A Nucleotide Phosphotransferase from *Escherichia coli*. Purification and Properties†

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ABSTRACT: The purification (500- to 700-fold) of a phosphotransferase, isolated from *Escherichia coli* W, is described. This enzyme transfers organically bound phosphoric acid esters to the 2′- or 3′-hydroxyls of nucleosides and their 5′-mono-, -di-, and -triphosphate derivatives. Since in most cases nucleotides are better acceptors than nucleosides, the designation nucleotide phosphotransferase is suggested. The enzyme is also able to phosphorylate the terminal 3′-hydroxyl of oligothymidylic acid (chain length 2–6). Among the most efficient phosphate donors may be mentioned thymidine 3′,5′-diphosphate, 2′- or 3′- guanylic acid, 3′-deoxyguanylic acid, 2′-adenylic acid, 3′-deoxyadenylic acid, as well as *p*-nitrophenyl phosphate. The Michaelis constants of several donors are reported. In the absence of a suitable acceptor, the phosphate donors are hydrolyzed. The enzyme has a particle weight of 29,000 and an isoelectric point of 7.1. It may be a useful tool for the synthesis of 2′- and 3′-nucleotides, in view of the ease of isolation of enzyme and of products and the high yields of the phosphorylation products (50–95%).

It has been known since the discovery of the nucleoside phosphotransferases (Brawerman and Chargaff, 1953) that these enzymes, which are widely distributed in living matter (Brawerman and Chargaff, 1955), represent one of the pathways through which cells may effect the phosphorylation of nucleosides. This group of enzymes brings about the synthesis of deoxyribo- and ribonucleotides by the transfer, from suitable donors, of organically esterified phosphoric acid to a nucleoside. The best known example is the nucleoside phosphotransferase of carrot whose purification, kinetics, and chemical and physical properties have been described in detail recently (Brunngraber and Chargaff, 1967, 1970a; Rodgers and Chargaff, 1972). This enzyme produces essentially 5′-nucleotides.

A different type of transferase was discovered in *Escherichia coli* (Brunngraber and Chargaff, 1970b). A preliminary survey with a partially purified enzyme preparation showed it to be capable of a low-energy transfer of organically bound phosphate to the 2′- and 3′-hydroxyls of nucleosides, 5′-nucleotides, and even certain nucleoside 5′-triphosphates. Only in the case of adenosine as the acceptor were appreciable quantities of 5′-adenylic acid formed. Thymidine and its derivatives were the best acceptors of phosphate groups. It had to be left undecided whether all these activities were to be ascribed to a single enzyme.

In this paper we describe a purification procedure for the *E. coli* enzyme which, for reasons to be discussed, we prefer to designate as *nucleotide phosphotransferase*, and discuss some of its properties. An initial exploration of one of the possible regulatory properties of this enzyme is reported in the accompanying paper (Brunngraber and Chargaff, 1973) in which the enzymic phosphorylation of NAD is described.

Experimental Section

Materials. The enzyme was isolated from *E. coli* W. The frozen cells, grown on a high peptone medium and harvested in the early, mid or late logarithmic phases, were supplied by General Biochemicals, Chagrin Falls, Ohio. Deoxyribonuclease 1 (EC 3.1.4.5) was obtained commercially as were the various nucleosides, nucleoside 5′-phosphates and *p*-nitrophenyl phosphate. Deoxynucleoside 3′-phosphates and thymidine 3′,5′-diphosphate were synthesized by incubating the corresponding deoxynucleoside or 5′-thymidylic acid (0.1 M) and *p*-nitrophenyl phosphate (0.4 M) in sodium acetate buffer (0.1 M, pH 5.2) with the enzyme described here (step 1-3) at 37° for the period (determined by previous assay) producing the maximum yield of product. The newly formed nucleotide was separated chromatographically on paper using a solvent system of 1-propanol–concentrated ammonia–H_2O (11:7:2, v/v). The thymidine oligonucleotides were a gift from Dr. John G. Moffatt. DEAE-cellulose Type 40 (Schleicher and Schuell) had a capacity of 0.93 mequiv/g. Sephadex G-75 and G-100 were supplied by Pharmacia. Proteins for markers in the determination of molecular weight were furnished by Schwarz/Mann.

Isolation of Enzyme. The phosphotransferase was prepared from 125 g of *E. coli* W cells. All operations were performed in the cold. The cells were ground in a mortar with 250 g of levigated alumina for 10–15 min. Approximately 75 ml of the extraction medium (0.05 M Tris-chloride–0.01 M $MgCl_2$–0.1 mM EDTA, pH 7.5) was added and the cells were ground for an additional 10–15 min. A second portion of 75 ml of the same medium and 0.5 ml of a 0.1% solution of deoxyribonuclease were then added to the mixture. After 30 min, the mixture was centrifuged (20,000*g*, 20 min) and the supernatant fluid was dialyzed first against two changes of 1 l. each of distilled H_2O and then against 1 l. of 0.01 M sodium acetate (pH 5.8). The dialyzed extract, designated as step 1-1, was applied to a DEAE-cellulose, Type 40, column (3.5 × 22 cm; 1 g of DEAE-cellulose/200 mg of protein) which had been previously equilibrated with 0.01 M sodium acetate (pH 5.8). The column was washed with about 750 ml of the same buffer and the phosphotransferase was then eluted with

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TABLE I: Purification of Phosphotransferase of *E. coli*.

