Nucleoside Phosphotransferase from Barley
CHARACTERIZATION AND EVIDENCE FOR PING PONG KINETICS INVOLVING PHOSPHORYL ENZYME*

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A nucleoside phosphotransferase has been purified to homogeneity from barley seedlings. Its M, is about 50,000 and it consists of two subunits of equal size. A tightly bound metal ion required for activity can be replaced by Mg, Ba, or Co ions, but not by divalent Cu, Ca, Cd, or Hg. The enzyme is capable of catalyzing the transfer of phosphate from nucleoside monophosphates to the 5'-hydroxyl of any other nucleoside, but shows a decided preference for purine deoxy nucleoside phosphate acceptors. A short lived phosphoryl enzyme intermediate has been trapped by rapid denaturation in presence of [32P]AMP.

The steady state kinetics of this enzyme is found to be fully consistent with a branched ping pong pathway involving a compulsory phosphoryl enzyme intermediate from which phosphate may be transferred to nucleoside or to water. With appropriately chosen substrates, parallel line reciprocal plots were obtained, provided that both products of the branched pathway were taken into account in the rate measurements. Conversely, when only transphosphorylation was measured, converging reciprocal plots were obtained. Accordingly, assays were devised for the measurement of three velocity components: transphosphorylation, hydrolysis, and the sum of both reactions. Competitive inhibition of the transferase reaction by higher concentrations of either substrate is attributed to deadend interactions. Instead of being inhibited by the formation of a putative E-P-AMP complex, however, the phosphatase reaction is accelerated almost 2-fold.

Steady state rate analyses of the nucleoside phosphotransferases from carrot (1) and fern (2) yielded converging initial rate patterns in double reciprocal plots. Such patterns are usually associated with sequential kinetic pathways, as distinguished from double displacement, i.e. ping-pong, pathways. These enzymes exhibit inherent nucleotidase activities, which complicates the interpretation of conventional plots of initial rate data; these complications were not taken into account in the earlier work.

A recent stereochemical study reported from these laboratories showed that [32P]phosphoryl transfer catalyzed by the nucleoside phosphotransferase from barley proceeds with net retention of configuration, suggesting a double displacement mechanism involving the compulsory formation of a phosphoryl enzyme as an intermediate (3). We now report the first direct chemical evidence for a covalently bonded [32P]phosphoryl enzyme form of the barley enzyme.

Our stereochemical findings prompted us to propose a modified ping-pong kinetic pathway for this enzyme, in which the phosphoryl enzyme could transfer its phosphoryl group either to a nucleoside (accounting for transferase activity) or to water to give nucleotidase activity (3). This kinetic pathway has previously been observed in the cases of several group-transferring enzymes which exhibit hydrolase activities (4-7). The basic pathway is represented for nucleoside phosphotransferase by Equations 1 to 3, in which N1MP and N2MP represent two nucleoside monophosphates, N1 and N2 represent the corresponding nucleosides, and E-P represents the intermediate phosphoryl enzyme. We have investigated the nucleoside phosphotransferase from barley and found the kinetics to be consistent with a modified ping-pong kinetic pathway involving a hydrolytically labile phosphoryl enzyme. We have also detected several enzyme-substrate interactions in addition to those represented by Equations 1 to 3.

N1MP + E = E-N1MP = E-P + N1 (1)
E-P + N2 = E-P-N2 = E + N2MP (2)
E-P + H2O = E + P (3)

EXPERIMENTAL PROCEDURES†

RESULTS

The following studies were carried with enzyme purified to apparent homogeneity from an acetone powder of 4-day barley

† Portions of this paper (including "Experimental Procedures," part of "Results," Table IV, Figs. 8-11, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20614. Request Document No. 81M-2464, cite the authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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Purification and Kinetics of Nucleoside Phosphotransferase

Nucleoside Specificity—As can be seen in Table I, barley nucleoside phosphotransferase is highly ordered in its nucleoside specificity. Although it phosphorylates all of the normal nucleic acid precursors, the apparent $K_m$ values vary over a range of 2 orders of magnitude, and the catalytic rate constants show almost a 10-fold variation. Wilkinson's weighted least squares linear regression analysis, adopted to a programmable calculator (8), was used to compute these two kinetic parameters and to fit straight lines to the kinetic data shown below.

