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ENZYMES OF THYMIDINE AND URIDINE PHOSPHORYLATION IN HIGHER PLANTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in The Ohio State University

By

Quey-ing F. Deng

* * * *

The Ohio State University
1973

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ACKNOWLEDGMENTS

The author wishes to express her sincerest appreciation to Dr. D. H. Ives, not only for his excellent guidance and efforts but also for his personal enthusiasm and stimulus, as the thesis adviser.

This investigation was conducted with financial support from N. I. H. grant No. CA-06913, and from the Department of Biochemistry, The Ohio State University.

Finally, she is very grateful to her husband, Zai-foo, for his patience and understanding during her graduate studies.
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Apparent Thymidine Kinase and True Uridine Kinase of Seedlings ".

FIELDS OF STUDY

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Studies in Enzyme Kinetics. Professor T. I. Diamondstone

Studies in Nucleic Acids. Professor D. H. Ives

Studies in Genetics. Professor V. L. House
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>1. Thymidine Kinase</td>
<td></td>
</tr>
<tr>
<td>2. Uridine Kinase</td>
<td></td>
</tr>
<tr>
<td>3. Nucleoside Phosphotransferase</td>
<td></td>
</tr>
<tr>
<td>EXPERIMENTAL PROCEDURE</td>
<td>20</td>
</tr>
<tr>
<td>1. Materials</td>
<td></td>
</tr>
<tr>
<td>2. Methods</td>
<td></td>
</tr>
<tr>
<td>A. Germination of seeds</td>
<td></td>
</tr>
<tr>
<td>B. Enzyme preparation</td>
<td></td>
</tr>
<tr>
<td>C. Enzyme assays</td>
<td></td>
</tr>
<tr>
<td>D. Protein determination</td>
<td></td>
</tr>
<tr>
<td>E. DNA extraction and determination</td>
<td></td>
</tr>
<tr>
<td>F. Identification of ATP phosphate moiety transferred to thymidine</td>
<td></td>
</tr>
<tr>
<td>G. Sepharose gel filtration</td>
<td></td>
</tr>
<tr>
<td>H. DEAE cellulose chromatography</td>
<td></td>
</tr>
<tr>
<td>RESULTS</td>
<td>34</td>
</tr>
<tr>
<td>PART I: THYMIDINE PHOSPHORYLATION MECHANISM IN BARLEY SEEDLINGS</td>
<td></td>
</tr>
<tr>
<td>1. General Survey</td>
<td>34</td>
</tr>
<tr>
<td>2. Ammonium Sulfate Fractionation of Barley Extract to Components P and T</td>
<td>37</td>
</tr>
<tr>
<td>3. Properties of P and T Fractions</td>
<td>39</td>
</tr>
</tbody>
</table>

iv
4. Nucleoside Acceptor Specificity of T Fraction 41
5. Phosphate Donor Specificity of Fraction T toward Thymidine Phosphorylation 41
6. Effects of Mg++ and EDTA on the Thymidine Phosphorylation Activity of Fraction T 44
7. Isotopic Analysis of Phosphate Transfer 48
8. The Incorporation of 3H-thymidine into DNA of Barley Seedling Roots 50

PART II: PARTIAL PURIFICATION AND PROPERTIES OF URIDINE KINASE FROM CORN SEEDS
1. Time Course Studies of Thymidine and Uridine Phosphorylating Activities during Germination 51
2. Evidence That the Early Appearing Uridine Phosphorylating Enzyme Is a True Uridine Kinase 54
3. Isolation and Partial Purification of Uridine Kinase from Corn Seeds 58
4. Properties of the Partially Purified Uridine Kinase from Corn Seed
   A. General assay
   B. Contaminating enzyme activity
   C. ATP and Mg++ optima
   D. pH optimum
   E. Nucleoside acceptor specificity
   F. Metal ion requirement
   G. Phosphate donor specificity
   H. Sulfhydryl dependence
   I. Effect of "protective agents" on uridine kinase activity at elevated temperature
5. Kinetics of Uridine Kinase
   A. Variable nucleosides at saturating ATP-Mg^{2+}
   B. Variable phosphate donor, ATP-Mg^{2+}, at subsaturating uridine concentration
   C. The reaction mechanism of uridine kinase

DISCUSSION 123

BIBLIOGRAPHY 131
# LIST OF TABLES

Table | Page
--- | ---
1. Paper chromatography solvent systems for testing the purity, or for purification, of radioactive nucleosides or deoxy-nucleosides | 22
2. Paper chromatography and paper electrophoresis methods—separation of nucleotides and nucleosides commonly used in this study. | 23
3. Relative levels of thymidine and deoxycytidine phosphorylating activities in several plant seedling extracts | 35
4. Effects of dTTP and AMP on thymidine phosphorylating activity | 36
5. Effect of nucleotides on thymidine phosphorylation by barley extract | 38
6. Superadditive effect of combined $(\text{NH}_4)_2\text{SO}_4$ fractions on apparent kinase activity | 40
7. ATPase activity of P and T components | 40
8. Nucleoside specificity of nucleoside phosphotransferase from barley seedlings | 44
9. Phosphate donor specificity toward thymidine phosphorylation of fraction T | 45
10. Effects of Mg++ and EDTA on the thymidine phosphorylating activity of fraction T | 45
11. Identification of phosphate moiety transferred to thymidine from ATP | 49
12. Dependence of the phosphate donor specificity toward nucleoside phosphorylation on age of corn seedlings | 55
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>13. Effects of Mg++ and EDTA on uridine phosphorylation by the 24-hr</td>
<td>55</td>
</tr>
<tr>
<td>corn extract</td>
<td></td>
</tr>
<tr>
<td>14. The effect of nucleotides on uridine kinase activity of corn</td>
<td>57</td>
</tr>
<tr>
<td>seed</td>
<td></td>
</tr>
<tr>
<td>15. Methods of extracting uridine kinase from corn seed</td>
<td>59</td>
</tr>
<tr>
<td>16. Summary of purification of uridine kinase from corn seed</td>
<td>59</td>
</tr>
<tr>
<td>17. Phosphate acceptor specificity of uridine kinase from corn seed</td>
<td>78</td>
</tr>
<tr>
<td>18. Nucleoside acceptor specificity of uridine kinase during</td>
<td>80</td>
</tr>
<tr>
<td>purification</td>
<td></td>
</tr>
<tr>
<td>19. Divalent cation specificity of uridine kinase from corn seed</td>
<td>83</td>
</tr>
<tr>
<td>20. Phosphate donor specificity of uridine kinase</td>
<td>84</td>
</tr>
<tr>
<td>21. Effect of sulfhydryl-specific reagents on uridine kinase activity</td>
<td>86</td>
</tr>
<tr>
<td>22. Several kinetic constants of uridine kinase from corn seeds</td>
<td>110</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. Time course study of ATP breakdown and dTMP formation</td>
<td>42</td>
</tr>
<tr>
<td>2. Effect of varying AMP concentration on the rate of thymidine phosphorylation</td>
<td>46</td>
</tr>
<tr>
<td>3. Thymidine and uridine phosphorylating activities during germination of corn</td>
<td>52</td>
</tr>
<tr>
<td>4. Elution profile of uridine kinase from DEAE column</td>
<td>63</td>
</tr>
<tr>
<td>5. Elution profile of uridine kinase from Sepharose 4B-200 column</td>
<td>66</td>
</tr>
<tr>
<td>6. Uridine kinase activity as a function of protein concentration</td>
<td>68</td>
</tr>
<tr>
<td>7. Time course of uridine phosphorylation at 30°C</td>
<td>71</td>
</tr>
<tr>
<td>8. ATP and Mg++ optima for uridine kinase</td>
<td>73</td>
</tr>
<tr>
<td>9. Effect of variation of the pH on the rate of uridine phosphorylation</td>
<td>76</td>
</tr>
<tr>
<td>10. Effect of uridine concentration on uridine phosphorylation; inhibition by cytidine</td>
<td>81</td>
</tr>
<tr>
<td>11. Heat inactivation of uridine kinase in the presence of &quot;protective agents&quot;</td>
<td>88</td>
</tr>
<tr>
<td>12. Effect of varying uridine concentration on the reaction rate of uridine phosphorylation, with ATP saturating</td>
<td>91</td>
</tr>
<tr>
<td>13. Effect of varying Cyd concentration on the reaction rate of Cyd phosphorylation, with ATP-Mg^2+ saturating</td>
<td>93</td>
</tr>
</tbody>
</table>
14. Effect of varying Urd concentration on the reaction rate, with ATP-Mg$^{2-}$-saturating; inhibition by UTP.

15. Effect of varying ATP-Mg$^{2-}$ concentration on the reaction rate, with Urd concentration nonsaturating; inhibition by UTP.

16. Effect of varying ATP-Mg$^{2-}$ concentration on the reaction rate, with Urd nonsaturating; inhibition by CTP.

17. The effect of CTP on uridine phosphorylation.

18. Effect of varying ATP-Mg$^{2-}$ concentration on the reaction rate of uridine phosphorylation, activation by GTP; inhibition by CTP and reversal of CTP inhibition by GTP.

19. Effect of varying GTP-Mg$^{2-}$ on the reaction rate of uridine phosphorylation.

20. Effect of varying uridine and ATP-Mg$^{2-}$ concentrations on the rate of uridine kinase.

21. Effect of varying ATP-Mg$^{2-}$ and Urd concentrations on the reaction rate of uridine kinase.

22. Effect of varying ATP-Mg$^{2-}$ concentration on the reaction rate, with Urd concentration nonsaturating; inhibition by UMP.

23. Effect of varying Urd concentration on the reaction rate, with ATP-Mg$^{2-}$ concentration nonsaturating; inhibition by UMP.

24. Effect of varying ATP-Mg$^{2-}$ concentration on the reaction rate, with Urd concentration nonsaturating; inhibition by ADP.

