml of 0.1 M sodium acetate (pH 5.2), either 21 μmol of 2′-NADP or a mixture of 10.4 μmol of 2′- and of 8.2 μmol of 3′-NADP; 33 μg of enzyme corresponding to purification step 1-4 (Brunngraber and Chargaff, 1973); and, when indicated, 20 μmol of thymidine. Incubation for 18 hr at 37°; analysis by paper and liquid chromatography in the Varian aerograph.

another suitable acceptor. Table III summarizes the results obtained when either 2′-NADP or a mixture of both NADP isomers is treated with the enzyme in the presence and the absence of the acceptor thymidine. Without acceptor, 45% of 2′-NADP and 54% of 2′- + 3′-NADP were hydrolyzed. It seems that the 3′ isomer is attacked preferentially, since only 22% of the 3′-NADP, but 60% of the 2′-NADP originally present survived prolonged incubation with the enzyme. The inhibition of hydrolase action in the presence of an acceptor, which was pointed out in the preceding paper (Brunngraber and Chargaff, 1973) is quite evident. Only 15% of 2′-NADP and 18% of 2′- + 3′-NADP were hydrolyzed under these conditions, with the 3′ isomer again being more susceptible: 88% of the 2′ isomer, but only 61% of the 3′ isomer were recovered at the end of the experiment. The quantities of 3′-thymidylic acid recorded in this experiment indicate the extent of phosphate transfer to thymidine. With 2′-NADP, 5.5% of the acceptor was phosphorylated, with 2′- + 3′-NADP the yield was 8%. The higher figure probably again points to a greater donor efficiency of the 3′ isomer.

Another feature of Table III deserves mention, namely, the observation that when 2′-NADP is treated with the enzyme in the absence of an acceptor, about 5% of the surviving NADP occurs as the 3′ isomer. This is certainly due to phosphate transfer from 2′-NADP to NAD formed by initial hydrolysis. Experiments with mixtures of 2′-NADP (25 mM) and NAD (20 mM) prove this point. Upon incubation with phosphotransferase for 2 hr (other conditions as in Table III), no net hydrolysis of NADP is observed, but 10% of the latter is found to exist as the 3′ isomer. On the basis of the proportions shown in Table I, this would mean that 12.5% of the acceptor NAD was converted to 3′-NADP and 9% to the 2′ isomer.

When the donor capacity of 2′-NADP was compared with that of other donors with respect to phosphate transfer to thymidine, it was found that 2′-NADP could produce 3′-thymidylic acid, but with less efficiency. Under identical conditions (6 mM donor, 20 mM thymidine), 2′-adenylic acid gave rise to 1.5 μmol/ml of 3′-thymidylic acid, *p*-nitrophenyl phosphate to 1.2 μmol/ml, and 2′-NADP to 0.6 μmol/ml.

Attempts were made to discover whether the enzyme could also in other respects act as pyridine nucleotide transphosphorylase, *viz.*, by transferring the phosphate residue from NADP to NADH. The procedure of Pastore and Friedkin (1961) for the separation of NAD^+, $NADP^+$, NADH, and NADPH on DEAE-cellulose was used to detect the formation of NADPH. Because of the lability of the reduced pyridine nucleotides (Lowry *et al.*, 1961) the phosphotransferase reaction was performed at pH 7.5; a pH above the optimum for this enzyme. No phosphate transfer was observed under these conditions.

Other Characteristics of Transfer Reaction. The K_m value of NAD for the nucleotide phosphotransferase is 2.2 ± 0.3 mM. The pH curve (Figure 5) shows a maximum activity between pH 5 and 7. With a nucleotide as phosphate donor, no metal cofactor appears to be required. When *p*-nitrophenyl phosphate, however, served as the donor the yield of NADP was tripled in the presence of 1 mM Cu^{2+}.