In situations where the $K_m$ and $k_{cat}$ figures seem to make opposing statements about the effective utilization of particular substrates, the catalytic efficiency ratios may be useful. However, these ratios do not greatly change the order based on $K_m$ alone. Purine nucleosides are clearly preferred, in the order of deoxyribose > ribose > arabinose. In all cases, the deoxyribonucleosides have lower $K_m$ values, but also lower $k_{cat}$ values than their respective ribonucleosides. The enzyme is 80 times more efficient in handling dAdo than dUrd, the poorest substrate in the list.

The phosphate donor specificity of the barley nucleoside phosphotransferase has been reported previously (3).

Evidence for a Phosphoryl Enzyme Intermediate—It could be predicted that the hydrolytic potential of the enzyme might result in relatively low steady state concentrations of the putative $E$-$P$ intermediate. Accordingly, it seemed essential that such an intermediate be trapped by rapid denaturation of the enzyme before reaching the steady state. As shown in Table II, addition of trichloroacetic acid to the reaction mixture within 2 to 3 s permits trapping about 60% of the enzyme protein as the phosphorylated intermediate, assuming an average molecular weight of 50,000. Preliminary experiments carried out under similar conditions, but not quenched until 15 s of reaction, resulted in the labeling of only 10% of the protein molecules, suggesting rapid catalyzed decay of the phosphoprotein intermediate after an initial burst of phosphorylation. A control experiment with $^{[3]}$HJUMP resulted in negligible labeling of enzyme, tending to rule out any sort of mechanism involving binding of the intact nucleotide to the denatured protein.

Aliquots of the labeled protein were subjected to SDS-PAGE. The stained gel was dried on a filter paper support, and the location of the radioactive protein was determined by x-ray film autoradiography and found to coincide with the protein band. It remains to be determined whether the labeling we observe is due to one phosphate group on each of 60% of the enzyme molecules or to two on each of 30% of these dimeric molecules.

Kinetics for Equations 1 to 3—In accord with the properties of nucleoside phosphotransferase described here and in a previous report (3), and the kinetic model defined by Equations 1 to 3, there are three different initial rates that can be measured: the transferase rate defined as $v_1 = d[NMP]/dt$, the nucleotidase rate defined as $v_2 = d[P]/dt$, and the sum of transferase and nucleotidase rates defined as $v_3 = -d[N]/dt$. The transferase reaction corresponds to Equations 1 and 2, the nucleotidase corresponds to Equations 1 and 3, and the sum of transferase and nucleotidase corresponds to all three equations. The substrate concentration dependencies of these rates are given in Equations 4 to 6, which refer to initial rates in the absence of products.

$$E_0 \rightarrow (k_1 + k_2 + k_3 + k_4) [NMP] + k_5 [NMP][N] + k_6 [NMP][N]$$

TABLE I

| Nucleoside | $K_m$ (mM) | $k_{cat}$ | $k_{cat}/K_m$ |
|------------|------------|-----------|---------------|
| dAdo       | 0.087      | 0.91      | 10.4          |
| dGuo       | 0.412      | 2.32      | 5.63          |
| Ado        | 0.540      | 1.52      | 2.81          |
| dThd       | 0.890      | 3.40      | 3.82          |
| Guo        | 2.13       | 4.73      | 2.22          |
| araA       | 3.83       | 4.73      | 1.24          |
| Urd        | 3.93       | 0.51      | 0.13          |
| dCyd       | 4.15       | 3.12      | 0.75          |
| Cyc        | 5.39       | 4.31      | 0.67          |

a Molar of NMP formed $\cdot$ min$^{-1}$ $\cdot$ mol enzyme$^{-1}$ $\times$ $10^{-5}$ assuming molecular weight of 50,800.
b Liters $\cdot$ mol$^{-1}$ $\cdot$ min$^{-1}$ $\times$ $10^{-9}$.