Expt	Step	Procedure	Vol (ml)	Protein (mg/ml)	Phosphotransferase Sp Act.[a]	Phosphotransferase Recov (%)
1	1-1	Extract after treatment with deoxyribonuclease	135	13.8	0.80	100
	1-2	DEAE-cellulose column	155	0.92	7.2	69
	1-3	Ammonium sulfate precipitate	10.0	2.60	35.0	61
	1-4	Sephadex G-75 column	21.0	0.052	526	39
2	2-1	As in expt 1	139	14.6	0.88	100
	2-2	As in expt 1	145	1.13	7.9	72
	2-3	As in expt 1	14.8	2.15	35.9	64
	2-4	Isoelectric focusing (pH 3–10)	13.6	0.30	240	55
	2-5	Isoelectric focusing (pH 6–8)	20.1	0.10	450	51

[a] The specific activity is expressed as micromoles of thymidine 3′,5′-diphosphate produced in 1 hr by 1 mg of protein in 1 ml of assay mixture.

0.06 M sodium acetate (pH 5.8). Fractions collected (flow rate 10 ml/25 min) were assayed and those with transferase activity were combined and designated as step 1-2. This solution was brought to 65% saturation with respect to ammonium sulfate by the addition of solid salt. The precipitate thus produced within 1 hr (collected at 20,000*g*, 20 min) was dissolved in distilled water and dialyzed against two changes of 1 l. each of distilled water. The enzyme, step 1-3, was stored at −20° and used in subsequent studies. Further purification utilized, alternatively, chromatography on Sephadex G-75 or preparative isoelectric focusing.

Chromatography on Sephadex G-75 employed 10 ml of step 1-3 enzyme dialyzed against either 0.05 M sodium acetate (pH 5.8) or 0.05 M triethanolamine hydrochloride–NaOH (pH 7.0). The enzyme was applied to a Sephadex G-75 column (2.8 × 52 cm) previously equilibrated with the corresponding buffer. A flow rate of 27 ml/hr was maintained and 7-ml fractions were collected. These were assayed for enzymic activity and protein. Fractions with transferase activity were pooled, dialyzed against 1 l. of 0.01 M sodium acetate (pH 5.8), and frozen. This preparation is designated as step 1-4.

Preparative isoelectric focusing (Svensson, 1962; Vesterberg and Svensson, 1966) utilized a 110-ml column and pH 3–10 and 6–8 carrier ampholytes (LKB Instruments, Stockholm) in the manner suggested by the manufacturer. The density gradient consisted of 0–50% (wt/v) sucrose with a final ampholyte concentration of approximately 1% (wt/v). First, using the pH 3–10 ampholytes, the step 2-3 enzyme (15–70 mg) was fractionated at 4° for 72 hr with a final voltage of 300 V after which 100–125 fractions were collected and analyzed. Those fractions with transferase activity were pooled and dialyzed against two changes of 500 ml each of distilled H_2O. This enzyme, step 2-4 (3–5 mg), was then fractionated with the use of the pH 6–8 ampholytes at 4° for 48 hr with a final voltage of 600 V. Fractions were collected, analyzed, and pooled (step 2-5).

Assay of Nucleotide Phosphotransferase. Phosphotransferase was assayed routinely with the use of a stock solution of 0.2 M *p*-nitrophenyl phosphate and 0.04 M 5′-thymidylic acid in 0.2 M sodium acetate (pH 5.2). Suitably diluted enzyme portions (0.1 ml) were incubated with 0.1 ml of stock solution for 30 min at 37°. The reaction was stopped by the addition of 0.05 ml of 1 M KOH. The analytical procedures for the determination of the newly formed nucleotides and inorganic phosphate as well as of total protein were those detailed previously (Brunngraber and Chargaff, 1970b). The specific activity of the nucleotide phosphotransferase is defined as the micromoles of thymidine 3′,5′-diphosphate produced in 1 hr by 1 mg of protein in 1 ml of assay mixture.

For studies of substrate specificity, solutions of potential donors and acceptors at specified concentrations were prepared in 0.2 M sodium acetate and the pH was adjusted to 5.2. The incubation mixtures usually consisted of 0.1-ml portions each of acceptor and donor and of 0.2 ml of enzyme solution. The assays were performed as above.

Other Determinations. The particle weight of the phosphotransferase was estimated by means of a Sephadex G-100 column (Andrews, 1964). Gel electrophoresis was performed with 7.5% gels at pH 4.3 and at pH 8.3. Enzyme preparations corresponding to step 1-4 in Table I were used.

Results

Enzyme Purification. Modifications of the procedure reported previously (Brunngraber and Chargaff, 1970b) gave enzyme preparations of higher specific activity than were available before. Two typical experiments are summarized in Table I. In expt 1, which terminated with an elution from Sephadex G-75, a 660-fold purification with a recovery of 39% was achieved. In expt 2, in which electrofocusing was applied, the purification was better than 500-fold and the final yield 51%.

