Purification and Properties of a Nucleoside Phosphotransferase from Carrot*

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SUMMARY

A simple procedure affording a 940-fold purification of the nucleoside phosphotransferase from carrot is described. Preliminary evidence indicates that, by an additional fractionation through a Sephadex column, a further 5- to 6-fold increase in activity can be effected. The particle weight of the enzyme varies with pH. At the pH optimum of 5.0 one species of particle weight 45,000, at pH 9.5 two species of 63,000 and 44,000 are observed. Disc electrophoresis shows only one band at pH 5.0, two bands at pH 9.5.

The enzyme is considered a nucleoside phosphotransferase which also exhibits hydrolase activity because (a) phosphate transfer is favored over hydrolysis; (b) transfer occurs specifically to nucleosides; (c) high yields of nucleotide are recorded. In the characterization of the transfer reaction four factors must be considered: (a) the nature of the phosphate donor; (b) the aglycone of the nucleoside acceptor; (c) the sugar moiety of the nucleoside; (d) the donor-acceptor pair. The main product of the enzymic transfer is a 5'-nucleotide, although upon long incubation other isomers can also be observed.

The presence in the enzyme of several active sites is made probable by studies of the effects of Mg$^{2+}$, Cu$^{2+}$, and Co$^{2+}$, of the dialysis against chelating agents, of the pH curves, and of aging properties.

The investigation of the hydrolytic functions of the enzyme leads to the conclusion that efficient donors for the transferase are hydrolyzed, although not all hydrolyzable substrates are efficient donors; and that poor acceptors for transferase are good inhibitors of hydrolase.

EXPERIMENTAL PROCEDURE

Materials

Nucleosides and nucleotides were commercial preparations supplied by Calbiochem and Sigma. 3'-Deoxyadenosine (cordycepin) was a gift from Dr. G. E. Boxer of Merck. Phenylphosphate was used as the disodium salt (Eastman Kodak). Diethylaminoethyl cellulose was purchased from Brown Company and Schleicher and Schuell. Sephadex G-100 was supplied by Pharmacia, the proteins used as reference markers in the determination of molecular weight were furnished by Mann.

Analytical Procedures

Phosphorus was determined according to King (8), protein by the method of Lowry et al. (9) or, in crude preparations, by ultraviolet absorption (10, 11).

Previous papers from this laboratory have reported the discovery (1), some of the properties (2, 3), and the partial purification (4, 5) of a group of enzymes, designated nucleoside phosphotransferases, capable of transferring organically esterified phosphoric acid specifically to nucleosides. As these enzymes are present in many plant and animal tissues and in microorganisms, exhibiting a considerably increased activity during periods of growth (3), it was suggested that they may have some part in the biosynthesis of nucleotides.

It is usually position 5' of the nucleoside that accepts the enzymically transferred phosphoric acid in the presence of enzymes of plant origin, whereas with preparations from animal tissues varying amounts of the 2' and 3'-nucleotides are also produced (2, 3). This distinction is, however, not absolute, as will be mentioned in the present communication. As regards the 5'-nucleotides, enzymes of the type discussed here have proved very useful for the synthesis of many unusual nucleotides that are otherwise not easily accessible (6, 7).

The present paper describes a simplified procedure for the isolation of nucleoside phosphotransferase from carrot and discusses some of the properties of the enzyme.

1 The Report of the Commission on Enzymes of the International Union of Biochemistry does not accord a separate entry to the nucleoside phosphotransferases, but states, in mentioning acid phosphatase (EC 3.1.3.2), that this enzyme also catalyzes transphosphorylation. In describing the separation of acid phosphatase from nucleoside phosphotransferase one would, hence, have to make the awkward statement that EC 3.1.3.2 was removed from EC 3.1.3.2.

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Enzyme Assay

Incubation Mixture 1—Fractions eluted from the columns were routinely assayed for both transferase and phosphatase activities by the incubation, at 37° for 1 hour, of equal volumes of (a) the enzyme solution, diluted when necessary to give a concentration hydrolyzing less than 25% of the donor; and (b) a stock solution which contained uridine (40 mM) and sodium phenylphosphate (200 mM) in 0.2 M sodium acetate of pH 5.0.