TABLE II

| Experiment | Cpm retained by filter | Net Cpm | Phosphate bound | Fraction of protein molecules phosphorylated |
|------------|------------------------|---------|-----------------|---------------------------------------------|
|            |                        |         |                 |                                             |
| 1          | 19,900                 | 16,300  | 0.0820          | 60.3                                       |
| 2          | 19,400                 | 16,000  | 0.0823          | 60.5                                       |
| Control    | 3,000                  | 0       |                 |                                             |

$$E_0 \rightarrow k_1 + \frac{k_2 + k_3}{k_{cat}[NMP]} + \frac{k_4 + k_5 + k_6 + k_7}{k_{cat}[NMP][N]}$$

$$E_0 \rightarrow k_1 + \frac{k_2 + k_3}{k_{cat}[NMP]} + \frac{k_4 + k_5 + k_6 + k_7}{k_{cat}[NMP][N]}$$

$$E_0 \rightarrow k_1 + \frac{k_2 + k_3 + k_4}{k_{cat}[NMP]} + \frac{k_5 + k_6 + k_7}{k_{cat}[NMP][N]}$$

Transferase Rate—The transferase rate is described by Equation 4 which has the form characteristic of equations for sequential bisubstrate pathways, although the kinetic model being considered is essentially a ping-pong pathway. This mathematical form results from the fact that the transferase rate is smaller than the rate of consumption of nucleotide substrate, i.e. a fraction of the $N$MP is hydrolyzed to $P_i$ and $N_i$. Since this fractional diversion to hydrolysis decreases with increasing acceptor concentration, its effect is to convert a basically parallel line pattern in a double reciprocal plot into a converging pattern. As shown in Fig. 1, in which AMP is $N$MP and dAdo is $N_5$, the transferase rate for nucleoside phosphotransferase is consistent with Equation 4. Inasmuch as Equation 4 is also consistent with a number of other reasonable kinetic models, Fig. 1 does not by itself identify the kinetic pathway. However, the data are consistent with the pathway of Equations 1 to 3.
Nucleotidase Rate—Equation 5 describes the dependence of nucleotidase activity on the concentrations of the nucleotide and nucleoside if Equations 1 to 3 represent the kinetic pathway. The effect of the nucleoside on the nucleotidase rate is as a noncompetitive inhibitor with respect to the nucleotide. According to Cleland’s rules, this is because the nucleoside interacts with \( E-P \) while the nucleotide interacts with \( E \), giving an intercept effect; and the two are connected by reversible steps in the presence of a nucleoside, giving a slope effect (9).

The effect of dThd on the nucleotidase rate with AMP as the donor substrate is illustrated in Fig. 2. Both slope and intercept effects are present in the primary plot; however, the intercept replot is not a straight line so the kinetics cannot be consistent with Equation 5. Note that the intercept replot rises with increasing [dThd] and reaches a plateau at the highest concentrations. Inasmuch as the intercepts are the reciprocals of apparent maximal nucleotidase activities and dThd is acting as a nucleotidase inhibitor, the plateau at high [dThd] must represent residual nucleotidase activity intrinsic to enzyme-dThd complexes which cannot be inhibited further by dThd. This could not occur in Equations 1 to 3 since increasing [dThd] would divert \( E-P \) into \( E-P-dThd \) which, according to Equation 2, produces only \( E \) and dTMP.