25. Effect of varying Urd concentration on the reaction rate, with ATP-Mg$^{2-}$ concentration nonsaturating; inhibition by ADP.
ABBREVIATIONS

The abbreviations used are:

A  Adenine
C  Cytosine
G  Guanine
T  Thymine
U  Uracil
Ado  Adenosine
dAdo  Deoxyadenosine
Cyd  Cytidine
dCyd  Deoxycytidine
araCyd  1-β-D-arabinofuranosyl cytosine
Guo  Guanosine
dGuo  Deoxyguanosine
riboThd  5-methyl-uridine
dThd  Deoxycytidylde
Urd  uridine
AMP  Adenosine-5'-monophosphate
dAMP  Deoxyadenosine-5'-monophosphate
ADP  Adenosine-5'-diphosphate
ATP  Adenosine-5'-triphosphate
dATP  Deoxyadenosine-5'-triphosphate
CMP  Cytidine-5'-monophosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>Cytidine-5'-monophosphate</td>
</tr>
<tr>
<td>dCMP</td>
<td>Deoxycytidine-5'-monophosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>Cytidine-5'-diphosphate</td>
</tr>
<tr>
<td>dGDP</td>
<td>Deoxycytidine-5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Cytidine-5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxycytidine-5'-triphosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine-5'-monophosphate</td>
</tr>
<tr>
<td>dGMP</td>
<td>Deoxyguanosine-5'-monophosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>dGDP</td>
<td>Deoxyguanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine-5'-triphosphate</td>
</tr>
<tr>
<td>dTMP</td>
<td>Thymidine-5'-monophosphate</td>
</tr>
<tr>
<td>dTDP</td>
<td>Thymidine-5'-diphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Thymidine-5'-triphosphate</td>
</tr>
<tr>
<td>UMP</td>
<td>Uridine-5'-monophosphate</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine-5'-diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine-5'-triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>Deoxyuridine-5'-triphosphate</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>pMB</td>
<td>p-mercuribenzoate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
</tbody>
</table>
INTRODUCTION

It has been well known for many years that thymidine is a precursor of DNA, and that the pathway for its incorporation into DNA involves the phosphorylation of this nucleoside first to deoxythymidine-5'-monophosphate and then to the deoxynucleoside triphosphate stage (1-4). In many instances, this first phosphorylation step is carried out by an enzyme, called thymidine kinase, which catalyzes the transfer of the γ-phosphate group of ATP to thymidine to form the product, thymidine-5'-monophosphate (5-7). However, in some organisms, such phosphorylation is carried out by another type of enzyme, called nucleoside phosphotransferase, which performs the nonspecific phosphorylation of nucleosides by reversible transfer of the phosphate from low energy phosphate donors, such as nucleoside monophosphates, to nucleosides (8-13).

Thymidine kinase has been relatively more extensively studied in bacteria (5,14) and in animal systems (6,15). One of its most interesting properties is the strong inhibition of the enzyme by its end product, dTTP (5-7).

An enzyme able to phosphorylate thymidine with ATP has been reported in several crude plant seedling extracts (16-18). However, the identity of the products formed has never been checked.
In addition, the assay methods commonly used are based on the conversion of radioactive thymidine to dTMP, followed by the separation of product from reactant by paper chromatography. In crude extracts, which contain higher levels of ATPase and phosphatase activities, and are capable of degrading ATP to AMP, this method may fail to identify the true phosphate donor, ATP or AMP, and therefore fails to distinguish between nucleoside kinase and phosphotransferase. In the case of the higher plants, this has led to particular confusion. In addition, in contrast to animal and bacterial systems, no regulatory mechanism for this enzyme in plants has yet been studied or reported.

Wanka et al (16) reported that thymidine kinase from germinating corn and wheat seedlings could be fractionated into two components, $P$ and $T$, by $(NH_4)_2SO_4$ precipitation. Neither the $P$ nor the $T$ component alone was active toward thymidine phosphorylation, but the phosphorylation activity was restored by combining them.

The above factors prompted the author to reinvestigate in detail the mechanism of thymidine phosphorylation in plants.

An investigation of the mechanism of thymidine phosphorylation during the first part of this work revealed that in plants thymidine is phosphorylated essentially by the combined actions of the ATP hydrolyzing enzyme and nucleoside phosphotransferase, catalyzing the
synthesis of nucleotides by transferring the phosphate moiety from nucleotides to nucleosides nonspecifically. Uridine was supposed to be phosphorylated in a similar way. However, several investigators reported the appearance of a uridine phosphorylating activity in seedlings a day or two before thymidine phosphorylation could be detected (19, 20). In this way, the "early" enzyme at least showed some degree of specificity in that it could only phosphorylate uridine and not thymidine. An attempt was then made to determine if this early enzyme is a true uridine kinase. Several preliminary experiments showed it was indeed a true uridine kinase, resembling those found in animal tissues (21, 22, 23) and protozoa (24). Since it is the first nucleoside kinase in plants showing control by the end product, the second part of this work was initiated, with the aim of partially purifying this enzyme and comparing its properties and kinetic characteristics with those of the uridine kinase found in other organisms.
Following the development of molecular biology in the 1950s, a
great number of investigations have been carried out on the synthesis
of DNA precursors. It is now generally known that in addition to the
de novo pathways of nucleotide synthesis, cells of most
organisms also synthesize nucleotides from purine and pyrimidine
bases and nucleosides, by the "salvage pathway" (25-28). Through
this pathway, exogenously supplied purine and pyrimidine bases and
nucleosides can be converted to nucleotides, and those bases or nucleo-
sides arising from breakdown of DNA and RNA, or from newly synthesized
nucleotides, can be reutilized. Thus, the cells in an organism can
synthesize DNA or RNA more economically by salvaging material from
dead cells that would otherwise have been discarded. Enzymes of this
"salvage pathway", include (29):
1) mononucleotide pyrophosphorylases
2) nucleoside phosphorylases
3) nucleoside or deoxynucleoside kinases
4) nucleoside phosphotransferases
5) trans-N-deoxyribosylases

These enzymes generally appear to operate at minimal levels in
cells that are not proliferating rapidly, but increase to significant
activity in rapidly growing tissues under conditions of increased demand
for nucleotide synthesis (27, 30, 31).
Among them, only thymidine kinase, uridine kinase, and nucleotide phosphotransferase will be reviewed briefly here.

**Thymidine Kinase**

Thymidine was first demonstrated to be a precursor of DNA by Reichard and Estborn (1) in studies in the rat and by Friedkin, Tilson, and Roberts (2) in experiments with chicken embryos. It soon became clear from the cell-free synthesis of DNA done by Kornberg et al that the pathway of its incorporation into DNA involved the phosphorylation of this nucleoside first to 5'-dTMP by an enzyme called thymidine kinase, utilizing ATP as phosphate donor, then to the triphosphate stage (3, 4).

Since then, thymidine kinase has been studied in many organisms. It has been partially purified from animal tumors (36), highly purified from *E. coli* B. by Okazaki et al (5) and recently purified to homogeneity by affinity chromatography on agarose columns by Rohde et al (37), also using extracts of *E. coli* B.

The enzyme is generally not very stable in solution and decays with a half-life of about 30 min. at 38°C; however, it can be stabilized by the presence of thymidine (32). By contrast, the supposed thymidine kinase from plants has been shown to be heat stable up to 60°C (17, 33). Thymidine kinase requires ATP and Mg²⁺ for activity and has a pH optimum around 8.0. The enzyme activity is sensitive to reagents
which attack the thiol moiety. The nucleoside specificity is quite limited, irrespective of the tissue of origin. Deoxyuridine and 5-substituted derivatives including thymidine and the halogenated derivatives are all substrates for the enzyme but other deoxynucleosides or ribonucleosides are not phosphorylated to any significant extent (32). However, the enzyme reported in plants shows much broader specificity. It is able to phosphorylate Urd, dUrd, Cyd and dCyd in addition to Thd (33-35).

Many other properties of this enzyme vary from source to source and will be mentioned categorically below.

a) Molecular weight and molecular forms

The molecular weight is greater than $10^5$ (36, 38), and the enzyme exists in a number of different states of aggregation. The enzyme from mammalian cells, for example, occurs in an aggregated form in dilute solution and dissociates into six subunits of molecular weight of $1.1 \times 10^5$ when the ionic strength of the solution is increased (15). In the *E. coli* system, the enzyme can be converted to a dimer form in the presence of feedback inhibitor (dTTP) or activators (dCDP, dATP, dGTP, etc.). The molecular weights of the enzyme monomer and dimer were estimated by gel filtration to be approximately 42,000 and 89,000 to 91,000, respectively. Good correlation has been found between the extent of activation or inhibition and the increase in the sedimentation rate found at various concentration of activators or inhibitors (14, 39). These results indicate that dimerization
is an essential change involved in both inhibitory and activating regulation of this enzyme by deoxynucleotides.

Okazaki et al further studied the effect of temperature on this enzyme and found that the enzyme is extremely temperature sensitive in the monomeric form and exists mostly in a less active state even at moderate temperature, but the dimer is temperature insensitive (40).

Bresnick et al compared the properties of Thd kinase partially purified from regenerating rat liver and E.coli and confirmed the finding of Okazaki et al that the sedimentation constant of the E.coli enzyme was markedly influenced by the presence of dTTP but that of regenerating rat liver enzyme stayed constant in the presence of dTTP (41).

In plants, partially purified thymidine phosphorylating activity from germinating corn and wheat seedlings has been separated by ammonium sulfate fractionation into two components, P and T, both enzymically inactive, but the activity is restored after recombination of the two components from the same or different sources (16). No significant difference in size exists between component P and component T, both being slightly smaller than bovine serum albumin (33).

b) Regulatory properties by nucleotides

The end product of a synthetic pathway is often found to inhibit
the activity of the initial enzyme in the sequence of enzymatic reactions, thus regulating its own synthesis (42). Thymidine kinase has been found to be one of these enzymes. It is subject to end product inhibition by dTTP in mammalian tissues (6, 7, 44, 45), in chick embryo (31), and in E.coli (5, 39). Nevertheless, in Tetrahymena pyriformis there is some evidence from experiments with crude extracts that thymidine kinase is not subject to feedback inhibition (46). In plants, no end product inhibition properties of this enzyme have yet been reported.

In addition to dTTP inhibition, the E.coli enzyme is markedly activated by dCDP and dCTP (5, 39) whereas the activity of the enzyme from mammalian sources (36) was not affected by these nucleotides. Furthermore, in both bacterial and mammalian systems, ATP serves in a dual capacity, as an activator and as a phosphorylating agent (14, 15, 39).

The kinetics of inhibition of thymidine kinase by dTTP have been extensively studied and differ in different organisms. In Novikoff hepatoma cells, Ives et al reported that dTTP is competitive with both dThd and ATP (6). Similar observations have been reported by Breitman (7). Bresnick and Thompson studied the Walker tumor cells and obtained a somewhat complicated result. Both forms of thymidine kinase, aggregated and disaggregated forms, are subject to dTTP inhibition, but the disaggregated form is more sensitive to the end pro-
duct inhibition. The different degrees of inhibition of the aggregated and the disaggregated enzymes are also reflected in the type of inhibition which is manifested, i.e., the inhibition by dTTP is simple competitive with respect to thymidine in the presence of the disaggregated form but it is noncompetitive in the case of the aggregated form (36).

In E.coli the inhibition by dTTP is competitive with the phosphate acceptor, dThd, but is noncompetitive with the phosphate donor, ATP, or with the activator, dCDP. This may suggest that dTTP binds to the same catalytic site as dThd, but from the fact that dTDP and dTMP or other dThd derivatives have no inhibitory effect, it seems more likely that the inhibitor site occupied by dTTP is not identical with that of dThd but rather overlaps it or competes with it sterically because of its proximity (14).

c) Occurrence of thymidine kinase activity

It has often been suggested that thymidine kinase is important in the control of DNA synthesis in various cells because increased activity of this enzyme is associated with rapid growth and increased rates of DNA synthesis.

Its activity is greatly enhanced in regenerating rat liver (47-51), in liver of rats on a high-protein diet (52) and in rapidly
proliferating tissue in general (47, 49, 53).

In addition, a periodic change in thymidine kinase activity has been observed in synchronously dividing cells of a slime mold (58, 59), sea urchin embryos (60), and mouse fibroblasts (61). In all these cells, the maximum kinase activity is reached by the end of mitosis and coincides with the onset of DNA replication.