Incubation Mixture 2—In the study of the properties of the purified enzyme preparations, each assay tube contained, unless specified otherwise, 20 mM nucleoside, 100 mM sodium phenylphosphate, and enzyme in a total volume of 1 ml of 0.1 M sodium acetate buffer of pH 5.0. The amount of enzyme to be added was determined in preliminary experiments so as to be within the linear range of product formation. The tubes were incubated at 37° for 1 hour.

Portions of 0.05 or 0.1 ml were taken for the estimation of inorganic phosphate. The enzymically formed nucleotides in 0.02-ml or 0.04-ml portions of the assay mixture were separated and estimated by paper chromatography in a solvent system (12) which consisted of 1-propanol, concentrated ammonium hydroxide, and water (11:7:2, v/v/v) followed by spectrophotometry in the ultraviolet. This system separated the 5'-nucleotides from the 2'- and 3'-nucleotides and also from nucleosides and inorganic phosphate. The enzymically formed nucleotides in the ultraviolet. This system separated the 5'-nucleotides from the 2'- and 3'-nucleotides and also from nucleosides and sodium phenylphosphate. When the 2'- and 3'-adenylic acids, which migrate together in this solvent, were to be examined, two-dimensional chromatography was used. The second solvent was either a solution of 16 g of ammonium bicarbonate in 100 ml of HzO (13) or saturated ammonium sulfate-water-isopropyl alcohol (79:19:2, v/v/v), adjusted to pH 6 with ammonia (14).

Expression of Activity—The transferase activity is expressed as micromoles per ml per hour of nucleotide formed, the phosphatase activity as micromoles per ml per hour of inorganic phosphate released. The specific activity is the activity per mg of protein. The phosphorylation ratio, defined previously (2), is the molar ratio of phosphate transferred to inorganic phosphate released.

Preparation of Nucleoside Phosphotransferase

All operations were performed in the cold.

Step I—Ten pounds of washed carrot root were converted to juice in a Sweden speed juicer (Seattle, Washington). Approximately 2.5 liters of juice were obtained and frozen. This extract was, after thawing, centrifuged (10,300 × g, 30 min) and the supernatant fluid was decanted through glass wool.

Step II—The filtrate was applied to a DEAE-cellulose column (15 g, standard type) previously equilibrated with 0.05 M sodium acetate solution of pH 5.0. After the column had been washed with 500 ml of the same solution, protein was eluted with 0.4 M sodium acetate (pH 5.0). Fractions of 15 ml each during a period of 45 min, were collected and assayed for protein, phosphotransferase, and phosphatase. After approximately 150 ml were collected, transferase activity usually began to appear in the eluates. The fractions that contained the enzyme were combined.

Step III—The solution (325 ml) was brought to 90% saturation with ammonium sulfate by the gradual addition, with stirring, of 216 g of (NH₄)₂SO₄. The mixture was left over-night and then centrifuged (16,300 × g, 15 min). The suspension of the sediment in 50 ml of water was subjected to dialysis overnight against several changes of distilled water (3 liters) and clarified by centrifugation (500 × g, 5 min).

Step IV—This solution was adjusted to a 0.05 M sodium acetate concentration by the addition of molar sodium acetate (pH 5.0) and applied to a column (1.2 × 14 cm) of 2 g of DEAE-cellulose (type 20) previously equilibrated with 0.05 M sodium acetate (pH 5.0). The column was washed with the same solution until the protein concentration of the effluent fell below 0.02 mg per ml. A pH gradient was used for subsequent elution of phosphotransferase activity. The mixing chamber contained 100 ml of 0.05 M sodium acetate, pH 5.0, and the reservoir contained 100 ml of 0.05 M sodium acetate, pH 4.0. A flow rate of 5 ml/25 min was maintained. Phosphotransferase was eluted at a pH of approximately 4.3. The fractions which contained the peak activity of this enzyme were combined and, after dialysis against distilled water overnight, stored in the frozen state at −20°. Enzyme preparations of this stage of purification were used in the subsequent experiments.