The basic kinetic pathway can be made consistent with this aspect of Fig. 2 if it is amended to include the production of \( P \), by the \( E-P-dThd \) complex according to Equation 7. When this process is included in the basic pathway, the new rate equation correctly describes the behavior of the intercept replot in reaching a plateau, but it fails to account for the continued linearity of the slope replot throughout the range of [dThd] used in Fig. 2. The continued slope effect signals the presence of an additional competitive inhibitory interaction by dThd, most probably an interaction with free enzyme according to Equation 8 where \( K_{N_e} \) is the dissociation constant for the dead-end-enzyme-acceptor complex. This type of interaction is characteristic of ping-pong kinetic pathways (9).

It was described and explained by Ray and Roscelli in their kinetic study of phosphoglucomutase-catalyzed transphosphorylation (10) and subsequently observed in several other laboratories reporting on the kinetics or enzymes which catalyze group transfer reactions by the basic Ping Pong Bi Bi pathway (11-14). This interaction also appears in the kinetics for \( u_3 \), the sum of nucleotidase and transferase rates described in the next section.

Equation 9 is the rate law for the kinetic model defined by Equations 1, 2, 3, 7, and 8. Equation 9 reduces to the rate law for the basic Ping Pong Bi Bi pathway if [dThd] is set equal to zero.
for nucleotidase
\[
E_0 = \frac{(k_3 + k_4)(k_6 + k_7 + k_{11}) + k_4(k_3 + k_{11})[N_2]}{k_3(k_6 + k_7 + k_{11}) + k_4[1][N_2]} + \frac{(k_2 + k_3)}{k_1}[N\text{MP}]
\]
\[
(1 + \frac{[N_2]}{K_{Nz}}) \left( \frac{k_4(k_3 + k_{11}) + k_6(k_7 + k_{11})[N_2]}{k_4(k_3 + k_{11}) + k_6(k_7 + k_{11}) + k_4[1][N_2]} \right)
\]
(9)
activity in the absence of acceptor when \([N_2] = 0\), i.e. the same equation as that resulting from setting \([N_2] = 0\) in Equation 5. It also accounts for the secondary plots of slopes and intercepts in Fig. 2. When \([N_2]\) is very large, i.e. \(k_2k_3/[N_2] > k_3(k_6 + k_7 + k_{11})\) and all \(E-\text{P}\) is in the form of \(E-\text{P} \cdot N_2\), Equation 9 reduces to Equation 10, according to which the intercepts
\[
E_0 = \frac{k_3 + k_4 + k_{11}}{k_3} + \frac{(k_2 + k_3)}{k_4[1][N\text{MP}]} \left( 1 + \frac{[N_2]}{K_{Nz}} \right) \left( \frac{k_3 + k_{11}}{k_1} \right)
\]
(10)
of double reciprocal plots of rate versus \([N\text{MP}]\) are independent of \([N_2]\), whereas the slopes are proportional to \([N_2]\).

The dependence of the intercept on \([N_2]\) at lower concentrations can also be explained by Equation 9 under conditions which appear to be satisfied by available data. Considering the first term on the right side of Equation 9, the effects of \([N_2]\) on the numerator and denominator are compensatory at large \([N_2]\). The degree to which they compensate at smaller \([N_2]\) depends upon the relative values of rate constants. If \(k_1 > k_3 + k_4\), the effects will be largely compensatory and little or no effect of \([N_2]\) on the intercepts will be seen. If \(k_1 < k_3 + k_4\), the effect of \([N_2]\) on the numerator will dominate and an intercept effect will be seen as in Fig. 2. The following independent evidence is consistent with the latter relationship of rate constants. The plateau in the intercept replot of Fig. 2 corresponds to a nucleotidase rate of about 13 to 14% of that observed in the absence of dThd, indicating that \(k_{11} < k_3\) as shown above (Table II), up to 60% of the enzyme was trapped as the phosphoryl enzyme, suggesting that \(k_3\) is comparable to or larger than \(k_4\) so that \(k_1 > k_3 + k_4\).