Several phosphatases having no transferase activity are removed in the course of the purification procedure. In step 2, for instance, though a single peak having both transfer and hydrolytic activities is eluted (compare Figure 1 in Brunngraber and Chargaff, 1970b), an acid phosphatase stays adsorbed on the DEAE-cellulose column. This enzyme can be eluted by lowering the pH of the buffer. Similarly, a phosphatase remains in the supernatant of the 65% ammonium sulfate solution (step 3). Isoelectric focusing in a pH gradient of 6–8 (step 2-5 in expt 2) separates three hydrolases, only one of which has transferase activity (Figure 1). Two hydrolases were seen in gel filtration on Sephadex G-75 (step 1-4 in expt 1); one of these peaks comprised the transferase activity (Figure 2). As will be noticed in the same figure, the

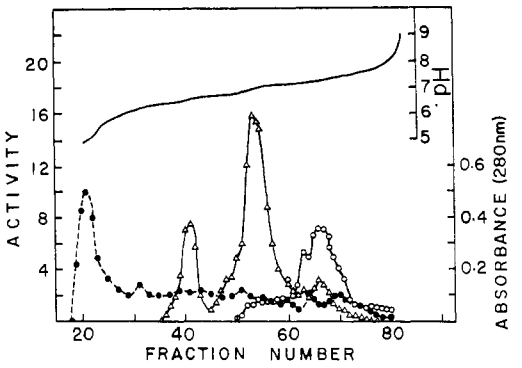

FIGURE 1: Purification of phosphotransferase (step 2-4 in Table I) by electrofocusing. The activity is expressed as μmol/ml of thymidine 3′,5′-diphosphate (transferase) or μmol/ml of inorganic phosphate (phosphatase) formed by 0.5 ml of enzyme in 30 min at 37°. Conditions as in Experimental Section: full circles, absorbance at 280 nm; open circles, phosphotransferase; triangles, phosphatase.

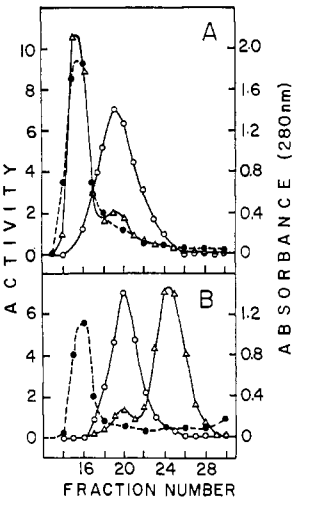

FIGURE 2: Chromatography of phosphotransferase (step 3 in Table I) on Sephadex G-75: (A) at pH 5.8; (B) at pH 7.0. Symbols and expression of activity as in Figure 1.

elution volume of the contaminating hydrolase varies with pH, whereas that of the transferase does not. The latter does, however, exhibit association at pH 5.

The gel filtration procedure (expt 1 in Table I) appears preferable. It is faster and yields a more stable enzyme preparation. When, in a purification run exemplified by expt 2, step 2-4 was omitted by going directly from step 2-3 to step 2-5, protein precipitation in the electrofocusing column disrupted the pH gradient. More importantly, the phosphotransferase isolated by electrofocusing lost 70% of its activity when subjected to dialysis.

In summary, if the enzyme is to be used for synthetic applications, step 3 of the purification procedure (Table I) is recommended; such preparations are fairly stable and can be stored for a few weeks at −20° without excessive loss of activity. If further purification is desired, the Sephadex G-75 column at pH 5.8 is suggested. At pH 5.0 the transferase is associated with the principal protein peak; at pH 7.0, the isoelectric point, the transferase is more labile. The increased lability at neutrality also became evident when attempts were made at further purification on CM-Sephadex C-50.

Physical Properties. The particle weight of the purified preparations of nucleotide phosphotransferase, at pH 5.8 and 7.0, was estimated at 29,000 ± 1600 daltons. The isoelectric point was found at 7.1 ± 0.1. The electropherograms obtained at pH 4.3 showed one major band (about 70%) and one not inconsiderable minor band (30%), those at pH 8.9 revealed one major component (about 80%) and several faint bands.

Stability of Nucleotide Phosphotransferase. This is a comparatively labile enzyme, becoming more so as purification progresses. When preparations corresponding to steps 1, 2, and 3 were stored at −20° for 8 weeks, losses of 7, 28, and 48% of activity were recorded, respectively. At neutrality, the purified enzyme is quite unstable. When gel filtration was performed at pH 7.0, only 53% of the activity yielded by this procedure at pH 5.8 was recovered.

Enzyme Levels during Growth. A comparison of the levels of nucleotide phosphotransferase in crude extracts of commercial specimens of *E. coli* W cells harvested at early-, mid-, and late-log phase showed no significant differences in the specific activity of the enzyme. The total activity in the early exponential phase, however, was twice that observed in the late phase, in measure with the increased protein content of these cells.