RESULTS

Purification of Nucleoside Phosphotransferase

Isolation—A typical experiment is summarized in Table I. Although the nucleoside phosphotransferase is adsorbed in its entirety on the first DEAE-cellulose column (Step II), only 4% of the phosphatase activity is retained. A large part of the acid

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Volume</th>
<th>Total activity</th>
<th>Phosphotransferase</th>
<th>Total protein</th>
<th>Phosphotransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Crude extract</td>
<td>ml</td>
<td>147,500</td>
<td>2500</td>
<td>12,500</td>
<td>0.03</td>
</tr>
<tr>
<td>II</td>
<td>DEAE-cellulose Column 1</td>
<td>325</td>
<td>6179</td>
<td>4400</td>
<td>479</td>
<td>0.7</td>
</tr>
<tr>
<td>III</td>
<td>(NH₄)₂SO₄ precipitate</td>
<td>62</td>
<td>2420</td>
<td>4530</td>
<td>136</td>
<td>1.9</td>
</tr>
<tr>
<td>IV</td>
<td>DEAE-cellulose Column 2</td>
<td>164</td>
<td>930</td>
<td>2300</td>
<td>7.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* In this preparation, 10 lb of carrots were processed.
* The activity is expressed as micromoles per ml per hour of inorganic phosphate (phosphatase) or of uridylic acid (phosphotransferase) produced under conditions stated in the text ("Incubation Mixture 2").
* Molar ratio of phosphate transferred to inorganic phosphate formed.
* Activity per mg of protein.
phosphotransferase, which corresponds to an approximately peak (see Fig. 2) were analyzed and did not vary much among phosphorylation ratio.

When different individual eluates which comprised the transferase activity were resolved on acrylamide gel electrophoresis with the use of Incubation Mixture 1.

The additional treatment of the preparations described here is shown in Fig. 1. More contaminating phosphatase activity is not adsorbed on the column; several additional phosphatase peaks are eluted by 0.4 M buffer before transferase activity appears. An elution diagram which represents Step II is shown in Fig. 1. More contaminating phosphatase is removed in Step III, which consists in the precipitation of the active protein fraction at 90% saturation with ammonium sulfate. A fractional precipitation of protein with (NH₄)₂SO₄ at this stage of purification resulted in the loss of a good part of the transferase with no appreciable enhancement of the phosphorylation ratio.

Much of the remaining protein and additional phosphatase activity are removed during the second adsorption on DEAE-cellulose (Step IV), which is shown in Fig. 2. Less than 1% of the original phosphatase activity remains associated with the column; several enzyme preparations: in four isolation experiments the final phosphorylation ratio varied between 2.1 and 2.8. This could signify that a single specific phosphatase capable of transferring phosphate to a nucleoside acceptor has been isolated.

The specific activity of the present preparations of nucleoside phosphotransferase, which corresponds to an approximately 940-fold purification, is much higher than that of our previous preparations (4, 5).

The purified enzyme (Step IV) is stable for at least 1 month when stored at -20°C. No measurable loss of either transferase or phosphatase activity was observed during this period. In solutions stored at 4°C for 1 week, 90% of the transferase activity was retained, with no loss of phosphatase activity. This is in contrast to the observation that the crude extract (Step I) stored under the same conditions (4°C, 1 week) lost the bulk of its phosphatase activity.

**Characteristics of the Transfer Reaction**

**Ribonucleosides and Deoxyribonucleosides as Acceptors**—The initial velocities of nucleotide synthesis, with the use of different nucleosides as acceptors, are listed in descending order in Table II. In general, when phenylphosphate serves as the donor, the pyrimidine nucleosides are better acceptors than the purine nucleosides. Within the pyrimidine series, the ribonucleosides are more efficient acceptors than the deoxyribonucleoside derivatives, whereas the opposite appears to hold for the purines. The importance of the sugar moiety is emphasized by the experiments assembled in Table III, which show that 3'-deoxyadenosine is more readily phosphorylated than the 2'-deoxynucleoside or adenosine. Both the aglycone and the sugar moiety of the acceptor must, hence, be considered in assessing the efficiency of the transfer reaction.