According to Equation 9, the slope replot might exhibit curvature at lower \([\text{dThd}]\) because of the appearance of \([N_2]\) in the complex factor of the second term. No curvature is apparent in Fig. 2. In this case, there is independent evidence for compensation in the numerator and denominator of the complex factor. The maximum transferase rate at pH 5 was found to be 10 to 20% of the maximum nucleotidase rate when \(\text{dThd}\) is the acceptor, suggesting that \(k_2\) is comparable to \(k_{11}\); therefore, the effects of \([N_2]\) in the denominator and numerator should be substantially compensatory. We note, however, that our data are not extensive enough to exclude the possibility of a small degree of curvature at low \([\text{dThd}]\).

Sum of Transferase and Nucleotidase Rates—The most characteristic feature of the kinetics for the basic pathway (Equations 1 to 3) is the substrate concentration dependence of the sum of the transferase and nucleotidase activities, i.e.
\[
v_0 = d[N]/dt = -d[N\text{MP}]/dt.
\]
The rate law is Equation 6, according to which a plot of \(v_0\) versus \([\text{AMP}]\) under conditions fixed \([N_2]\), including \([N_2] = 0\), should give a family of parallel straight lines. The situation differs in several respects from the conventional Ping-Pong Bi Bi system. In the first place, no rate could be detected in the absence of co-substrate in the conventional pathway, while in the present case, the sum of rates becomes the nucleotidase rate in the absence of co-substrate. Secondly, the intercepts may either increase or decrease with \([N_2]\), depending upon whether the maximum nucleotidase or transferase rate is the faster. In the present case, it is known that the nucleotidase rate is the faster, and this is also true of the carrot enzyme (1), so the intercepts should increase with increasing \([N_2]\). Third, when plotted with \(N_1\) as the variable substrate and \(N\text{MP}\) as the changing fixed substrate, the data should produce a family of curves rather than straight lines.

Double reciprocal plots of \(v_0\) are presented in Fig. 3, in which \(N_1\) is AMP and \(N_2\) is dAdo plotted in the two ways cited above and are seen to behave as predicted by Equation 6, with the exception that the slopes of the plot of \(v_0\) versus \([\text{AMP}]\) begin to increase at high \([\text{dAdo}]\). This latter behavior is a characteristic of ping-pong pathways when the acceptor substrate interacts with the free enzyme to form a dead-end complex according to Equation 8. When this interaction is incorporated into the basic kinetic model defined by Equations 1, 2, and 3, the rate equation for \(v_0\) is Equation 11, which differs from Equation 6 by the presence of the multiplier in the \([N_1]\) MP term. At \([N_2] \ll K_{Nz}\), this multiplier is 1 and the lines are parallel. As \([N_2]\) approaches \(K_{Nz}\), the multiplier grows and begins to increase the slopes.

\[
E_0 = \frac{1}{k_1} + \frac{(k_3 + k_4)}{k_3} + \frac{(k_2 + k_3)}{k_4[1][N\text{MP}]} \left( 1 + \frac{[N_2]}{K_{Nz}} \right)
\]
(11)

![Fig. 3. Effect of varying AMP and dAdo concentrations on combined velocities of phosphotransferase and hydrolase reactions. A, reciprocal plot with AMP as the varied substrate; B, reciprocal plot with dAdo as the varied substrate. Assays were carried out as described under "Experimental Procedures," with 10 ng of Fraction IV enzyme/ml of reaction mixture. Lines were fitted as in Fig. 1.](http://www.jbc.org/Downloadedfrom)
This secondary interaction is characteristic of Ping Pong pathways (9-14).

The parallel part of the pattern in Fig. 3 is not seen with all acceptor substrates. Another set of data is presented in Fig. 4 in which dThd was used as the acceptor. Again, the inhibition is seen at high [dThd], but at the lower concentrations, where a parallel pattern was approached with dAdo, there is no dependence of \( v_0 \) on [dThd]. This is because, below 0.1 mM dThd, almost all of \( v_0 \) is nucleotidase activity. dThd apparently does not bind as well as dAdo to E-P; note that its \( K_{\text{m}} \text{app} \) is about 10 times that for dAdo (Table I). Moreover, dThd evidently does not discriminate significantly between E and E-P for binding so that at concentrations at which it can interact productively with E-P to give a transferase rate, it also interacts in dead end fashion with E to give inhibition. A replot of the slopes in Fig. 4 versus [dThd] gives a value of 14 mM as the \( K_{\text{v}} \) for dThd.