The nature of such an increase in thymidine kinase activity has been studied by many investigators by means of inhibitors of protein and RNA synthesis, such as actinomycin D, 8-azaguanine, cycloheximide, puromycin, and chloramphenicol, all of which were capable of preventing the elevated thymidine kinase activity (51, 54, 55, 56). Therefore, it has been concluded that the increase in thymidine kinase activity is due to new protein synthesis and is not the result of a change in the turnover rate of enzyme protein (57).

Uridine Kinase

The occurrence of an enzyme which catalyzes the phosphorylation of uridine with ATP was first demonstrated in extracts from mammalian liver (62) and Ehrlich ascites tumor (63), and later in extracts from a variety of different tissues (21, 24, 30, 64, 65).

This enzyme has been partially purified from Ehrlich and Novikoff ascites tumor (22, 23), relatively more extensively purified from *Tetrahymena pyriformis* GL cells (24) and from the
obligate thermophile, *Bacillus stearothermophilus* (65).

The properties of this enzyme have been studied in various organisms. Like thymidine kinase, it shows an absolute requirements for ATP and $\text{Mg}^{2+}$ and a broad pH optimum ranging from 7.4 to 8.5. It has a broad phosphate donor specificity in that almost all of the physiological nucleoside triphosphates except CTP and UTP can serve as phosphate donors, but it exhibits strict stereospecific demands for its phosphate acceptor. While uridine and cytidine can be readily phosphorylated (24), none of the following compounds, 2'-deoxyuridine, 2'-deoxycytidine, 5'-methyluridine, 5'-fluoro-2'-deoxyuridine can serve as a phosphate donor. From the fact that the phosphorylation ratio of uridine to cytidine remains constant throughout the purification, although this ratio may vary from tissue to tissue, ranging from 1.14 to 2, and the fact that the two substrates are mutually competitive in kinetic studies, it has been suggested that the phosphorylation of both pyrimidine ribonucleosides is catalyzed by a single enzyme (22 - 24).

The molecular weight of this enzyme has been determined to be 195,000 in *Tetrahymena pyriformis* GL cells. In the rat liver system, two species of uridine kinase, with molecular weights of approximately 120,000 (I) and 30,000 (II) have been obtained (66). Species I predominates in the 7 day postnatal and adult rat liver while species II is the predominant form in the foetal rat liver and accounts for
40% of the total activity in the rapidly growing Novikoff ascites hepatoma.

In addition to the above mentioned properties, uridine kinase attracted great attention by being feedback-inhibited by the end products UTP and CTP, and many investigators believe that this enzyme catalyzes a rate-limiting step in the anabolism of uridine to the successive nucleotide derivatives. The terminal products, UTP and CTP have been shown to serve as potent and specific inhibitors to this enzyme from tumor cells (21), sea urchin embryos (64), human lymphocytes (67), Tetrahymena (24), and several bacterial strains (21, 65). Again, no such instance has been demonstrated in a plant system. The inhibition by CTP can be partially reversed by high concentrations of either uridine or ATP (21, 23, 24) or by GTP or dGTP (64). However, the possible modes of action of the modifiers cannot be presumed until more kinetic experiments have been done.

Several experiments seem to indicate that uridine kinase is a sulfhydryl enzyme in many organisms. The enzyme in Tetrahymena lost activity gradually during cold storage but could be reactivated by addition of dithiothreitol or mercaptoethanol (24). The enzyme from Ehrlich ascites tumors was inhibited by p-chloromercuribenzoate but was protected by glutathione (22). In mouse leukemic cells, the treatment with p-chloromercuribenzoate resulted in an enhanced feedback inhibition by CTP possibly due to conformational
or structural changes of the enzyme induced by p-chloromercuribenzoate (68). The uridine kinase partially purified from mouse leukemic cells made resistant to 5-azacytidine in vivo was found to be considerably more stable toward heating and p-chloromercuribenzoate and showed a smaller degree of CTP inhibition than in 5-azacytidine-sensitive cells.

Although there are reports of an enzyme capable of phosphorylating uridine in several plant extracts (18, 20, 33) very little study has been given to the properties of this enzyme in plants. Wanka et al (33, 35) found there were two species of uridine phosphorylating enzyme existing during germination of Zea mays, of which only one is active in vitro during the initial hours of germination. The early-appearing enzyme is less heat stable and of higher molecular weight than the later one (33). They also presented some evidence that the second enzyme is identical with thymidine kinase, consisting of two dissociable components and that the enzyme exhibits broad nucleoside specificity, being capable of phosphorylating nearly all physiological nucleosides. The gel filtration data on Sephadex further revealed that the molecular sizes of the two components are the same as of the components of thymidine kinase (33).

The emergence of a uridine phosphorylating enzyme before thymidine phosphorylation takes place has also been shown in germinating peanut seedlings (20).
Phosphotransferase

About two decades ago, Brawerman and Chargaff first discovered in malt extracts a type of enzyme capable of transferring organically esterified phosphoric acid to nucleosides, thereby effecting the synthesis of nucleotides by a low energy phosphate donor (69). For such a type of enzyme the authors proposed the general designation "nucleoside phosphotransferase". Since then, similar enzymes have been found in a variety of organisms including rat liver, human prostate (70), higher plants (71), various bacteria (11, 72), chick embryo (8), fowl leukemic myoblasts (12), and human placenta (73).

Nucleoside phosphotransferase has been most extensively studied in Chargaff's laboratory. Sixteen years after they first attempted to purify this enzyme from carrot, they were finally able to purify it to homogeneity (98). Some of the properties of this enzyme have been studied in various organisms and will be summarized below:

a) Nature of the acceptor:

The transferase found in most organisms has a phosphate acceptor specificity strictly limited to nucleosides; even D-ribose can not be phosphorylated. The specificity toward nucleoside acceptors is very broad but may vary from organism to organism to a slight extent. Generally speaking, in carrots, 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. As to the sugar moiety, 3'-deoxyadenosine is more readily phosphorylated than 2'-deoxynucleosides or adenosine (10). In chick embryo, the deoxyribose-containing substrates are better acceptors than ribose-containing ones, with TMP as phosphate donor (8), and the pyrimidine deoxynucleosides seems to be better acceptors than purine deoxynucleoside. However, such specificity differences between organisms probably have little significance because the difference in pH optima (5.2 in carrots, and 9.0 in chick embryo) and the different phosphate donors used would both have influenced the specificity toward the acceptor.

Moreover, in addition to utilizing the common nucleosides as the phosphate acceptors, the partially purified carrot enzyme has been demonstrated to be able to transfer the phosphate to several unusual nucleosides, such as the ribosides of purine, 2,6-diaminopurine, 5-bromouracil, and the riboside or arabinoside of adenine to form the corresponding 5'-nucleotides. It was also able to phosphorylate cyclic or 2'-adenosine nucleotides to form adenosine 2',5'-diphosphate and cyclic adenosine (2',3')-5'-diphosphate. The latter could be further hydrolyzed to 2',5' and 3',5' - diphosphates of adenosine (74.).

The transferase found in E.coli was capable of a low energy transfer of organically bound phosphate to nucleoside 5'-phosphates
and deoxyribonucleoside-5'-triphosphate in addition to nucleosides. Because 5'-nucleotides are converted in high yields to the nucleoside 3',5' or 2',5'-diphosphates, the enzyme was also regarded as a nucleotide phosphotransferase (72).

b) Nature of phosphate donor

Nucleoside phosphotransferase seems to be able to transfer the phosphate nonspecifically from all nucleotide monophosphates. Sugar phosphates, such as glucose-1-phosphate, glucose-6-phosphate, 3-glycerophosphate and ribose-5-phosphate can not serve as donors.

For carrot enzyme, phenylphosphate is the preferred donor, better than all nucleoside monophosphates tested (70). However, in chick embryo, the preferred donors of phosphate to the pyrimidine deoxyribonucleosides appear to be the pyrimidine deoxyribonucleotides (8). In lower organisms, protozoa as well as bacteria, phenylphosphate and 5'-inosinic acid are equally effective donors (70). Again this specificity difference in different organisms may not be significant since in crude extracts this difference may due to extraneous factors such as phosphatase action on the donors used or the newly formed nucleotide rather than true difference in specificity.

Maley et al further established the specific group transferred by utilizing selective sugar labeling experiments which demonstrated that the transfer reaction merely involved a transfer of phosphate.
from the phosphate donor and not the entire sugar phosphate moiety (66).

c) Nature of the product formed:

It is usually position 5' of the nucleoside sugar that accepts enzymically transferred phosphoric acid in the presence of enzyme of plant origin, whereas with preparations from mammalian tissues and microorganisms varying amounts of the 2'- and 3'-nucleotides are also produced in addition to 5'-nucleotides (10). The human prostate phosphotransferase showed somewhat unusual properties in that large amount of 2'- and 3'-nucleotides were formed (71).

d) Catalytic properties and characterization of catalytic sites:

Since the enzymes first isolated from various organisms were all able to show both phosphate-transfer activity and phosphatase or nucleotidase activity, there has been great perplexity for quite a while as to the catalytic nature of this enzyme, as to whether only one enzyme takes part in both hydrolytic and transfer reactions or if two separable enzymes are involved in the two types of reaction. If it is one bifunctional enzyme, then it is actually a nucleotidase with transferase function or is a transferase capable of acting as a hydrolase under suitable condition.

Since in crude extracts many factors would complicate the experiment, a definite answer would be hard to obtain unless one could
succeed in purifying the enzyme. The carrot enzyme was extensively purified and finally purified to homogeneity by Brunngraber and Chargaff (10, 98). Several lines of experimental evidence from the pure enzyme suggested that with one protein this enzyme could perform both transfer and hydrolytic functions. The enzyme should be regarded as a nucleoside phosphotransferase capable of acting as a hydrolase under suitable conditions, rather than as an acid phosphatase capable of phosphate transfer, on the grounds that phosphate transfer is favored over hydrolysis; transfer occurs specifically to nucleosides, and high yields of nucleotides are recorded (10, 98).

From the kinetic studies, the nucleoside phosphotransferase of carrot is considered an enzyme possessing two hydrolytic sites binding the phosphate donor, and one transfer site engaging the nucleoside acceptor (98).

More properties of the homogeneous enzyme have been described recently (98, 75). The enzyme has a molecular weight of 44,000 with two subunits of identical size. The two subunits after treatment with urea could be separated by electrofocusing and showed substantial differences in their amino acid composition. The amino acid analysis of the protein showed it contained a single residue of histidine and no tyrosine. The amino terminus has been identified as aspartic acid. Glycine and serine probably represented the carboxyl terminus.
The isoelectric point is 4.1. The Michaelis constants for the transfer reaction have been determined with phenylphosphate as the donor, \( K_m = 3.5 \) mM and uridine as the acceptor, \( K_m = 3.5 \) mM.