**Simultaneous Transfer to Two Acceptors**—The enzymic phosphate transfer to an equimolar mixture of two acceptors is shown in Table IV. It can be seen that the formation of 5'-uridylic acid from uridine is reduced in the presence of deoxycytidine and very much diminished in the presence of adenosine. The less efficient acceptor controls the rate of phosphorylation.
The I- and B-phosphates of glucose and β-glycerophosphate are involved in the synthesis of the different nucleotides. This strengthens the suggestion made before (4) that the same enzyme site. Preliminary experiments (Table VI) show this not to be the case. Ribose 5-phosphate does not depress the initial velocity of the formation of 5'-UMP by phenylphosphate; it does have, however, an inhibitory effect on the phosphatase activity.

### Table II

**Activities of transfer and hydrolytic functions of nucleoside phosphotransferase in presence of various nucleosides as acceptors and phenylphosphate as donor**

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Activity&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Phosphorylation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphotransferase</td>
<td>Phosphatase</td>
</tr>
<tr>
<td>Cytidine</td>
<td>3.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Uridine</td>
<td>2.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Deoxyctydine</td>
<td>2.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Ribothymidine</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>1.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Deoxyguanosine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Guanosine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Deoxyadenosine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Adenosine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The activity is expressed as μmoles of nucleotide or inorganic phosphate per ml "Incubation Mixture 2" per hour at 37°.

<sup>b</sup> Saturated solutions of these nucleosides were added to the incubation mixtures. Under these conditions the amounts of enzyme were still limiting.

### Table III

**Different adenine nucleosides as acceptors for nucleoside phosphotransferase**

Each tube contained 20 mM nucleoside, 400 mM phenylphosphate, and 40 μg of protein, in a total volume of 1 ml of 0.1 M sodium acetate buffer (pH 5.0).

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>5'-Nucleotide synthesized during hours of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.6</td>
</tr>
<tr>
<td>2'-Deoxyadenosine</td>
<td>0.9</td>
</tr>
<tr>
<td>3'-Deoxyadenosine</td>
<td>1.5</td>
</tr>
</tbody>
</table>

This strengthens the suggestion made before (4) that the same enzyme is involved in the synthesis of the different nucleotides.

**Various Organic Phosphates as Donors**—The abilities of different organic phosphates to act as donors in the transfer of phosphate to uridine are listed, in descending order, in Table V. The 1- and 6-phosphates of glucose and β-glycerophosphate are nearly or entirely inactive in phosphate transfer. With ribose 5-phosphate as the donor, a small quantity of 5'-uridylic acid is formed. As was also found before (2, 6), certain nucleotides are efficient phosphate donors. This is, however, not true of (2' + 3')-uridylic acid and probably also of 2'-adenylic acid. On the other hand, the 5'- and 3'-adenylic acids exhibit about 70% of the effectiveness of phenylphosphate. With 3'-AMP as the donor, the formation of small amounts of adenosine serves to make available a second acceptor nucleoside to the enzyme, as shown by the appearance of some 5'-AMP. The sum of the two nucleotides thus synthesized approaches the amount of 5'-uridylic acid produced with phenylphosphate as the donor. If adenosine is used as the only acceptor, 3'-adenylic acid proves a much more efficient donor than phenylphosphate.

For instance, in an experimental series with limiting amounts of enzyme and with assay mixtures which contained 25 mM donor and 5.8 mM adenosine in 1 ml of 0.1 M sodium acetate of pH 5.0, incubation (1 hour, 37°) produced 0.2 μmole of 5'-adenylic acid and no inorganic phosphate with phenylphosphate as the donor, 1.2 μmoles of 5'-adenylic acid and 1.3 μmoles of inorganic phosphate with 3'-adenylic acid as the donor. With the acceptor omitted, 3'-AMP produced, under the same conditions, 1.0 μmole of 5'-AMP and 2.5 μmoles of inorganic phosphate.

It was of interest to see whether an efficient and a poor phosphate donor (compare Table V) would compete for the same enzyme site. Preliminary experiments (Table VI) show this not to be the case. Ribose 5-phosphate does not depress the initial velocity of the formation of 5'-UMP by phenylphosphate; it does have, however, an inhibitory effect on the phosphatase activity.