Use of the Enzyme as a Tool for the Synthesis of 2′- and 3′-Nucleotides. Since the hydrolases removed in step 1-4 or in steps 2-4 and 2-5 exhibit no nucleotidase activity, enzyme preparations at step 3 are sufficiently pure to be used routinely for the synthesis of nucleotides phosphorylated at the 2′- and 3′-hydroxyls that are otherwise difficultly accessible. To give two examples: with 20 mM 5′-thymidylic acid as the acceptor and 100 mM *p*-nitrophenyl phosphate as the donor, thymidine 3′,5′-diphosphate was obtained in a yield of 80–90% within 2 hr; with 20 mM thymidine and 100 mM *p*-nitrophenyl phosphate the yield of 3′-thymidylic acid amounted to 75% within 24 hr.

Survey of Acceptors. Our previous paper (Brunngraber and Chargaff, 1970b) offered a qualitative survey of a large selection of acceptors. It seemed, however, of interest to compare the acceptor potential of the various ribo- and deoxyribonucleosides and nucleotides under strictly comparable conditions. This is done here in Table II in which the rates of phosphorylation are correlated. It will be seen that all classes of compounds tested can be phosphorylated, although some of the ribonucleotides, namely, adenosine 5′-di- and -triphosphates and guanosine and cytidine 5′-triphosphates are very sluggish acceptors. Deoxyribose derivatives are, in general, more prone to accept phosphate than are ribose derivatives; among them, the pyrimidine nucleoside monophosphates prove the most accessible to phosphorylation. After as short an incubation time as 30 min, 65% of deoxycytidylic acid and 58% of thymidylic acid are converted to the corresponding 3′,5′-diphosphates.

Phosphorylation of 5′-Guanylic Acid. In our preliminary description of the nucleotide phosphotransferase we reported the distribution of nucleoside diphosphate isomers formed by enzymic phosphate transfer to all 5′-ribonucleoside monophosphates with the exception of 5′-guanylic acid as acceptor for which no quantitative data were then available (Table III in Brunngraber and Chargaff, 1970b). We can now supply the missing information. Whereas with 5′-adenylic acids as acceptor 42% of 2′,5′-adenosine diphosphate and 58% of the 3′,5′-diphosphate are formed, 5′-guanylic acid gives rise almost exclusively to the 3′,5′-diphosphate isomer.

As the first step 2′,5′-guanosine diphosphate was pre-

TABLE II: Comparative Efficiencies of Enzymic Phosphorylation of Ribo- and Deoxyribonucleosides and Their 5′-Mono-, -Di-, and -Triphosphates.[a]

Acceptor	Phosphorylated Products (μmol/ml)[b]							
	Adenine		Guanine		Cytosine		Uracil	Thymine
	Ribonucleoside	Deoxyribonucleoside	Ribonucleoside	Deoxyribonucleoside	Ribonucleoside	Deoxyribonucleoside	Ribonucleoside	Deoxyribonucleoside
Nucleoside	1.4	1.6	1.7[c]	0.6	1.9	3.5	1.4	2.8
Nucleoside 5′-monophosphate	0.9	1.4	1.2	2.8	1.9	6.5	1.4	5.8
Nucleoside 5′-diphosphate	nd[d]	1.2	0.3	3.5	0.4	5.8	0.7	5.5
Nucleoside 5′-triphosphate	nd[d]	0.5	0.1[e]	1.3	0.1[e]	3.7	0.2[e]	2.9

[a] The composition of the assay mixtures (total volume, 0.4 ml) was: 0.1 M sodium acetate (pH 5.2), 10 mM acceptor, 100 mM *p*-nitrophenyl phosphate, and 4 μg of purified phosphotransferase, corresponding to step 1-4 in Table I. Incubation was at 37° for 30 min. Portions of 30 μl of the mixture were analyzed by paper chromatography in 1-propanol–ammonia–water (11:7:2, v/v). [b] In the case of the ribonucleoside acceptors, the values reported for the phosphorylation products comprise both 2′- and 3′-phosphates. Data on the distribution of individual products will be found, for many of the acceptors used, in our previous paper (Brunngraber and Chargaff, 1970b). [c] This acceptor was not entirely dissolved at the start of incubation. [d] nd = not demonstrable. After incubation at 37° for 18 hr, adenosine 5′-diphosphate yielded a phosphorylation product amounting to about 0.3 μmol/ml and adenosine 5′-triphosphate one of about 0.2 μmol/ml. For the separation of these compounds the chromatographic system was changed slightly: 1-propanol–ammonia–water (11:2:7, v/v). [e] After incubation at 37° for 18 hr, the yields of phosphorylation products from 5′-triphosphates as acceptors were higher: GTP, 0.6 μmol/ml; CTP, 0.4; UTP, 1.0.