The biological significance of this enzyme is not entirely certain. However, the wide distribution of this enzyme, its occurrence in higher plants, bacteria, protozoa, and vertebrates as well as its marked increase in activity in germinating wheat and regenerating muscle (71) strongly suggest that this enzyme activity is one of the attributes of growing cells, and it must have some part in the biosynthesis of nucleotides.
EXPERIMENTAL PROCEDURE

1. Materials

Barley, corn, and wheat seeds were provided from stock maintained by the Ohio State University, Department of Agronomy. Peanuts were obtained from the Krema Company, Columbus, Ohio, broad beans (Vicia faba) were purchased from W. Attee Burpee and Mung beans (Phaseolus aureus) were a gift from Dr. G. A. Barber. The remaining seed was purchased from the Livingston seed company, Columbus, Ohio. None of the seed used had been treated with mercurial fungicides.

ATP-8-\(^{14}\)C (2 mCi/mole), \(3^\text{H}-\text{dThd}\) (20 Ci/mole), \(3^\text{H}-\text{dCyd}\) (8.15 Ci/mole), \(3^\text{H}-\text{dGuo}\) (1.8 Ci/mole), \(3^\text{H}-\text{dAdo}\) (19.2 Ci/mole), \(\text{\(^{14}\)}\text{C-dUrd}\) (23.2 mCi/mole), \(\text{\(^{14}\)}\text{C-5-meUrd}\) (20 mCi/mole), \(3^\text{H}-\text{ATP}\) (14.1 Ci/mole), \(3^\text{H}-\text{Cyd}\) (17 Ci/mole), \(3^\text{H}-\text{araCyd}\) (8.9 Ci/mole), \(\text{\(^{32}\)}\text{P-ATP}\) (5.9 Ci/mole), \(3^\text{H}-\text{CTP}\) (28.4 Ci/mole), and \(\text{\(^{33}\)}\text{P}_{\text{O}}\text{~}\text{4}\) (carrier free) were purchased from New England Nuclear Corp., \(3^\text{H}-\text{Urd}\) (28 Ci/mole), \(3^\text{H}-\text{Guo}\) (23.4 Ci/mole) were purchased from International Chemical & Nuclear Corp., \(\gamma-\text{\(^{33}\)}\text{P-ATP}\) was synthesized by the method of Glynn and Chappel (76). \(3^\text{H}-\text{UMP}\) was prepared by the enzymatic phosphorylation of \(3^\text{H}-\text{Urd}\) with uridine kinase partially purified from corn seed, and then purified by paper chromatography. Unlabeled nucleosides and nucleotides were obtained from P-L biochemicals, Sigma Chemical Company and CalBiochem.
The purities of radioactive compounds were checked periodically, and then repurified by paper chromatography through one or two different solvent systems if they proved to be less than 95% pure (Table 1 & Table 2).

Unlabeled nucleoside and nucleotide solutions were made up by dissolving in water, neutralizing with NaOH where necessary and measuring concentrations spectrophotometrically (81). The purity of nucleotide phosphates used was checked occasionally by anion-exchange thin layer chromatography on PEI-cellulose which had been successively prewashed with 10% NaCl and deionized water and kept in the dark in the refrigerator until use (82).

Tris, N-ethylmaleimide, β-mercaptoethanol, calcium phosphate gel, imidazole, EDTA, lysine, 3-phosphoglycerate kinase (type IV, 1600 units/mg protein), glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle, 95 units/mg protein), 3-phosphoglycerate, RNase B (from bovine pancreas, type III-B, 100 Kunitz units/mg protein), Sepharose 4B-200, Sepharose 6B-100, and L-cysteine were all obtained from the Sigma Chemical Company. DEAE cellulose (DE 23), DEAE-cellulose paper (DE 81) and Amerlite ion-exchange resin loaded paper sheets (grade SB-2) were purchased from Reeve Angel Company. Streptomycin sulfate, p-mercuribenzoate, and dithiothreitol were obtained from the Calbiochem Company. Triton-X100, RNase B (550,000 units/mg protein), and crystalline BSA were purchased from Rohm and
TABLE 1

PAPER CHROMATOGRAPHY SOLVENT SYSTEMS FOR TESTING THE PURITY OR FOR
PURIFICATION OF RADIOACTIVE NUCLEOSIDES OR DEOXYNUCLEOSIDES

After application of the compounds to Whiteman #1 paper, the
chromatograms were developed by descending chromatography for 8-12
hrs. In case of purification, solvent was generally allowed to run off
the end of the paper to get greater separation.

<table>
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<th>#3</th>
<th>#4</th>
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<td>dGuo</td>
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<td>36</td>
<td>24</td>
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</table>

#1 upper phase from EtOAc : H₂O : HCOOH (60 : 35 : 5).
#2 t-butanol : MEK : H₂O : NH₄OH (4 : 3 : 2 : 1). (77)
#3 isopropyl alcohol : H₂O : conc. HCl (65 : 18.4 : 16.6).
#4 n-butanol : formic acid : H₂O (77 : 10 : 13). (85)
### TABLE 2

PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS METHODS FOR SEPARATION OF NUCLEOSIDES AND NUCLEOTIDES COMMONLY USED IN THIS STUDY

<table>
<thead>
<tr>
<th>COMPOUNDS</th>
<th>METHOD (A) (Rf)</th>
<th>METHOD (B) (Rf)</th>
<th>METHOD (C) (Rp)</th>
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<td>SOLVENT #1</td>
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<td>SOLVENT #3</td>
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<td>ATP</td>
<td>0.21</td>
<td>0.02</td>
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<td>ADP</td>
<td>0.59</td>
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<tr>
<td>AMP</td>
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<td>0.26</td>
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<tr>
<td>Ado</td>
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<td>0.62</td>
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<td>dTTP</td>
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<td>0.29</td>
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<tr>
<td>UMP</td>
<td></td>
<td>0.35</td>
<td>0.51</td>
</tr>
<tr>
<td>Urd</td>
<td></td>
<td>0.61</td>
<td>0.86</td>
</tr>
<tr>
<td>dUMP</td>
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<td>Cyd</td>
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</tr>
<tr>
<td>P</td>
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</tr>
<tr>
<td>Piric Acid</td>
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</table>

<sup>a</sup> Piric acid as standard

<sup>b</sup> Ado moved toward the cathode, because it was positively charged at pH 3.7.

**METHOD (A):** Compounds were spotted on 1" strips of Whatman DE-81 (DEAE-cellulose anion-exchange) paper prewashed with 1 M formic acid and developed for about 3.5 hrs until the solvent front reached the bottom of the strip.
Solvent #1 = 4N formic acid-0.1N ammonium formate (6)
Solvent #2 = 0.1M ammonium formate pH 4.0 (78)

METHOD (B): Compounds were spotted on Whatman #1 paper strip prewashed with 1M formic acid and deionized water, and developed for about 20 hrs.
Solvent #3 = 1M ammonium acetate (pH 7.5) : 95% alcohol (3:7). (79)

METHOD (C): Compounds were spotted on strips of Schleicher and Schuell Orange Ribbon 589 paper, and the electrophoresis was carried out in 0.037 M ammonium formate buffer (pH 3.7) at 4000 V. for 40 min. (80)

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Haas, Miles Laboratories, and Pentex, respectively.

2. Methods

A) Germination of Seeds

The seeds were sprouted by scattering them on a fine screen kept moist by a continuous mist of tap water controlled at room temperature. They were allowed to germinate in the dark for various periods of time.

B) Enzyme preparations

a) Preparation of enzyme extracts from germinating seedlings:

After germinating for the desired period of time, the seedling roots were excised and ground, with a pestle in a chilled mortar, in 3 vol. of 0.2 M sodium-potassium phosphate buffer, pH 7.0, for 5 min. Larger quantities were homogenized in a blender for 30 sec. Homogenates
were centrifuged at 27,000 g for 20 min, and the supernatant fractions were quick-frozen and stored until needed.

b) Preparation of uridine kinase extract from corn seed:

Corn seed was washed thoroughly with deionized water and dried at room temperature with a fan. It was then ground to a powder with a Wiley mill (Arthur H. Thomas Co.) using 2 mm mesh screen.

Acetone-dried powder was then prepared by first blending the corn powder with 5 volumes of chilled acetone in a 3,8 liter Waring blender for 30 seconds at high speed. The resulting slurry was filtered through a Büchner funnel and washed with an excess of chilled acetone until the filtrate was colorless, and the residue was spread out on filter paper and allowed to dry at room temperature.

The dried acetone powder can be stored in an air-tight jar at -20°C for several months without losing enzyme activity.

Enzyme extract from acetone-dried powder was simply prepared by first blending with 4 volumes of cold 0.2 M sodium potassium phosphate buffer (pH 7.0) in a Waring blender for 30 seconds at high speed. The homogenate, after passing through double layers of cheese cloth, was then centrifuged at 23,000 g for 30 minutes. The supernatant was fast-frozen and kept at -60°C until needed.

C) Enzyme Assays

a) Thymidine Kinase and Phosphotransferase:

The standard assay mixture consisted of the following components:

\[ ^3H-dThd \] (or other tritiated or \[^{14}C\]-nucleosides or deoxynucleo-
sides) (60-125 mCi/m mole), 0.02 mM ATP (AMP), 5 mM MgCl₂, 2.5 mM; Phosphate buffer, 0.1 M (pH 7.0) and an amount of enzyme which will give less than 25% conversion of the substrates to the products during 30 min. of incubation time at 37°C. The reactions were terminated by immersing the incubation tubes in boiling water for 2 min. After cooling the tubes in ice, 2 volumes of H₂O were added to each reaction tube and the tubes were centrifuged briefly to collect the condensate and sediment the denatured protein. The radioactive nucleotides formed were determined by the ion-exchange disc method of Ives et al. (83) by spotting 50 µl of the supernatant solution on 17 mm SB-2 paper discs and washing away the unreacted nucleosides or deoxynucleosides, while the newly formed radioactive nucleotides or deoxy-nucleotides remained on the disc.