### Table IV

**Simultaneous phosphate transfer to two acceptors**

Concentrations: 20 mM, of each acceptor were tested in a total volume of 1 ml under conditions stated in the text ("Incubation Mixture 2"). The activity is expressed as micromoles of nucleotide or inorganic phosphate per ml per hour.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Acceptor 1</th>
<th>Acceptor 2</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphotransferase</td>
<td>Phosphatase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Product 1</td>
<td>Product 2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Uridine</td>
<td>Deoxyctydine</td>
<td>3.2 UMP</td>
</tr>
<tr>
<td></td>
<td>Uridine</td>
<td>Deoxyctydine</td>
<td>1.2 UMP</td>
</tr>
<tr>
<td>2</td>
<td>Uridine</td>
<td>Adenosine</td>
<td>2.2 UMP</td>
</tr>
<tr>
<td></td>
<td>Uridine</td>
<td>Adenosine</td>
<td>0.1 UMP</td>
</tr>
</tbody>
</table>

### Table V

**Transfer and hydrolytic functions of nucleoside phosphotransferase in presence of various organic phosphates as donors**

Each assay tube contained, in 1 ml of 0.1 M sodium acetate solution of pH 5.0, 100 mM phosphate donor, 11 μg of enzyme, and (where indicated) 20 mM uridine as acceptor. The activity is expressed as micromoles of uridylic acid or inorganic phosphate produced per ml per hour.

<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphotransferase</td>
</tr>
<tr>
<td>Phenylphosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>3'-Adenylic acid</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5'-Adenylic acid</td>
<td>1.8</td>
</tr>
<tr>
<td>Ribose 5-phosphate</td>
<td>0.7</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>0.4</td>
</tr>
<tr>
<td>(2' + 3')-Uridylic acid</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.2</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> In this experiment, 0.3 μmole of 5'-adenylic acid was also formed.
TABLE VI

Effect of simultaneous presence of two phosphate donors

Concentrations of phosphate donors, 100 mM, (in a total volume of 1 ml of 0.1 M sodium acetate, pH 5.0) were incubated (1 hour, 37°) with limiting amount of enzyme and 20 mM uridine as acceptor. The activity is expressed as micromoles of 5'-uridylic acid or inorganic phosphate per ml per hour.

<table>
<thead>
<tr>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylphosphate</td>
<td>Ribose 5-phosphate</td>
<td>2.5**</td>
</tr>
<tr>
<td>Phenylphosphate</td>
<td>Ribose 5-phosphate</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* The same values were obtained when the assay mixture contained 20 mM ribose.

which becomes more noticeable with longer periods of incubation. Neither reaction is influenced by the addition of ribose.

Nucleotide Isomers Produced in Transfer Reaction—With plant phosphotransferases of the state of purity then available, the only products of the transfer reaction that could be seen previously were the 5'-nucleotides (2, 3). This was also observed with the present preparations up to Step III. With more highly purified enzyme preparations (Step IV), however, small amounts of other isomers are observed after an incubation period of 18 hours. A few orienting experiments may be cited. When adenosine is thus phosphorylated, with phenylphosphate as the donor, the product has the following composition: 80.5% of 5'-adenylic acid, 17.2% of 3'-adenylic acid, 2.3% of 2'-adenylic acid. With 2'-deoxyadenosine as the acceptor, 69% of the product is the 5'-nucleotide, 32% the 3'-nucleotide. All deoxy-nucleosides tested also yield small quantities (less than 5%) of the 3',5'-diphosphates.

Characteristics of Phosphatase Activity of Phosphotransferase Preparations

The rates at which various organic phosphates are hydrolyzed by the purified enzyme (Step IV) in the absence of an acceptor are shown in Fig. 3. The rates of the dephosphorylation of the three isomeric adenylic acids, 5'-AMP, 3'-AMP, and 2'-AMP, are compared in Fig. 4. It will be seen that those substrates that are efficient phosphate donors in the transfer reaction (compare Table V) are hydrolyzed rapidly. At a 20 mM concentration, phenylphosphate, 5'-uridylic acid, and ribose 5-phosphate are hydrolyzed at approximately the same initial velocity; a rate not influenced by excess substrate. 5'-Adenylic acid is hydrolyzed slightly faster than the 3'-isomer, and 2'-adenylic acid very slowly. β-Glycerophosphate and (2' + 3')-uridylic acid are hydrolyzed to a lesser degree, and glucose 1-phosphate is degraded only slightly.