TABLE III: Enzymic Phosphate Transfer to Oligothymidylic Acid Preparations.[a]

Acceptor			Product			
Composition	Total (nmol)	Rel Mobility[b] (R_F)	Composition	Total (nmol)	Rel Mobility[b] (R_F)	Yield (% of Acceptor)
T	480	9.57	Tp	480	3.58	100
pT	440	3.58	pTp	440	1.00	100
TpT	440	8.50	$(Tp)_2$	440	2.52	100
$(pT)_2$	560	2.55	$(pT)_2p$	400	0.63	71
$(pT)_3$	520	1.31	$(pT)_3p$	360	0.38	69
$(pT)_4$	440	0.71	$(pT)_4p$	280	0.19	64
$(pT)_5$	360	0.37	$(pT)_5p$	280	0.10	78
$(pT)_6$	440	0.22	$(pT)_6p$	320	0.07	73

[a] Conditions as in Table II, except for the acceptor concentrations specified here. [b] Relative to pTp taken as 1.00, in the system consisting of 1-propanol–ammonia–water (11:7:2, v/v).

pared by the action of the nucleoside phosphotransferase of carrot (Brunngraber and Chargaff, 1967) on authentic 2′-guanylic acid with *p*-nitrophenyl phosphate as donor. Under these conditions, the 3′-guanylic acid isomer failed to accept phosphate in the 5′ position, in analogy with the behavior of the adenylic acid isomers (Tunis and Chargaff, 1960).

Next, guanosine 5′-phosphate was treated with *E. coli* nucleotide phosphotransferase and *p*-nitrophenyl phosphate, in the standard arrangement described above, for 4 hr at 37°. The products of phosphate transfer were characterized by paper chromatography in saturated ammonium sulfate–water–isopropyl alcohol (79:19:2, v/v). Only one product was seen which was not identical with authentic guanosine 2′,5′-diphosphate. The relative mobilities were: 5′-GMP, 1.00; 2′,5′-GDP (synthesized by carrot enzyme), 2.3. The only visible, newly formed spot, presumably 3′,5′-GDP, synthesized by the *E. coli* enzyme, had a mobility of 1.6.

Analysis of the reaction products by liquid chromatography in the Varian Aerograph LCS 1000 showed a major peak (94%) and a minor peak (6%). The latter coincided with 2′,5′-GDP. It is, therefore, most likely that the *E. coli* transferase synthesizes mainly the 3′,5′ isomer.

Phosphorylation of Oligonucleotides. The phosphotransferase appears capable of inserting a phosphate group in the terminal 3′-hydroxyl of an oligonucleotide. So far, one series of deoxy oligonucleotides have been investigated, namely, the oligothymidylic acids $(pT)_2$ to $(pT)_6$. This experiment is summarized in Table III which shows that the oligonucleotides are converted to their 3′-phospho derivatives with a yield of 65–80% under conditions in which thymidine, 5′-thymidylic acid, and thymidylyl-(3′→5′)-thymidine are phosphorylated completely. Within the limits tested, there is no evidence of the influence of chain length on the accessibility of the terminal 3′-hydroxyl. The behavior of oligoribonucleotides and of RNA preparations is under investigation.

Nucleotides as Phosphate Donors. Although *p*-nitrophenyl phosphate was employed routinely as phosphate donor in the

course of the purification of the enzyme because of ease of analysis, it is not the only, and not even the most efficient, donor in enzymic phosphate transfer. Among the many organic phosphoric acid esters and anhydrides that were tested, the following were inactive: deoxyribose 5-phosphate, glucose 1- and 6-phosphates, glycerophosphate, phenyl phosphate, *O*-phosphoserine, *O*-phosphothreonine, ADP, ATP, and cytidine cyclic 2′,3′-monophosphate. Nucleotides were, however, efficient phosphate donors; they were tested with 5′-thymidylic acid as acceptor. A selection of these experiments is presented in Table IV with regard to both ribo- and deoxyribonucleotides as donors. The most effective donors are the purine 2′ and 3′-nucleotides, with the exception of 3′-AMP. The order of activity apparently is: 3′-dGMP > 2′-AMP > 2′-GMP > 3′-GMP > 3′-dAMP, of which the first three surpass *p*-nitrophenyl phosphate. The pyrimidine nucleotides and the purine 5′-nucleotides show weak donor capacity, although it should be noted that the incubation of a single 5′-nucleotide with the enzyme gives rise to the formation of some nucleoside 3′,5′-diphosphate, presumably by intermolecular phosphate transfer which is, of course, accompanied by the appearance of an equimolar quantity of the respective nucleoside.

That 2′-adenylic acid is indeed a more efficient donor than *p*-nitrophenyl phosphate regardless of whether nucleoside mono-, di-, or triphosphates serve as acceptors could be shown in a series of comparative experiments with thymidine and its 5′-mono-, di-, and triphosphates as acceptors. With 2′-AMP as donor the yields of pT, pTp, ppTp, and pppTp were, respectively, 2.2, 2.0, 1.4, and 2.3 times greater than those recorded with *p*-nitrophenyl phosphate (compare Table II).

The purine nucleotides mentioned before could very well play a physiological role as phosphate sources in the enzymic transfer reaction. Since, on the other hand, pyrimidine 5′-nucleotides are among the best phosphate acceptors (see Table II), it appeared of interest to see whether the donor capacity of a poor donor, such as 3′-thymidylic acid (see Table IV), could be enhanced by the introduction of a second phosphate group in position 5′. This appears to be the case; as is shown in Table V, thymidine 3′,5′-diphosphate is able to act as an effective phosphate donor. The relative order of accessibility to phosphorylation is the same as that shown in Table II for *p*-nitrophenyl phosphate as donor, but it is noteworthy that under identical conditions the latter compound is less effective as phosphate source: only 16% of deoxycytidine 5′-phosphate was converted to the 3′,5′-diphosphate with *p*-nitrophenyl phosphate, but 33% with thymidine 3′,5′-diphosphate.