The discs were then dropped into 20 ml-scintillation vials and eluted with 1 ml HCl/KCl (0.1 M/0.2 M) solution by shaking in a divided paper vial box tied on top of a Laboratory Rotator (Model G-2, New Brunswick Scientific Company) 300 rpm for 20 min. Then 10 ml of modified Triton-toluene scintillation solvent (84) (two parts toluene, one part Triton-X100, 0.5% PPO and 0.05% bis-MSB), were added to each vial; the vials were tightly capped and shaken for another 30 min. After this, the emulsion was generally completely clear and the discs were fully permeated with solvent and the vials were ready for counting.
A Packard Model 3320 was used during most of the counting process. To correct for any irreversible adsorption of unreacted substrate, a disc spotted with unreacted assay mixture was washed along with the other discs, counted, and this count was subtracted as part of the background. A disc spotted with the same volume of the reaction mixture without washing was also counted along with other samples. Thus,

\[
\% \text{ conversion} = \frac{\text{cpm in sample} - \text{cpm washed control}}{\text{cpm unwashed control}} \times 100
\]

b) ATPase assay:

The final concentrations of the components in the reaction mixture were; \(^{14}\text{C}-\text{ATP} \ (200 \mu\text{Ci/mole}) \) or \(^{3}\text{H}-\text{ATP} \ (310 \mu\text{Ci/mole})\), 5 mM MgCl\(_2\), 5 mM phosphate buffer, pH 7.0, 0.1 M; and enzyme preparations. The incubation was carried out at 37°C for 30 min. or for another specific time period. After the reaction was stopped by boiling 2 min. and the denatured protein was centrifuged down, the supernatant together with cold standards were applied to Whatman DE-81 strips pre-washed with 1 M formic acid and developed by descending chromatography in the 4N formic acid-0.1 M ammonium formate solvent system (Table 2). The products were located by UV absorption and by a radiochromatogram scanner. The substrate and the product areas were cut out of the strips, cut into 1 cm segments, placed into scintillation vials and eluted with 1 ml of 0.1 M HCl- 0.2 M KCl solution. Ten ml of the scintillation solvent mentioned above was added to each vial and the samples were counted in a liquid scintillation counter.
c) Cytidine deaminase assay:

The reaction mixture contained the following components: ATP, 5 mM; MgCl₂, 5 mM; Tris-HCl, 0.1 M (pH 8.0 at 4°C); ³H-Cyd, 0.02 mM (250 mCi/mM); and enzyme preparations. After incubation at 30°C for 30 min., the reaction was stopped by boiling. The supernatant of the centrifuged reaction mixture, together with standard uridine and cytidine, were spotted on DE-81 strips and developed in the 0.03 N formic acid solvent system (29). The areas corresponding to Urd and Cyd were cut out and counted for the radioactivity as mentioned above.

d) Nucleotide monophosphatase assay:

The assay mixture contained: ³H-UMP, 1 mM (10 mCi/m mole); MgCl₂, 5 mM; Tris-HCl, 0.1 M (pH 8.0 at 4°C) and enzyme solution. The incubation was carried out at 30°C for 30 min. and the product was separated similarly from the reactant by DEAE ion-exchange paper chromatography using 0.1 M ammonium formate (pH 4.0) as the solvent system (Table 2). The radioactivity in the areas corresponding to UMP and Urd were located by UV absorption and counted as in b).

e) Uridine phosphorylase assay:

The final concentrations of the components in the reaction mixture were: ³H-Urd, 0.02 mM (125 mCi/m mole); MgCl₂, 5 mM; phosphate buffer, 50 mM (pH 7.0) and enzyme preparation. After incubation at 30°C for 30 min., the reaction was stopped and the supernatant from
the centrifuged reaction mixture was spotted on Whatman #1 paper and
developed by descending chromatography with the n-Butanol: Formic acid : H₂O (77: 10 : 13) solvent system (Table 1). The areas corresponding
to uridine and uracil were cut out and counted for radioactivity as
in b).

f) Uridine monophosphate kinase assay:

The assay mixture contained the following components: ³H-UMP,
0.02 mM (10 mCi/mmole); ATP, 10 mM; MgCl₂, 10 mM; Tris-HCl, 0.1 M (pH 8.0
at 4°C) and enzyme solution. It was incubated at 37°C for 30 min.
After the reaction was stopped by boiling and centrifuged to remove
the denatured protein, the supernatant solution, together with some un-
labeled UTP, UDP, UMP and Urd, was spotted on Whatman #1 strips and developed
in the 1 M ammonium acetate (pH 7.5) : 95% alcohol (3 : 7) solvent
system for 19 hrs. The areas corresponding to UTP, UDP, UMP and Urd
as located by UV absorption, were cut out and the radioactivity in each
area was counted as mentioned in b).

g) Uridine kinase and cytidine kinase assay:

The assay technique is basically the same as that used for the
thymidine kinase assay. In most cases, the following standard assay
conditions were used, employing duplicate assay tubes from which duplic-
cate discs were spotted. Eppendorf and Oxford microliter pipettes were
utilized wherever possible. The final concentrations of reagents in
the general assay were: ATP, 5 mM; MgCl₂, 2.5 mM; phosphate buffer,
0.1 M (pH 7.0); \textsuperscript{3}H-Urd, 0.02 mM (250 mCi/mmole). However, for most assays associated with the purification procedures and kinetic characterization studies, the following final concentrations of reagents were: ATP, 5 mM; MgCl\textsubscript{2}, 5 mM; Tris-HCl, 50 mM (pH 8.0 at 4°C); \textsuperscript{3}H-Urd, 0.02 mM (250 mCi/mmole), or \textsuperscript{3}H-Cyd, 0.02 mM(250 mCi/mmole) and dithiothreitol, 10 mM. The incubations were carried out at 30°C for 20 min. Enzyme concentrations were adjusted to give not more than 20% conversion of substrate to product during the incubation. The subsequent assay procedures were performed as described in a).

D) Protein Determination

The determinations of protein concentration were carried out by the method of Lowry et al (86). However, due to the high content of phenolic compounds in plants, and other interfering substances such as NH\textsubscript{4}+, β-ME, DTT in some enzyme solutions, protein was generally first precipitated with an equal volume of 10% trichloroacetic acid and washed twice with 10% TCA to remove interfering substances. The pellets were taken up in 0.1 M NaOH before following Lowry's procedure. Bovine serum albumin was used for making a standard curve.

E) DNA Extraction and Determination

DNA and RNA fractions were extracted mainly by Holdgate and Goodwin's modification of the Schmidt-Thannhauser method (87). DNA was determined by Burton's modification of the diphenylamine assay (88) and the base composition of barley embryo DNA was used to
predict the weight of DNA from the reactive purine-bound sugar (89). A standard curve was constructed with various concentrations of dAMP.

F) Identification of ATP Phosphate Moiety Transferred to Thymidine

In these experiments $^3$H-thymidine was phosphorylated by ATP labeled either in the $\alpha$- or the $\gamma$-phosphate group. The doubly labeled dTMP was purified first by descending chromatography on acid washed Whatman 3 MM paper developed with 1 M ammonium acetate (pH 7.5)-95% ethanol (30:70, v/v) (79). The dTMP was located by UV absorption and the radiochromatogram scanner, eluted with water and concentrated under a vacuum. To remove possible orthophosphate contamination the concentrate was spotted on a strip of Schleicher and Schuell Orange Ribbon 589, and electrophoresis was carried out in 0.037 M ammonium formate buffer (pH 3.7) at 4000 V for 40 min. (80). The radioactive region was cut out, dropped into a scintillation vial and eluted with 1 ml of solution containing 0.1 M HCl and 0.2 M KCl. Using standard double label counting techniques with a Packard Model 3320 scintillation spectrometer, the relative amount of $^3$H and $^{32}$P or $^{33}$P were determined. From the known specific activities of each precursor, it was possible to calculate the amount of dTMP formed using both the $^3$H data and the radiophosphorus data and to compare the results. Since both labels appeared in a single sample, quantitative recovery of dTMP was not essential.

G) Sepharose Gel Filtration
Sepharose Gel 6B-100 or 4B-200 was already in the swollen state in 0.02% sodium azide when purchased. The gel was washed in fresh buffer (50 mM Tris-HCl, pH 8.0 at 4°C - 10 mM β-mercaptoethanol -20% glycerol) to remove the azide, deaerated under reduced pressure and further equilibrated in the buffer overnight at 4°C. It was then packed on a 2.5 cm x 50 cm glass column fitted at the lower end with a fritted glass disc followed the standard packing technique described in "Gel Chromatography" (90). Although the hydrostatic pressure was not supposed to be a limiting factor, according to the manufacturer's manual, it has been my experience that the hydrostatic pressure used in packing and elution had better not exceed 20 cm of water if good flow rates are to be obtained. From an attached reservoir, the column was generally washed with a total of 3 bed volumes of equilibrating buffer before use. The protein sample was applied to the column using a standard layering method in which sufficient glycerol was added to make the sample more dense than the buffer before layering it on the column. After the sample settled into the gel, it was eluted at a flow rate of 15 ml per hour, and fractions of 6 ml were collected using a Research Specialties Company fraction collector fitted with an automatic drop counter.

H) DEAE cellulose Chromatography

Whatman DE 23 cellulose was thoroughly washed on a Büchner funnel through an alkali- acid - alkali cycle (91), after each run. After the last alkaline wash, the adsorbent was rinsed with distilled water
until the filtrate was free of alkali. Then the filter cake was resuspended in 2 or 3 volumes of the selected starting buffer and titrated with a relatively concentrated solution of the acidic component of the buffer until the pH of the suspension reached the desired pH. The adsorbent was then washed with starting buffer again and hydraulically sized if fine particles were present. It was then degassed under reduced pressure and equilibrated at 4°C overnight before packing. The column was washed with starting buffer again until fully equilibrated.
RESULTS

PART I: THYMIDINE PHOSPHORYLATION MECHANISM IN BARLEY SEEDLINGS

1. General Survey

Root extracts from higher plant seedlings of a number of species were prepared and assayed for their thymidine and deoxycytidine phosphorylation activities. As shown in Table 3, such activities were found in every plant tested. While in each plant seedling extract the levels of thymidine and deoxycytidine phosphorylation activities were almost equal, the phosphorylation activities varied widely among different seedlings. The specific activities of barley and wheat were more than 100 times greater than the least active lima beans. In most systems dTTP stimulated the phosphorylation of thymidine (Table 4), contrary to what had been shown in animal tumor and bacterial systems where dTTP serves as an potent feedback inhibitor of thymidine phosphorylation (5, 6). When AMP was added to the reaction mixture to replace ATP as a phosphate donor, thymidine was phosphorylated at least to the same extent as with ATP and in some cases AMP was a much better phosphate donor than ATP (Table 4).

Several nucleotides and deoxynucleotides were also added to the assay mixture to test their effects on thymidine phosphorylation in barley extract which has the highest level of thymidine phosphorylation activity. The results in Table 5 show that all nucleotides and deoxynucleotides had a promoting effect on thymidine phosphorylation.
TABLE 3

RELATIVE LEVELS OF THYMIDINE AND DEOXYCYTIDINE PHOSPHORYLATING ACTIVITY IN SEVERAL PLANT SEEDLING EXTRACTS

Preparation of extracts and enzyme assays were carried out as described in Methods, except that \(^{3}H\)-dCyd (125 mCi/mmole), 0.02mM replaced \(^{3}H\)-dThd in the right-handed column. ATP (5 mM) was the phosphate donor.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>dThd phosphorylation</th>
<th>dCyd phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley (var Harrison)</td>
<td>34.1</td>
<td>35.1</td>
</tr>
<tr>
<td>Wheat</td>
<td>17.1</td>
<td>18.2</td>
</tr>
<tr>
<td>Corn</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Bean (Vicia faba)</td>
<td>3.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Peanut (Spanish)</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Pea (extra Earley Alaska)</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Peanut (Redskin)</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Mustard</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Mung bean (Phaseolus aureus)</td>
<td>0.16</td>
<td>0.2</td>
</tr>
<tr>
<td>Carrot</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Bean (Bush Lima)</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>
TABLE 4

EFFECTS OF dTTP AND AMP ON THYMIDINE PHOSPHORYLATING ACTIVITY

Preparations of extracts and enzyme assays were carried out as described in Materials and Methods, except that nucleotides were added or substituted as shown (ATP and AMP were 5 mM, and dTTP was 1 mM).