Discussion

So far as the phosphorylation of NAD is concerned, one might be inclined to concede a certain curiosity interest to the role of the nucleotide phosphotransferase; but when its potential biological function in the production of NADP is considered, one would have to classify it as a nuisance. For here we have an enzyme that, though phosphorylating NAD readily through phosphate transfer from a nucleotide (Table II), makes a high proportion of what generally would be considered as the wrong isomer (Table I). The mere fact of the existence of such an enzyme must, however, surely outweigh these arguments, unless we wish to engage in the futile task of speculating whether an enzyme isolated from a cell exists in that cell. It is, moreover, not impossible that the 3′ isomer of NADP will eventually be assigned a biological function.

In any event, it is a fact that the nucleotide phosphotransferase acts *in vitro* on NAD in such a way as to produce nearly 60% of the 3′ and only 40% of the biologically active

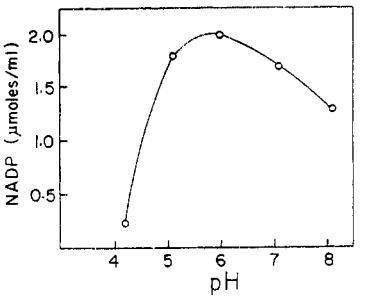

FIGURE 5: Effect of pH on the synthesis of NADP by *E. coli* nucleotide phosphotransferase. The reaction mixture consisted of *p*-nitrophenyl phosphate (100 mM), NAD (20 mM), and the step 3 *E. coli* enzyme in either 0.1 M sodium acetate buffer (pH 4.2, 5.1, and 6.0) or 0.1 M Tris-HCl buffer (pH 7.1 and 8.1).

2′ isomer of NADP (Table I). In an analytical survey Kaplan (1960) quotes F. E. Stolzenbach as having found the following distribution of pyridine nucleotides in *E. coli:* NAD, 57%; NADP, 10.3%; unknown, 32.7%. The latter may have included the α isomer of NAD. The occurrence of 3′-NADP in *E. coli* does not seem to have been recorded. It should be of interest to perform the chemical characterization of NADP samples isolated from different *E. coli* strains, in order to ascertain whether they contain some 3′-NADP. Such experiments are planned.

In the experiments presented here, thymidine 3′,5′-diphosphate was the most efficient donor for the enzymic phosphorylation of NAD, followed by 3′-thymidylic acid (Table II). As has already been observed with other phosphate acceptors (Brunngraber and Chargaff, 1973), the phosphate donor capacity of a 3′-phosphate residue is enhanced by the presence of a second phosphate at the 5′-hydroxyl of the donor nucleotide.

It should also be noted that the two NADP isomers themselves can function as phosphate donors. This is in line with the observation that 2′- and 3′-adenylic acids transfer their phosphate residues readily (Brunngraber and Chargaff, 1973). Thus, 2′-NADP can phosphorylate NAD, with the production of a mixture of the 2′- and 3′-NADP isomers, as is the case with other phosphate donors. It apparently fails, however, to transfer its phosphate residue to NADH.

As we have pointed out (Brunngraber and Chargaff, 1973), both the hydrolysis of a donor and the phosphate transfer to an acceptor by the phosphotransferase are depressed in the presence of ATP. This is also observed with NAD as acceptor and NADP, pTp, or *p*-nitrophenyl phosphate as donors. The inhibition is approximately 50%, but the proportions of the 2′ and 3′ isomers of NADP produced remain unaffected.

One could ask whether the phosphorylation of NAD *via* a nucleotide as phosphate donor and nucleotide phosphotransferase as catalyst could play a physiological role when compared with ATP as donor and NAD kinase as catalyst. It is not yet possible to answer this question, but a decision could hardly be based on a comparison of the respective Michaelis constants. With the NAD-kinase of *E. coli* K-12 the K_m value of NAD has been reported as 1.0 mM (Imsande and Pardee, 1962); in the present study, the K_m of NAD with the nucleotide phosphotransferase of *E. coli* W was found as 2.2 mM. The two enzymes could, hence, compete for the available NAD substrate, thus providing alternative pathways for its phosphorylation. These studies are being continued.

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