As is shown in Table V for several organic phosphates, the hydrolytic function of the purified transferase is inhibited by uridine. No correlation appears to exist between the extent of inhibition and the efficacy of the particular substrate as the donor in the transfer reaction. For example, uridine inhibits the hydrolysis of phenylphosphate and of ribose 5-phosphate to nearly the same extent, but only the first is an efficient donor. On the other hand, whereas the substrates 3'-adenylic acid and phenylphosphate are approximately equal in their ability to transfer the phosphoryl group to uridine, this nucleoside inhibits the hydrolysis of phenylphosphate by 86%, that of 3'-AMP by only 43%.

When the data in Table II are considered together with those in Table V, one gains the impression that the ability of a nucleoside to act as an acceptor is inversely related to its efficiency as an inhibitor of phosphatase action, e.g. adenosine is much more effective in preventing the hydrolysis of phenylphosphate than is uridine.

Comparison of Transfer and Hydrolytic Functions of Phosphotransferase

Dependence Upon pH—The pH optimum of the purified transferase preparations is about pH 5.0 with either uridine or deoxycytidine as the acceptor and phenylphosphate as the donor.

![Fig. 3. Time curves of hydrolysis of phosphoric acid esters (all 20 mM) by nucleoside phosphotransferase (Step IV) at 37°.](image)

![Fig. 4. Time curves of hydrolysis of adenylic acid isomers (all 25 mM) by nucleoside phosphotransferase (Step IV) at 37°.](image)
At pH 5, but also at 6 to 6.5.

The following cations had no or almost no activity of the preparations described here, it was observed that copper and zinc ions inhibited a large part of the contaminating phosphatases (4). Quite different metal effects (tested in 0.01 mm concentrations) were obtained with the present enzyme preparations. The following cations had no or almost no effect on either the transfer or the phosphatase functions of the enzyme: Na\(^+\), K\(^+\), (NH\(_4\))\(^+\), Zn\(^2+\). Ammonium molybdate also was ineffective. Ca\(^2+\), Mn\(^2+\), and Pb\(^2+\) were equally inhibitory to both functions, Fe\(^2+\) and Fe\(^3+\) were more inhibitory with regard to the transfer reaction. Mg\(^2+\), Cu\(^2+\), and Co\(^2+\) enhance the phosphatase function considerably, the transfer activity to a small extent.

Sodium fluoride has no effect on either activity at concentrations below 10 mM. With 0.25 mM NaF, the phosphatase is inhibited by less than 10%, the transferase enhanced by about 45%.

**Inactivation and Reactivation**—Glutathione (2.5 mM) or cysteine (2.5 mM) depress both transferase and phosphatase activities by about 25%. EDTA or 8-hydroxyquinoline (oxine), both in 0.625 mM concentration, exert a slightly stronger inhibition, which is reversed by 5 mM MgCl\(_2\). When the enzyme solution is subjected to dialysis overnight against distilled water, it loses no activity. Dialysis, first against EDTA or oxine and then against water, produces, however, an inhibition which results in the recovery of only 36% of the initial phosphotransferase activity and 65% of the initial phosphatase activity. As can be seen in Table VII, copper and cobalt ions reactivate the enzyme system. In the case of the treatment with EDTA, magnesium ions also have a limited effect.