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Specific activity (nmoles dTNP per mg protein per 30 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>Barley (var Harrison)</td>
<td>34.1</td>
</tr>
<tr>
<td>Wheat</td>
<td>17.1</td>
</tr>
<tr>
<td>Corn</td>
<td>6.2</td>
</tr>
<tr>
<td>Bean (Vicia faba)</td>
<td>3.3</td>
</tr>
<tr>
<td>Peanut (Spanish)</td>
<td>1.5</td>
</tr>
<tr>
<td>Pea (extra Early Alaska)</td>
<td>1.5</td>
</tr>
<tr>
<td>Peanut (Redskin)</td>
<td>1.2</td>
</tr>
<tr>
<td>Mustard</td>
<td>0.6</td>
</tr>
<tr>
<td>Mung bean (Phaseolus aureus)</td>
<td>0.16</td>
</tr>
<tr>
<td>Carrot</td>
<td>0.09</td>
</tr>
<tr>
<td>Bean (Bush Lima)</td>
<td>0.06</td>
</tr>
</tbody>
</table>
nucleotides tested stimulated thymidine phosphorylation, but in inverse relation to the number of phosphate groups on the nucleotides; i.e. nucleoside monophosphates were the most stimulatory. Taken together, these results suggested that the enzyme phosphorylating thymidine here is a nucleoside phosphotransferase rather than a true kinase.

2. Ammonium Sulfate Fractionation of Barley Extract to Components P and T

With the possibility that a phosphotransferase rather than a true kinase had been dealt with, the \((\text{NH}_4)_2\text{SO}_4\) fractionation experiments of Wanka et al. (16) were therefore repeated with barley extract, which had the most active thymidine phosphorylation activity of the plants tested. The fractionation procedures were primarily the same as those of Wanka et al. Following their parlance, component T represents the protein fraction precipitating between 0 and 40\% \((\text{NH}_4)_2\text{SO}_4\) saturation, and component P represents that precipitating between 60 and 90\% \((\text{NH}_4)_2\text{SO}_4\) saturation. The apparent thymidine kinase activity was assayed in the P and T fractions independently and in the combined fractions. As shown in Table 6, when using ATP as the phosphate donor, neither fraction by itself was very active toward thymidine phosphorylation. However, when fractions P and T were combined the activity for thymidine phosphorylation was stimulated many fold. When the activity of phosphotransferase was assayed, however, component T was found to be capable of phosphorylating thymidine rapidly by utilizing
### TABLE 5
**EFFECT OF NUCLEOTIDES ON THYMIDINE PHOSPHORYLATION BY BARLEY EXTRACT**

Assays were carried out as described in Methods, except the ATP used was 0.5 mM, and all nucleotides added were 2.5 mM.

<table>
<thead>
<tr>
<th>Nucleotides added</th>
<th>dThd phosphorylated, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>8.8</td>
</tr>
<tr>
<td>dTTP</td>
<td>13.7</td>
</tr>
<tr>
<td>dTDP</td>
<td>17.0</td>
</tr>
<tr>
<td>dTMP</td>
<td>26.7</td>
</tr>
<tr>
<td>dGTP</td>
<td>14.9</td>
</tr>
<tr>
<td>dCDP</td>
<td>22.7</td>
</tr>
<tr>
<td>dCMP</td>
<td>30.9</td>
</tr>
<tr>
<td>CDP</td>
<td>17.4</td>
</tr>
<tr>
<td>dATP</td>
<td>25.1</td>
</tr>
<tr>
<td>ADP</td>
<td>24.4</td>
</tr>
<tr>
<td>dGTP</td>
<td>16.8</td>
</tr>
<tr>
<td>GTP</td>
<td>14.6</td>
</tr>
</tbody>
</table>

* in the absence of any nucleotides other than ATP.
AMP as the phosphate donor in the absence of ATP (Table 6).

3. Properties of P and T Fractions

In order to arrive at an interpretation of the recombination experiment, and to find out what role each of the components play in the overall mechanism of thymidine phosphorylation, more experiments were carried out.

Starting with the facts that component T could phosphorylate thymidine in the presence of AMP but not ATP, but that when component P was added along with component T thymidine could be readily phosphorylated in the presence of ATP, it was not hard to imagine that component P must be somehow capable of generating AMP from ATP. Components P and T were therefore assayed for their ATPase activity by using (8)-$^{14}$C-ATP as the substrate. The result in Table 7 showed that fraction P had a very high level of ATPase activity which could hydrolyze 98% of the ATP in 30 min. or less, with 94% being degraded all the way to nucleoside or base. By contrast fraction T showed only slight ATP hydrolyzing capacity; it hydrolyzed only 15% of the ATP in the standard assay mixture after 30 min. of incubation, and then mostly only as far as ADP.

In addition, a time-course study of ATP breakdown and dTMP formation was carried out in parallel experiments with either $^{14}$C-ATP or $^3$H-dThd
TABLE 6

SUPERADDITIVE EFFECT OF COMBINED \((\text{NH}_4)_2\text{SO}_4\) FRACTIONS ON APPARENT KINASE ACTIVITY

Conditions were as described in Methods, except that AMP replaced ATP where stated.

<table>
<thead>
<tr>
<th>Enzyme fractions</th>
<th>n moles thymidine phosphorylated/ mg protein/30 min.</th>
<th>Apparent kinase activity(with ATP)</th>
<th>Nucleoside phosphotransferase activity(with AMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P (100 µg)</td>
<td>0.32</td>
<td>0.12*</td>
<td></td>
</tr>
<tr>
<td>T (80 µg)</td>
<td>0.12</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>P (50 µg) + T (40 µg)</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* More dilute samples of P were shown to contain substantial levels of phosphotransferase which otherwise tended to be masked by the very active nucleotidase which hydrolyzes the product, dTMP.

TABLE 7

ATPase ACTIVITY OF P AND T COMPONENTS

Assays were carried out as described in Methods. The reaction proceeded for 30 min. at 37°C.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>(^{14}\text{C-ATP})</th>
<th>(^{14}\text{C-ADP})</th>
<th>(^{14}\text{C-AMP})</th>
<th>(^{14}\text{C-Ado} + ^{14}\text{C-A})</th>
</tr>
</thead>
<tbody>
<tr>
<td>P (100 µg)</td>
<td>2.5</td>
<td>1.5</td>
<td>2.2</td>
<td>93.5</td>
</tr>
<tr>
<td>T (80 µg)</td>
<td>85.2</td>
<td>12.2</td>
<td>0.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>
in each experiment. The breakdown of ATP was followed by means of 
$^{14}$C-ATP and DEAE ion exchange paper chromatography of the products with 
the 4M formic acid-0.1 M ammonium formate system, as described in 
Methods. dTMP formation was followed by the disc assay method as 
described previously, using $^3$H-dThd as the substrate. The result in 
Fig. 1 showed that ATP was degraded very rapidly to ADP and then to 
AMP and adenosine. After 15 min. when most of the ATP was degraded to 
AMP, with less than 10% of the original ATP left, there was still 
quite a high rate of dTMP formation. This seems to suggest that 
AMP is the phosphate donor. However, the events of the initial 5 
min. are ambiguous, since it is unclear whether or not dTMP accumulation 
parallels the lag in AMP formation. This could probably have been 
resolved either by inserting more time points before 5 min, or by the 
use of a more dilute enzyme preparation. After 30 minutes, phosphatase 
oreactions are seen to proceed faster than phosphorylation reactions.

4. Nucleoside Acceptor Specificity of T Fraction

Fraction T is rather nonspecific concerning its nucleoside 
acceptor specificity. It does not seem to be able to distinguish 
between ribo- and deoxyribo-nucleosides or between purine or 
pyrimidine nucleosides (Table 8). In this respect it resembles a 
phosphotransferase rather than a kinase.

5. Phosphate Donor Specificity of Fraction T on Thymidine Phosphorylation

Component T is also equally nonselective with respect to phosphate
Fig. 1. TIME COURSE STUDY OF ATP BREAKDOWN AND TMP FORMATION

The experimental conditions were as described in Text and Methods, except a 2.5 mM final concentration of ATP, in a total reaction volume of 80 µl was used here, and 20 µg of Fraction P was used.

•—•, $^{14}$C-ATP; ■—■, ADP; ▲—▲, $^{14}$C-AMP; ○—○, $^3$H-TMP.
TABLE 8

NUCLEOSIDE SPECIFICITY OF NUCLEOSIDE PHOSPHOTRANFERASE FROM BARLEY SEEDLINGS

Conditions were as described in Table 3, except that tritiated or ¹⁴C-nucleosides (0.02 mM, 60-125 mCi/m mole) were substituted for thymidine as shown, and 80 μg of fraction T was used, in a final reaction volume of 100 μl.

<table>
<thead>
<tr>
<th>Acceptor nucleoside</th>
<th>Nucleotide formed / 30 min. ( nmoles / mg protein )</th>
</tr>
</thead>
<tbody>
<tr>
<td>dThd</td>
<td>4.65</td>
</tr>
<tr>
<td>dCyd</td>
<td>4.56</td>
</tr>
<tr>
<td>dGuo</td>
<td>5.50</td>
</tr>
<tr>
<td>dAdo</td>
<td>2.80</td>
</tr>
<tr>
<td>Urd</td>
<td>4.50</td>
</tr>
<tr>
<td>5-methyl-Urd</td>
<td>3.81</td>
</tr>
<tr>
<td>Ado</td>
<td>6.78</td>
</tr>
</tbody>
</table>

donors, as shown in Table 9. Of these donors, AMP was found to have an apparent Km of about 0.54 mM (Fig. 2).

6. Effects of Mg++ and EDTA on the Thymidine Phosphorylating Activity of Fraction T

It has been found that thymidine kinase in many organisms has either an absolute requirement for Mg++ or that the activity is greatly stimulated by Mg++ (5, 6). However, nucleoside phosphotransferase
**TABLE 9**

**PHOSPHATE DONOR SPECIFICITY TOWARD THYMIDINE PHOSPHORYLATION**

Conditions were as in Table 3, except that various nucleoside monophosphates replaced ATP, and 80 µg of fraction T was used in a final volume of 100 µl.

<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>Nucleotide formed (nmoles/mg protein/30 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>6.30</td>
</tr>
<tr>
<td>CMP</td>
<td>3.30</td>
</tr>
<tr>
<td>UMP</td>
<td>4.70</td>
</tr>
<tr>
<td>dTMP</td>
<td>5.4</td>
</tr>
<tr>
<td>dCMP</td>
<td>6.4</td>
</tr>
<tr>
<td>dGMP</td>
<td>7.0</td>
</tr>
<tr>
<td>dUMP</td>
<td>5.2</td>
</tr>
<tr>
<td>dAMP</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**TABLE 10**

**EFFECTS OF Mg++ AND EDTA ON THE THYMIDINE PHOSPHORYLATING ACTIVITY OF FRACTION T**

Conditions were the same as in Table 3, except MgCl₂ was omitted unless otherwise stated, and EDTA was added when stated. 80 µg of the dialyzed fraction T was used.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.7</td>
</tr>
<tr>
<td>Control + 2 mM EDTA</td>
<td>23.4</td>
</tr>
<tr>
<td>Control + 2.5 mM MgCl₂</td>
<td>25.0</td>
</tr>
</tbody>
</table>
Fig. 2  EFFECT OF VARYING AMP CONCENTRATION ON THE RATE OF THYMIDINE PHOSPHORYLATION

Experimental conditions were as described in Methods, except the AMP concentration was varied as shown. The phosphate acceptor was $^3$H-dThd (125 mCi/mmole), 0.02 mM.
has been reported to be completely lacking in the metal requirement 
(8, 10, 11, 12). As to the metal requirement of fraction T toward 
thymidine phosphorylation, Table 10 shows that the addition of 2.5 
mM MgCl₂ had absolutely no effect on the activity of the dialyzed 
enzyme. The enzyme was still fully active in the presence of 2 mM 
EDTA. This provides additional evidence that fraction T is actually 
a phosphotransferase.