Michaelis Constants of Phosphate Donors. It was of interest to determine the K_m values of several donors with the *E. coli* enzyme. The acceptors used were all tested in a 20 mM concentration. With either thymidine or thymidine 5′-phosphate as acceptor, 2′-adenylic acid had a K_m of 1.2 mM, whereas the K_m of *p*-nitrophenyl phosphate was found as 23 mM. Thymidine 3′-phosphate and thymidine 3′,5′-diphosphate were compared as phosphate donors with 5′-deoxycytidylic acid as acceptor. The Michaelis constant of thymidine 3′-phosphate was found as 0.6 mM, that of thymidine 3′,5′-diphosphate as less than 0.3 mM. The greater donor capacity of the diphosphate is also evident from the velocities observed when the enzyme is saturated with the acceptor substrate. At a 24 mM donor concentration, the initial velocity of phosphate transfer from the diphosphate was twice that from the monophosphate.

Hydrolytic Activity of Nucleotide Phosphotransferase. There exists a fairly strict correlation between the donor capacity of the phosphoric acid derivatives studied here and their hydrolysis in the absence of an acceptor. The organic phosphates listed before as unable to function as phosphate donors are not hydrolyzed by the enzyme. On the other hand, the nucleotides that are effective donors, *viz.*, the purine 2′- and 3′-nucleotide, are hydrolyzed when no acceptor is offered, whereas the pyrimidine 2′-nucleotides are not. The 5′-nucleotides are attacked to a minimal extent.

In the presence of a suitable acceptor the phosphatase activity of the transferase is greatly, if indeed not completely, inhibited. The action of the enzyme on 3′-deoxyadenylic and 3′-deoxyguanylic acids in the presence of the acceptor, 5′-thymidylic acid, is illustrated in Table VI. The following will

TABLE IV: Nucleotides as Donors in Enzymic Phosphate Transfer to 5′-Thymidylic Acid.[a]

Nucleotide as Phosphate Donor		pTp Produced, as % of Product with pNphP as Donor	
Aglycone	Position of P on Sugar	Ribo Donor[b]	Deoxyribo Donor[c]
Adenine	2′	110	
	3′	45	85
	5′	45	30
Guanine	2′	105	
	3′	90	140
	5′	30	30
Cytosine	2′	30	
	3′		40
	5′	30	10
Uracil	2′	20	
	3′		
	5′	50	
Thymine	3′		60
	5′		30
Control, with donor omitted		30[d]	

[a] Conditions as in Table II, with the exception that the concentration of the donors was 25 mM and that of 5′-thymidylic acid as acceptor was 15 mM. [b] With 5′-guanylic acid as donor, small amounts of pGp (0.4 μmol/ml) were also found. With 5′-adenylic or uridylic acids, no pAp or pUp was seen in the presence of acceptor, but about 0.5 μmol/ml of either diphosphate in its absence. No pCp was seen in either case. [c] With 5′-deoxyguanylic acid as donor, also 0.5 μmol/ml of pdGp was formed in the presence of acceptor. In its absence, all 5′-deoxynucleotides gave rise to 3′,5′-diphosphates; pdAp, pdCp, and pTp were formed in small quantities, pdGp in considerable quantity (1.3 μmol/ml). On incubation for 12 hr, the yield of pTp with 3′-deoxyguanylic acid as donor went up to 220%, with 3′-deoxyadenylic acid to 140%, whereas there was no increase with *p*-nitrophenyl phosphate. [d] This value represents the quantity of pTp formed when 15 mM 5′-thymidylic acid, present as acceptor in all assays, is incubated in the absence of any of the listed donors.

TABLE V: Thymidine 3′,5′-Diphosphate as Phosphate Donor in the Enzymic Phosphorylation of Ribo- and Deoxyribonucleosides and Their 5′-Monophosphates.[a]

Acceptor	Phosphorylated Products (μmol/ml)[b]						
	Adenine		Guanine		Cytosine		Uracil
	Ribonucleoside	Deoxyribonucleoside	Ribonucleoside	Deoxyribonucleoside	Ribonucleoside	Deoxyribonucleoside	Ribonucleoside
Nucleoside	0.9[c]	0.5[c]	0.7[d]	0.2[c]	1.2[d]	1.3[c]	0.7[d]
Nucleoside 5′-monophosphate	0.6[c]	0.9[d]	0.5[d]	0.8[d]	1.0[d]	3.3[d]	0.8[d]