To be strictly correct, the process of Equation 7 should also be taken into account for \( v_0 \) when dThd is the acceptor, since the secondary plot of intercepts in Fig. 2 indicates the nucleotidase rate for E-P-N2 could be 13 to 14% of that of E-P. The inclusion of this component would further complicate the rate equation for \( v_0 \) without either altering the mathematical form of its dependence on \([N, M, P]\) or \([N_1]\) or significantly clarifying the basic kinetic pathway. In a study to evaluate rate constants and the quantitative partitioning of intermediates, this process would have to be taken into account.

**Kinetic Parameters** — The maximum rates referring to the transferase, nucleotidase, and their sum differ since they refer to different enzymatic processes. The maximum transferase rate may be defined from Equation 4 as \( V_1 = k_5k_9[E_0](k_3 + k_7) \). With dAdo as the acceptor, it is the limiting intercept in Fig. 1. The maximum nucleotidase rate may be defined from Equation 5 or 9 as \( V_2 = k_5k_9[E_0](k_5 + k_7) \) and is calculated from the intercept of the line in Fig. 2 corresponding to the absence of acceptor. Maximum velocity for the sum of transferase and nucleotidase rates is not a useful parameter. From Equation 11, it can be defined as \( k_5k_9[E_0](k_3 + k_5) \) in the absence of acceptor, i.e. \( V_n \), or as \( k_5k_9[E_0](k_5 + k_7) \) at saturating acceptor, i.e. \( V_1 \). No definite value exists at intermediate acceptor concentrations.

For similar reasons, the Michaelis constant for a given substrate must be expected to depend upon which rate is being measured. The Michaelis constant for AMP in the transferase reaction may be defined from Equation 4 as \( K_{\text{AMP}}^{\text{app}} = k_5/k_1(k_3 + k_7) \). In the nucleotidase reaction, a Michaelis constant may be defined from Equation 5 or 9 as \( K_{\text{AMP}}^{\text{app}} = k_5(k_3 + k_8)/k_5(k_3 + k_8) \). A Michaelis constant referring to the sum of nucleotidase and transferase rates has no simple meaning. However, the two limiting states of zero and saturating acceptor permit \( K_{\text{m}} \) of AMP to be defined as \( k_5(k_3 + k_8)/k_5(k_3 + k_8) \) for the limit of [acceptor] = 0, i.e. \( K_{\text{m}} \), or, at saturating acceptor, \( K_{\text{AMP}}^{\text{app}} = k_5(k_3 + k_8)/k_5(k_3 + k_8) \). These latter two Michaelis constants can be numerically the same, either when \( k_5 \) and \( k_8 \) \( \gg \) 1 or when \( k_5 \approx k_8 \). A Michaelis constant for the nucleoside acceptor can be sensibly defined only for the transferase rate \( v_1 \). It may be derived from Equation 4 for dAdo as

\[
K_{\text{Ado}} = \frac{(k_3 + k_5)(k_1 + k_8)}{k_5(k_3 + k_8)}
\]

when the maximal velocities and Michaelis constants for AMP are as defined above. The nucleoside acceptor is an inhibitor rather than a substrate of the nucleotidase reaction, and in the sum of transferase and nucleotidase rates, it is simultaneously a substrate for the transferase component and an inhibitor of the nucleotidase component. In these cases, a Michaelis constant for the acceptor has no physical significance, as is made clear by Equations 5, 6, and 9.