7. Isotopic Analysis of Phosphate Transfer

From the above data the role of fractions T and P in thymidine 
phosphorylation seemed to be clear. However, there was still a 
possibility that true kinase activity might be masked, especially in 
fraction P, since when diluted, it showed some thymidine phosphorylation 
activity. In order to distinguish between, or to estimate the relative 
amounts of true kinase and phosphotransferase activities, the following 
approach was chosen: ATP was labeled either in the terminal or ester 
phosphate moiety, and the relative contributions of these groups to 
the product, dTMP, was observed. So that it would be possible to 
determine accurately the quantity of product formed, the thymidine 
also was labeled with tritium. The experimental design is illustrated 
in the diagram which depicts ATP labeled with $^{32}$P in the $\alpha$-phosphate 
and $^{33}$P in the $\gamma$-phosphate. (In the actual experiments ATP was 
labeled in only one position or the other, not in both simultaneously.)
Ado-\(^{32}\text{P} - \text{P} - {^{33}\text{P}}\) + dT* \xrightarrow{kinease} \(\frac{1}{2}\) \(\text{dT}^{32}\text{P}\) + ADP

\(3^{2}\text{P-AMP} + \text{dT}^* \xrightarrow{\text{Nucleoside phosphotransferase}} 3\text{dT}^{32}\text{P}\) + Ado

The results (Table 11) clearly show that the \(\alpha\)-phosphate of ATP is the sole phosphate donor for thymidine phosphorylation under these experimental conditions. None of the \(\gamma\)-phosphate appeared in the dTMP and it may be concluded that the mechanism of thymidine phosphorylation here is by Reactions 2 and 3, a combination of ATP degradation and nucleoside phosphotransferase activities.

### Table 11

**Identification of phosphate moiety transferred to thymidine from ATP**

| Reactions conditions: \(^3\text{H-thymidine}, 0.025 \text{mM (366 mCi/mole)}; \text{MgCl}_2, 2.5 \text{mM}; \text{Tris-HCl}, 65 \text{mM} (\text{pH 7.0, at 25°C}); [\(\gamma\text{-}{^{33}\text{P}}\)]\text{ATP, 3.92 mM (1 mCi/mole)}, or [\(\alpha\text{-}{^{32}\text{P}}\)]\text{ATP, 3.92 mM (3.1 mCi/mole)}; and 50 \text{µg of fraction P of barley in a final total volume of 0.4 ml. The method of data analysis was described in Methods.}|

<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>Radioactive dTMP formed, n moles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^3\text{H-dTMP})</td>
</tr>
<tr>
<td>Expt 1 ATP-(\gamma\text{-}{^{33}\text{P}})</td>
<td>1.44</td>
</tr>
<tr>
<td>Expt 2 ATP-(\alpha\text{-}{^{32}\text{P}})</td>
<td>1.30</td>
</tr>
</tbody>
</table>
8. The Incorporation of $^3$H-thymidine into DNA of Barley Seedling Roots

Excised roots of barley seedlings (germinated 48 hrs.) were immersed in $^3$H-thymidine (10 µCi/ml, 6.7 Ci/m mole) for 2 hrs. at room temperature and shaken gently. The roots were then rinsed and the DNA extracted according to the procedure described in Methods. The DNA concentration was then estimated by Burton's method (88), and the radioactivity incorporated into DNA was counted in the scintillation counter. The DNA was found to have a specific activity of 540 dpm/µg while the RNA fraction was negligibly labeled.
PART II: PARTIAL PURIFICATION AND PROPERTIES OF URIDINE KINASE FROM CORN SEEDS

1. Time Course Studies of Thymidine and Uridine Phosphorylation Activities During Germination

The appearance of uridine and thymidine phosphorylation activities of corn seeds during germination has been examined for periods ranging from 0 to 84 hours. As shown in Fig. 3, uridine phosphorylation activity exists even in dry seeds. The initial activity is relatively high but starts to decrease following the germinating process, decreasing to a minimum level at 36 hrs, then rising steadily during the later germination period. Unlike uridine phosphorylation, there was a very low level of thymidine phosphorylation activity up to a 36-hr. germination period. But from then on, the activity increases rapidly. This finding is essentially similar to what has been reported for germinating corn by Wanka et al. (19) and for germinating peanut axes by Schwarz et al. (20), where in each case uridine phosphorylation decreased to a minimum after two or three days of germination, although this timepoint may not be exactly the same in each case. However, the reason for the decrease is still not clear. One possibility would be that two different uridine phosphorylating enzymes appear, and the minimum activity point might correspond to the transition point where the first phosphorylating enzyme disappeared and the second enzyme just started
Fig. 3. THYMIDINE AND URIDINE PHOSPHORYLATING ACTIVITIES DURING GERMINATION OF CORN

After the germination times indicated, 20 seedlings each (or seeds in the 0- and 12-hr. germination time-points before shoots emerged) were homogenized with 10 ml of 0.2 M phosphate buffer, pH 7.0 in a pre-chilled mortar and pestle. The homogenates were then centrifuged at 27,000 g for 20 min. The supernatants were then assayed for enzyme activities as described in Methods, using ATP as the phosphate donor.

•—• uridine phosphorylation activity; ○—○, thymidine phosphorylation activity.
to emerge. Since the early-appearing enzyme could only phosphorylate uridine but not thymidine, it must show at least some sort of specificity toward its nucleoside acceptor. This early-appearing enzyme is therefore different from the nonspecific phosphotransferase which phosphorylates thymidine and uridine equally well discussed in the former part of this thesis. The next experiment concerns an attempt to determine if the early-appearing enzyme is a true uridine kinase.

2. Evidence That the Early Appearing Uridine Phosphorylating Enzyme Is a True Uridine Kinase

Firstly, the phosphate donor specificities of ATP and AMP toward thymidine and uridine phosphorylation in both early- and late-stage enzyme extracts were compared. As shown in Table 12, ATP was shown to be the preferred phosphate donor for uridine phosphorylation by extracts of 24-hr seedlings. By contrast, AMP is the better donor with 84-hr seedling extracts. Moreover, thymidine is not readily phosphorylated by the 24-hr extract, so the early enzyme is at least somewhat specific for uridine.

Secondly, the requirement for metal ion and the effect of EDTA on uridine phosphorylation by the early-stage enzyme were tested. The result obtained showed that the omission of MgCl₂ and the addition
TABLE 12

DEPENDENCE OF THE PHOSPHATE DONOR SPECIFICITY TOWARD NUCLEOSIDE PHOSPHORYLATION ON AGE OF CORN SEEDLINGS

Conditions were as described in Materials and Methods, with nucleotides and radioactive nucleosides substituted as shown, and 50 μl of extract from the roots of 24 hrs. and 84 hrs. corn seedlings, in a total volume of 100 μl.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Age of seedlings (hrs)</th>
<th>Nucleoside phosphorylated (%) by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMP</td>
</tr>
<tr>
<td>Uridine</td>
<td>24</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>84</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.3</td>
</tr>
<tr>
<td>Thymidine</td>
<td>24</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>84</td>
<td>20.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.4</td>
</tr>
</tbody>
</table>

TABLE 13

EFFECTS OF Mg++ AND EDTA ON URIDINE PHOSPHORYLATION BY THE 24-hr CORN EXTRACT

Conditions were as described in Materials and Methods, except MgCl₂ was omitted unless otherwise stated, and EDTA was added when stated, and 50 μl of the 24-hr corn extract was added in a total volume of 100 μl.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.3</td>
</tr>
<tr>
<td>Control + 1 mM EDTA</td>
<td>1.4</td>
</tr>
<tr>
<td>Control + 5 mM MgCl₂</td>
<td>18.9</td>
</tr>
</tbody>
</table>
of 1 mM EDTA inhibits uridine phosphorylation more than 90% in reaction mixtures containing ATP and the 24-hr corn extract. The residual activity is about the level of thymidine phosphorylation by the same extract. (Table 13). This might be well due to low levels of nucleoside phosphotransferase beginning to emerge at this time in the life of the seedling.

Finally, the effect of the end product, UTP, CTP and other nucleotides toward uridine phosphorylation was studied in the early-stage extract. In contrast to the total lack of end product inhibition of thymidine phosphorylation in the 4-day old barley seedling extract in the earlier part of this dissertation (Table 5), uridine and cytidine nucleotides effectively inhibited uridine phosphorylation. However, to minimize complications due to phosphatases, the uridine phosphorylating enzyme was partially purified by (NH₄)₂SO₄ precipitation and gel filtration (procedures to be described in the latter part of this dissertation) before being used in this experiment. As revealed in Table 14, this enzyme was inhibited specifically by the di- and tri-phosphates of uridine and cytidine, while the analogous deoxynucleotides are negligibly inhibitory at this concentration. GTP and dGTP stimulate uridine phosphorylation very strongly. Therefore, from the specificities both of inhibition and stimulation by nucleotides, the uridine phosphorylating activity from corn appears to resemble the
TABLE 14

THE EFFECT OF NUCLEOTIDES ON URIDINE KINASE ACTIVITY OF CORN SEED

Conditions were as described in Methods. Besides 5 mM ATP, nucleotides at 0.5 mM were added as shown. The enzyme was extracted from dry seed and partially purified. Under these conditions 9% of the \(^7\)H-uridine was phosphorylated in the control tube and was taken as 100% activity.

<table>
<thead>
<tr>
<th>Nucleotides added</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>CTP</td>
<td>25</td>
</tr>
<tr>
<td>GDP</td>
<td>29</td>
</tr>
<tr>
<td>CMP</td>
<td>83</td>
</tr>
<tr>
<td>UTP</td>
<td>61</td>
</tr>
<tr>
<td>UDP</td>
<td>54</td>
</tr>
<tr>
<td>UMP</td>
<td>103</td>
</tr>
<tr>
<td>AMP</td>
<td>100</td>
</tr>
<tr>
<td>dCTP</td>
<td>93</td>
</tr>
<tr>
<td>dUTP</td>
<td>95</td>
</tr>
<tr>
<td>dTTP</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>199</td>
</tr>
<tr>
<td>dGTP</td>
<td>179</td>
</tr>
</tbody>
</table>
uridine/cytidine kinase of animal tissues (21, 64). As a check against apparent inhibition due to radioactive substrate dilution by hydrolysis products of the nucleotides, reaction mixtures containing either $^3$H-CTP without $^3$H-uridine, or unlabeled UTP were chromatographed. The breakdown of CTP beyond CDP proved to be insignificant in both cases, so isotope dilution could not account for the observed inhibition by CTP.