[a] The composition of the assay mixtures (total volume, 0.4 ml) was: 0.1 M sodium acetate (pH 5.2), 10 mM acceptor, 25 mM thymidine 3′,5′-diphosphate, and 4 μg of purified phosphotransferase (see step 1-4 in Table I). Incubation at 37° for 30 min. Portions of 30 μl were used for chromatographic analysis. [b] Under identical conditions, 25 mM *p*-nitrophenyl phosphate and 10 mM deoxycytidine 5′-phosphate yielded 1.6 μmol/ml of deoxycytidine 3′,5′-diphosphate (compare Table II). [c] Solvent system for chromatography: isobutyric acid–1 M NH_4OH–0.02 M EDTA (100:60:0.8, v/v). [d] Two-dimensional chromatography: I, 1 propanol–concentrated ammonia–water (11:7:2, v/v); II, isopropyl alcohol–concentrated HCl (650:167, v/v; brought to 1 l. with H_2O).

be observed: (1) in the presence of the acceptor, all the phosphate from the donor is transferred to the acceptor and none is released as H_3PO_4; (2) the rate at which the phosphate donor is attacked by the enzyme is greater in the presence of acceptor than in its absence; (3) as pointed out before, an intermolecular phosphate transfer between two 5′-TMP molecules occurs with no simultaneous release of H_3PO_4. Such an intermolecular migration of phosphate residues appears to take place also with the other 5′-nucleotides, possibly with the exception of cytidylic acid. (Compare the footnotes of Table IV.) The inhibition of the hydrolysis of a donor in the presence of an acceptor is much more pronounced in the case of the enzyme discussed here than with the nucleoside phosphotransferase of carrot (Brunngraber and Chargaff, 1970a).

Inhibiting Effect of ATP. The hydrolytic function of a phosphotransferase is depressed in the presence of an effective acceptor (Brunngraber and Chargaff, 1970a, and also Table VI of the present paper). ATP, however, behaves anomalously; although a poor acceptor (Table II), it inhibits both hydrolysis and transfer significantly. For instance, in the presence of ATP the hydrolysis of 2′-adenylic acid was inhibited completely after a 15-min incubation and depressed 70% after 30 min; the phosphate transfer to thymidine was diminished by 35%. The effect of ATP on the hydrolysis of *p*-nitrophenyl phosphate is shown in Table VII.

Effects of Metals, etc. All reagents were tested in 1 mM concentration with *p*-nitrophenyl phosphate as phosphate donor. Co^{2+}, Cu^{2+}, and Zn^{2+} enhanced the formation of 3′-thymidylic acid and of thymidine 3′,5′-diphosphate by about 20%. EDTA depressed the formation of the former by 40% and that of the latter by 70%. Both syntheses were inhibited 70% by Mn^{2+}. The phosphate transfer to 5′-thymidylic acid was also depressed by Mg^{2+} (20%), Pb^{2+} (40%), and NaF (30%); dithiothreitol had a similar effect (40% inhibition). All these agents had no effect on the phosphorylation of thymidine.

TABLE VI: Hydrolytic Action of Nucleotide Phosphotransferase on 3′-Phosphates of Deoxyadenosine and Deoxyguanosine in the Presence and Absence of Acceptor (5′-Thymidylic Acid).[a]

P Donor or Hydrolytic Substrate	5′-Thymidylic Acid as Acceptor	Products of Phosphate Transfer or Hydrolysis		
		Thymidine-3′,5′-P_2 (μmol/ml)	Nucleoside	μmol/ml
Deoxyadenosine 3′-phosphate	+	1.1	Deoxyadenosine	1.0[b]
	−	−	Deoxyadenosine	0.7
Deoxyguanosine 3′-phosphate	+	1.8	Deoxyguanosine	1.9[b]
	−	−	Deoxyguanosine	0.6
−	+	0.6	Thymidine	0.7

[a] The concentration of deoxyadenosine 3′-phosphate was 6.1 mM, that of deoxyguanosine 3′-phosphate 6.4 mM, that of thymidine 5′-phosphate, when used, 15 mM. Other conditions as in Table II. [b] No thymidine was observed.

Discussion

The enzyme whose isolation from *E. coli* W cells[1] is described here would, had its transfer function not been recognized, have been classified as a purine 2′- or 3′-ribonucleotidase. Since in most instances nucleotides are phosphorylated with greater or equal readiness than are nucleosides—adenosine being the only exception (Table II)—we have preferred to designate it as a nucleotide phosphotransferase. This must not be taken as implying its real biological function which, as with many other enzymes able to act on an entire series of substrates, will remain arguable. We deal, hence, with an enzyme capable of introducing a phosphate residue into the 2′ or 3′ position of a nucleoside or of a nucleoside 5′-mono-, -di-, or -triphosphate. The differences between the several acceptors will be clear from an inspection of Table II which

[1] The abundance of the phosphotransferase appears to vary considerably in different specimens of the same strain and possibly also in different strains. Thus, seven different batches of *E. coli* W cells yielded crude extracts (step 1-1 or 2-1 in Table I) with specific activities of the enzyme varying from 0.8 to 1.2. Although no systematic study has been made, the values from *E. coli* B cells were much lower, varying from traces of activity to one-quarter of the specific activity exhibited by *E. coli* W.