The substrate inhibition constant for dThd, defined as \( K_{\text{N}} \), in Equation 8, is obtained from the slope and intercept of a secondary plot of the slopes in Fig. 4 versus [dThd] (see Equation 11). This and the other kinetic parameters defined above are assembled in Table III.

**Product Inhibition** — According to the proposed kinetic pathway, two different acceptors should compete for the E-P intermediate. For example, with AMP as the donor substrate, the product adenosine should inhibit the production of dTMP competitively with respect to dThd as the acceptor. This is shown to be the case in Fig. 5.

**Effect of High [AMP] on Nucleotidase Activity** — In a pathway such as the one proposed here, the isolation of the covalently bonded intermediate is complicated by its hydrolytic lability. We, therefore, explored the possibility of stabilizing it by making use of one of the types of substrate inhibition that is characteristic of ping-pong pathways, the dead-end association of the phosphoryl group donor substrate AMP with the intermediate E-P. This should block transferase activity to the co-substrate, and it was thought that it might block the nucleotidase activity by stabilizing the E-P toward hydrolysis. We measured nucleotidase rates over a large range of [AMP] in search of inhibition reflecting the formation of E-P-AMP. We observed instead, as illustrated...
The expanded pathway consists of Equations 1 to 3, 7, and 12. Under the conditions of Fig. 7, Equation 8 does not affect the kinetics because the [dThd] is well below its dissociation constant of 14 mM for the formation of the dead end complex. The transferase rate with AMP as the donor and dThd as the acceptor is given by Equation 13,

\[
\frac{E_{\text{p}}}{v_{\text{p}}} = \frac{(k_{10} + k_{11})}{k_{12}(k_{11} + k_{13})} + \frac{(k_{23} + k_{13})}{k_{12}(k_{11} + k_{13})(k_{14} + k_{15})([\text{dThd}])} + \frac{k_{23}(k_{12} + k_{13})(k_{2} + k_{14} + k_{15})([\text{AMP}])}{k_{12}(k_{11} + k_{13})(k_{14} + k_{15})([\text{dThd}])}
\]

according to which high concentrations of AMP inhibit the transferase activity. When [AMP] is large, the second term in Equation 13 becomes very small; under such conditions, AMP becomes a competitive inhibitor with respect to dThd as shown in Fig. 7. The kinetics is, therefore, consistent with the interpretation that the observed effects of AMP at high concentrations are attributable to its interaction with E-P. This does not exclude the possibility of the presence of another nucleotidase, but such a contaminant could not account for Fig. 7.

**DISCUSSION**

The kinetic data are consistent with the basic kinetic model, expanded to include Equations 7 and 8 which we had proposed on the basis of our stereochemical experiments (3). This pathway appears to be the simplest one that is consistent with the observation of retention of stereochemical configuration in the transferase reaction (3), the isolation of a covalently bonded [32P]phosphoenzyme, and the steady state rate data reported in this paper. This pathway has not been given consideration by other investigators of nucleoside phosphotransferase from other sources (1, 2), but it cannot be excluded on the basis of available information. The enzymes from other sources may be found to be mechanistically similar to the barley phosphotransferase. We suspect that the failure to detect significant concentrations of phosphorylated carrot phosphotransferase (15) may have been due to rapid turnover at 37 °C, such that levels of the intermediate dropped quickly to relatively low steady state levels. Even at 0–4 °C, it takes...
only a few seconds for the barley enzyme to drop from an initial 60% labeling with \[^{32}P\]AMP to only 10 or 15%.

In view of the inherent nucleotidase activity associated with nucleoside phosphotransferase, it is pertinent to consider whether its primary biological function is as a 5'-nucleotidase or as a phosphotransferase. Although this question cannot at present be answered with certainty, two properties of the enzyme lend support for the latter function. First, the strong preference for purine deoxynucleoside acceptors (Table I) contrasts with the lack of specificity exhibited by most acid phosphatases. It is interesting that a distinct uridine/cytidine kinase has been characterized in seeds (16), given the fact that these two nucleosides are among the poorest substrates for the barley phosphotransferase. Second, its mechanism of action must differ from at least one hydrolase, namely venom 5'-nucleotidase, if the kinetic pathway proposed here is correct. This is because the stereochemical course of transferase action by nucleoside phosphotransferase involves retention of configuration (3), whereas that of nucleotidase action by venom 5'-nucleotidase involves inversion (17). It will be of interest to determine whether the stereochemistry of nucleotidase action by nucleoside phosphotransferase confirms the proposed catalytic pathway.