**Effect of Cations and Anions**—With the crude enzyme preparations available before, which had only 2% of the specific activity of the preparations described here, it was observed that copper and zinc ions inhibited a large part of the contaminating phosphatases (4). Quite different metal effects (tested in 0.01 to 10 mM concentrations) were obtained with the present enzyme preparations. The following cations had no or almost no effect on either the transfer or the phosphatase functions of the enzyme: Na\(^+\), K\(^+\), (NH\(_4\))\(^+\), Zn\(^2+\). Ammonium molybdate also was ineffective. Ca\(^2+\), Mn\(^2+\), and Pb\(^2+\) were equally inhibitory to both functions, Fe\(^2+\) and Fe\(^3+\) were more inhibitory with regard to the transfer reaction. Mg\(^2+\), Cu\(^2+\), and Co\(^2+\) enhance the phosphatase function considerably, the transfer activity to a small extent.

Sodium fluoride has no effect on either activity at concentrations below 10 mM. With 0.25 mM NaF, the phosphatase is inhibited by less than 10%, the transferase enhanced by about 45%.

**Activity**

The present communication has two principal purposes: (a) to describe a facile preparation of the nucleoside phosphotransferase of carrot of a much higher degree of purification than has been available heretofore; and (b) to draw attention to some interesting and, perhaps, still baffling properties of this enzyme. As this enzyme has proved a convenient preparatory tool for the synthesis of nucleotides that are otherwise accessible only with difficulty, the availability of specimens of high activity may be of some value. It will, however, not be easy to obtain large quantities of this protein, unless richer sources are found, since it can be estimated that the pure enzyme hardly amounts to more than 0.02% of the carrot proteins.

**Discussion**

It would not be profitable—especially before kinetic evidence, which we hope to provide in a subsequent paper, is available—to discuss whether this enzyme should be regarded as a nucleotidase which also has transfer properties or as a nucleoside phosphotransferase capable of acting as a hydrolase under suitable conditions. We incline to the second view, although the possibility that a small part of the hydrolytic activity of our preparations is due to a contaminant cannot yet be ruled out entirely. Enzyme phosphate transfer (17-19) to alcohols...
and sugars has long been known to be catalyzed by certain acid (20, 21) and alkaline phosphatases (22–26). The transfer system discussed here is, however, distinguished from these phosphatases by the high ratio (about 2.5) of phosphoric acid ester transferred to phosphoric acid ester hydrolyzed and by its strict limitation to one type of acceptor, namely, nucleosides. Not even d-ribose is phosphorylated. Moreover, a very high yield of product (up to 45% of the nucleoside acceptor) can be achieved under the conditions studied here.

The most active preparations of the nucleoside phosphotransferase corresponded, in two experiments, to a specific activity of 1730 and 1780. With phenylphosphate as the donor and under the conditions defined before, the molecular activity of the transferase, i.e. the number of uridine molecules converted to UMP per min by 1 mole of enzyme (assuming a molecular weight of 45,000), is found as 1340. At pH 5.0, i.e. at the pH optimum for the transfer reaction, the enzyme shows a single electrophoretic boundary and passes as a single component corresponding to a particle weight of 45,000 through a Sephadex column. At pH 5.5, two components are seen in electrophoresis and two active fractions, of particle weights 63,000 and 44,000, pass through Sephadex. It would be premature to consider the existence of a subunit of molecular weight 21,000 to 22,000.

The enzymic product seen under normal conditions is exclusively a 5'-nucleotide. With highly purified enzyme preparations and long periods of incubation, however, smaller proportions of the 2'- and 3'-isomers are also formed. It will be remembered that even crude preparations of prostate trans-ferase catalyze the formation of 5'-, 3'-, and 2'nucleotides (2).

When the requirements of the transfer reaction are considered, it will be seen that at least four factors must be distinguished.

(a) The donor: with the exception of phenylphosphate, all efficient donors are 3'- or 5'-nucleotides (Table V). (b) The aglycone of the acceptor: for instance, with the rest of the variables kept constant, cytidine is a more efficient acceptor than ribothymidine, and deoxyguanosine better than deoxy-

This multiplicity of interrelationships will explain why we consider it as yet not advisable to speculate on the number and types of active sites (donor site, acceptor site, water site, and so on) which should be postulated. That different sites exist and that they may, in fact, require different cofactors is not improbable. To mention one example, when the enzyme is partially inactivated by dialysis against a chelating agent, there is evidence that the hydrolase function requires Mg ions, the transferase function copper or cobalt ions, for reactivation (Table VII).

These studies are being continued.

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