TABLE VII: Effect of ATP on Hydrolysis of *p*-Nitrophenyl Phosphate.[a]

p-Nitrophenyl Phosphate	ATP	Reaction Products (μmol/ml) after Stated Incubation Times: 15 min *p*-Nitrophenol	15 min P_i	30 min *p*-Nitrophenol	30 min P_i
+	−	2.8	2.9	5.2	5.0
+	+	0.7	0.6	1.2	1.1
−	+		0		0

[a] The assay mixtures were 100 mM with respect to *p*-nitrophenyl phosphate and 12.5 mM with respect to ATP. Other conditions as in Table II.

supplements the information given before (Brunngraber and Chargaff, 1970b).

The purification procedure described here makes available enzyme preparations that may prove valuable for the facile synthesis of compounds otherwise not easily accessible, *viz.*, the 2′- and 3′-phosphate derivatives of nucleosides and 5′-nucleotides. For preparatory purposes, it is sufficient to stop at step 3 of the isolation procedure (compare steps 1-3 and 2-3 in Table I). Enzyme specimens of this degree of purity have been used profitably for the synthesis of 3′-deoxynucleotides and of deoxynucleoside 3′,5′-diphosphates in yields of 50–95%. Though at this stage of purification the enzyme specimens retain some acid phosphatase activity, they are free of nucleotidase; the synthesis of 3′-nucleotides proceeds unimpaired as long as an excess of the donor, *p*-nitrophenyl phosphate, is present. This phosphate donor is recommended for purposes of synthesis since the reaction products are separated easily on paper; moreover, if 2′- or 3′-nucleotides are used as donors, the nucleoside to which they give rise during the transfer reaction would eventually compete with the acceptor substrate employed in the experiment.

In our previous studies on the nucleoside phosphotransferase of carrot (Brunngraber and Chargaff, 1967, 1970a) one unexpected feature became evident, namely, that it made little sense to rank acceptors by their readiness to be phosphorylated, unless the donor also was considered at the same time; in other words, the factor that had to be taken into account always was the donor–acceptor pair. The same consideration seems also to apply to the phosphotransferase studied here. A comparison of the results in Table II (*p*-nitrophenyl phosphate as donor) and Table V (thymidine 3′,5′-diphosphate as donor) brings to light at least one outstanding example; with *p*-nitrophenyl phosphate, dA and pdA are phosphorylated to nearly the same extent; with pTp as donor, pdA is phosphorylated almost twice as well as is dA. Another example of the importance of the relationship between a particular donor and a particular acceptor has been given previously in a comparison of *p*-nitrophenyl phosphate and 3′-adenylic acid (Brunngraber and Chargaff, 1970b).

This relationship between donor and acceptor may also be the cause of the failure of ADP and ATP to be phosphorylated significantly under the conditions depicted in Table II; a failure the more surprising in view of the ready phosphorylation of the deoxyriboside triphosphates. It is possible that other donors will be found more effective. One observation should, however, be mentioned. In the presence of ATP, the dephosphorylation of phosphate donors, such as 2′-adenylic acid or *p*-nitrophenyl phosphate, is depressed considerably in the absence of acceptors, as is also the phosphate transfer to an acceptor. This is reminiscent of the inhibiting effect on the hydrolysis of donors observed generally in the presence of acceptors and points to the possibility that ATP, although itself a poor acceptor, ties up the active center of the enzyme. Other effects of ATP are, however, not excluded. These questions still are under investigation.

One could ask whether the transfer to nucleosides, nucleotides, and nucleoside polyphosphates is performed by the same protein. Most observations favor an affirmative answer. For instance, throughout the isolation procedure (Table I) the ratio of the formation of two independently determined products, pTp/pT, remains constant; the same Michaelis constant is found for *p*-nitrophenyl phosphate (23 mM) or 2′-adenylic acid (1.2 mM) regardless of whether thymidine or 5′-thymidylic acid is used as the acceptor. In this connection, it may be noted that the nucleoside phosphotransferase from carrot, a protein whose homogeneity has been amply demonstrated (Rodgers and Chargaff, 1972), is also capable of phosphorylating both nucleosides and nucleotides (Tunis and Chargaff, 1960).

As is true of other phosphotransferases, the *E. coli* enzyme also functions as a hydrolase in the absence of a suitable acceptor; an action that is limited to those phosphate derivatives that can function as donors in the transfer reaction. The hydrolytic function of the *E. coli* enzyme is, however, completely inhibited in the presence of an acceptor (Table VI), whereas that of the carrot phosphotransferase is not, even at infinite acceptor concentrations.

We have listed, in our preceding communication (Brunngraber and Chargaff, 1970b), several biological systems on which an enzyme of the type described here could be assumed to have a regulatory effect. We shall limit ourselves here to one facet of the problem, namely, the biosynthesis of the nucleic acids. The observations presented in this paper point to two possible modes of interference by the nucleotide phosphotransferase: (a) modification of the nucleic acid precursors by the insertion of a phosphate residue into the 3′ position of a deoxynucleoside triphosphate (see Table II); (b) inactivation of the primer, in polymerase reactions requiring such a compound, by phosphorylation of the terminal 3′-hydroxyl required for chain growth(see Table III).

Another example of a possible biological role of this enzyme is presented in the following paper (Brunngraber and Chargaff, 1973). Additional studies with this enzyme and related ones will be presented in due course.

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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).