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Additional references will be found on p. 4939.
RESULTS

Am

1.8

A

1.0

0.6

0.2

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Purification and Kinetics of Nucleoside Phosphotransferase

EXPERIMENTAL PROCEDURE

Materials. All nucleoside, nucleotide, and sugar phosphates were purchased from Sigma Chemical Co. (St. Louis, MO). The nucleosides were obtained from the manufacturer's specified batches: 2′,3′-cyclic [4 (C15:0)] and 3′-cyclic [4 (C15:0)]. 2′,3′-cyclic [4 (C15:0)] and 3′-cyclic [4 (C15:0)] were purchased from E. Merck (Darmstadt, Germany). 3′-cyclic [4 (C15:0)] and 3′-cyclic [4 (C15:0)] were obtained from Merck, Pharmacia Fine Chemicals, 5′-adenosine 5′-triphosphate (ATP), and 5′-adenosine 5′-triphosphate (AMP) were purchased from New England Nuclear (Boston, MA). 5′-adenosine 5′-triphosphate (ADP) was purchased from Boehringer Mannheim (Mannheim, Germany).

All reagents were of analytical grade. The following reagents were utilized in the paper electrophoresis: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the gel electrophoresis: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the PAGE: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate.

Physico-chemical analyses were performed on the following reagents: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the PAGE: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the PAGE: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the PAGE: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the PAGE: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate. The following reagents were utilized in the PAGE: 8% acrylamide, 10% acrylamide, 3.5% methylene bisacrylamide, 0.5% SDS, and 0.05% ammonium persulfate.
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Figure 9. Optimization of phosphotransferase and phosphodiesterase activities with active particle band during differential electrophoresis in gel containing various concentrations of polyacrylamide. Phosphatase activity was determined as described in the Experimental Procedure, and for phosphodiesterase activity was carried out under Table 1, below.

Another estimate of the molecular weight was obtained by gel filtration (111). Both the nucleoside phosphotransferase and phosphodiesterase activities emerged in the same elution volume, with an estimated molecular weight of 45,000-50,000.

We found that some preparations which appeared homogeneous by disc electrophoresis failed to yield a single band of a major protein (m.w. = 25,700) and a small band of about 50,000 mol. wt. in 2.5% polyacrylamide gels. The recent discovery that the enzyme produces a positive periodic-acid-Schiff stain for carbohydrates suggests that the active site may be glycosidically linked to the enzyme molecule.

The rate constants for the phosphorylation of the enzyme at various pH values are shown in Table 10. Although both functions exhibit broad maxima, that of transphosphorylase, is at pH 7.4, is about one unit higher than that of the phosphatase. The relative efficiency of phosphate transfer to nucleoside (relative to total turnover of the reaction) is optimum above pH 6.0 and approaches 50% for both activities fall off sharply below pH 5.0.

Figure 10. Effects of pH on phosphotransferase and phosphodiesterase activities with 0.01 M Tris-KOH, (pH 7.0) and 0.01 M citrate (pH 6.0) buffer, with a constant concentration of 0.1 M NaCl. The pH optima for the phosphatase are 7.0, 7.2, 6.0, and 6.0, respectively, and for the phosphodiesterase are 6.8, 7.0, and 6.8, respectively. The effects of temperature and ionic strength on these two activities are shown in Figure 11. The optimum temperature for both activities is 37°C. Phosphatase activity is fairly constant over a wide range of ionic strength (0.01-0.1 M NaCl).

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