3. Isolation and Partial Purification of Uridine Kinase from Corn Seed

a) Preparation of crude extract

Since dry corn seed contains a high level of uridine kinase and can easily be ground to powder by the electric mill in large quantities within a short period of time, (3kg corn seeds/2.5 hrs.), it was chosen as the enzyme source for purification purpose.

In a preliminary experiment, both direct buffer extraction and indirect buffer extraction methods, i.e., making dry acetone powder first and then extracting the enzyme from the acetone powder, have been compared. As to the buffer used for extraction, phosphate buffer and Tris-HCl buffer at pH 7.0, 4°C were compared. The highest specific activity of enzyme in the crude extract was obtained by direct extraction with phosphate buffer (Table 15). However, the crude extract obtained by this method contained some fat and gum or resin-like materials which tended to interfere with the later
TABLE 15
METHODS OF EXTRACTION OF URIDINE KINASE FROM CORN SEED POWDER

The corn powder or dry acetone powder were extracted with 4 vol. of buffer in a Waring blender as described in Methods. Homogenates were centrifuged, and the uridine kinase activity and protein concentration were determined in the supernatant extracts as described in Methods.

<table>
<thead>
<tr>
<th>Buffer used</th>
<th>Specific Activity (nmole uridine phosphorylated per mg protein/30 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Direct Extraction</strong></td>
<td></td>
</tr>
<tr>
<td>(from corn powder)</td>
<td></td>
</tr>
<tr>
<td>phosphate buffer, pH 7.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Tris-HCl, pH 7.0 at 4°C</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>Indirect Extraction</strong></td>
<td></td>
</tr>
<tr>
<td>(from acetone powder)</td>
<td></td>
</tr>
<tr>
<td>phosphate buffer, pH 7.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Tris-HCl, pH 7.0 at 4°C</td>
<td>1.6</td>
</tr>
</tbody>
</table>

ammonium sulfate fractionation step. So in the standard isolation procedure, acetone powder was always prepared before extraction with phosphate buffer (Fraction I). The detailed procedures for acetone powder preparation and extraction were described in Methods.

b) Purification

Preliminary investigation of conditions suitable for the purification of uridine kinase from corn seed led to the following conclusion:

i) Since the enzyme is sulfdryl dependent, a sulfhydryl protecting agent should be present in the buffer at all time.

ii) The nucleic acid content is very high in the crude extract. Vari-
ous nucleic acid-removing agents have been tried to precipitate the nucleic acid from the crude extract. Streptomycin sulfate was found to give better results than protamine sulfate and MnCl$_2$ in terms of recovery and nucleic acid removal.

iii) Ammonium sulfate precipitation provided an excellent initial purification step.

iv) The enzyme loses most of its activity when desalted through a Bio-gel P-6 column, but the presence of either 1 mM (NH$_4$)$_2$SO$_4$ or 20% glycerol in the buffer used helps to preserve most of its activity. The enzyme also loses much activity through membrane ultrafiltration, but the enzyme can be concentrated by (NH$_4$)$_2$SO$_4$ precipitation without losing activity.

v) Calcium phosphate gel adsorbed the enzyme very well in 20 mM Tris-HCl (pH 8.0 at 4°C) buffer. After washing with 1 M Tris-HCl (pH 8.0 at 4°C) buffer and 1 mM K$_2$HSO$_4$-0.2 M Tris-HCl (pH 8.0 at 4°C), the enzyme could be eluted in 0.1 M and 0.2 M phosphate buffer (pH 7.4) fraction. Although through this step, the enzyme activity was increased 4 folds, the total enzyme recovery was only 25-30%. Neither glycerol nor ethylene glycol improved enzyme recovery when added to the buffers to a final concentration of 30% and to preequilibrate the gel followed the method of Sonoda et al (92). This procedure was therefore rejected.

vi) Uridine kinase binds very firmly to DEAE cellulose. A small amount of ATP has to be present in the eluting buffer to improve the
recovery of the enzyme. The use of a continuous concentration gradient seems to give the best purification.

Uridine kinase was excluded from Bio-Gel P 300 and Sepharose 6B columns, but Sepharose 4B provided useful step for purification by removing a large amount of a high molecular weight protein and nucleic acid present in the enzyme fraction eluted from the DEAE column. However, 20% glycerol has to be present in all buffers used for equilibration and elution of the column in order to get good recovery of the enzyme.

Making use of these conclusions, an example of the purification procedure is presented. The temperature was maintained at 4°C throughout all of the purification procedures.

To the crude extract of acetone powder (Fraction I), 0.1 volume of streptomycin sulfate (titrated to pH 7.0 with 1 N NaOH) was added over a period of 10 min. with continuous stirring. After a further 20 min. of stirring, the supernatant solution (Fraction II) was collected by centrifugation at 23,000 g for 20 minutes.

A suitable amount of solid (NH₄)₂SO₄ was then slowly added to Fraction II with constant stirring to bring the final concentration to 35% saturation according to the nomograph of Di Jeso (93). The solution was stirred further in the cold for 40 min., the precipitate
was collected by centrifugation at 23,000 g for 20 min., and it was dissolved in a minimum volume of 0.05 M Tris-HCl (pH 8.0 at 4°C) - 10 mM β-ME buffer (Fraction III).

Enzyme fraction III, after being diluted ten-fold with 50 mM Tris-HCl (pH 8.0 at 4°C) - 10 mM β-ME buffer, was applied to a column (2.5 x 30 cm) of DEAE-cellulose (DE-23) equilibrated with 50 mM Tris-HCl (pH 8.0 at 4°C) - 10 mM β-ME - 1 mM (NH₄)₂SO₄ buffer. After the column had been washed with 50 ml of the equilibrating buffer, the enzyme was eluted by a linear concentration gradient of 250 ml of 1 mM (NH₄)₂SO₄ - 2.5 mM ATP - 10 mM β-ME - 50 mM Tris-HCl (pH 8.0 at 4°C) and 250 ml of 250 mM (NH₄)₂SO₄ - 2.5 mM ATP - 10 mM β-ME - 50 mM Tris-HCl (pH 8.0 at 4°C) buffers (Fig. 4). Fifteen ml fractions were collected, and those containing the highest specific activity were pooled. The enzyme was precipitated from the solution obtained by adding solid (NH₄)₂SO₄ to bring the final concentration to 45% saturation over a period of 10 min.. It was then stirred for another 40 min. The precipitate was collected by centrifugation at 27,000 g for 20 min. and then redissolved in 10 mM Tris-HCl (pH 8.0 at 4°C) - 10 mM β-ME - 50% glycerol (Fraction IV).

Five ml of Fraction IV was applied to a Sepharose 4B column (2.5 x 50 cm) preequilibrated with 50 mM Tris-HCl (pH 8.0 at 4°C) - 1 mM (NH₄)₂SO₄ - 10 mM β-ME - 20% glycerol buffer. The column was then eluted with the same buffer, and 6 ml fractions were collected. The
Fig. 4. ELUTION PROFILE OF URIDINE KINASE FROM THE DEAE COLUMN

The conditions for the elution are described in the Text.

--- absorbancy at 280 mu, — uridine kinase activity.

Assay conditions were described in Methods.
URIDINE PHOSPHORYLATED, %

FRACTION NUMBER

A 280

10  20  30  40  50
elution profile obtained is shown in Fig. 5. The fractions corresponding to the enzyme peak were pooled and concentrated by (NH$_4$)$_2$SO$_4$ precipitation as described above. The precipitate was redissolved in 10 mM Tris-HCl (pH 8.0 at 4°C) - 10 mM β-ME - 50% glycerol (Fraction V).

Fraction IV and V were stable for months when kept unfrozen at -20°C and remained unfrozen. The summary of the results of the individual purification steps is presented in Table 16.

**TABLE 16**

SUMMARY OF PURIFICATION OF URIDINE KINASE FROM CORN SEED

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>250</td>
<td>1050</td>
<td>2520</td>
<td>2.4</td>
<td>100</td>
</tr>
<tr>
<td>II. Streptomycin sulfate</td>
<td>270</td>
<td>850</td>
<td>2240</td>
<td>2.8</td>
<td>89</td>
</tr>
<tr>
<td>III. (NH$_4$)$_2$SO$_4$</td>
<td>25</td>
<td>195</td>
<td>3939</td>
<td>20.2</td>
<td>156</td>
</tr>
<tr>
<td>IV. DEAE-cellulose</td>
<td>5</td>
<td>37.5</td>
<td>2738</td>
<td>73.0</td>
<td>108</td>
</tr>
<tr>
<td>V. Sepharose 4B-200</td>
<td>4</td>
<td>9.0</td>
<td>1539</td>
<td>171.0</td>
<td>61</td>
</tr>
</tbody>
</table>

*a 1 unit of the enzyme is defined as the quantity of enzyme that catalyzes the formation of 1 nmole of UMP in 30 min. at 30°C.

4. Properties of the Partially purified Uridine Kinase from Corn Seed

a) General assay

UMP formation was proportional to the amount of Fraction V pro-
Fig. 5. ELUTION PROFILE OF URIDINE KINASE FROM SEPHAROSE 4B-200 COLUMN

The conditions for running the column are described in the Text. --- absorbancy at 280 mu, — enzymatic activity. Enzyme assay methods were described in Methods.
tein added to 100 μl of the standard assay mixture up to 4.5 μg, which catalyzed conversion of about 26% of the substrate to the product (Fig. 6).

Uridine kinase activity was also linear for at least 80 min, under standard assay conditions (Fig. 7).

b) Contaminating enzyme activity

The final preparation, Fraction V appears to be free of ATPase, uridine phosphorylases, nucleoside monophosphate kinase, nucleoside phosphotransferase and cytidine deaminase activities. There was insignificant amount of nucleoside monophosphatase contamination when assayed in the presence of less than 0.2 mM UMP, the highest concentration which is likely to be produced by the uridine kinase reaction.

c) Optimal concentrations of ATP and Mg

When ATP and Mg⁺⁺ concentrations were varied independently, optimal activity resulted when the Mg⁺⁺ to ATP ratio was approximately 1.25 if the fixed component was present in a concentration of 10 mM (Fig. 8A). This was similar to what had been found for deoxycytidine kinase in calf thymus (29). However, it was quite different from what has been reported for most other ATP and Mg⁺⁺-dependent enzymes in which the optimal ATP : Mg⁺⁺ ratios are usually the integrals 1 or 2 (94, 95, 96). If ATP and Mg⁺⁺ were varied together with a ratio of 1:1, optimum enzyme activity appeared around 10 mM. Above this
Fig. 6. URIDINE KINASE ACTIVITY AS A FUNCTION OF PROTEIN CONCENTRATION

The indicated amount of Fraction V protein was added to 100 μl of the standard assay mixture, and the uridine kinase activity was assayed in Methods.
URIDINE PHOSPHORYLATED, %

µg PROTEIN
Fig. 7. TIME COURSE OF URIDINE PHOSPHORYLATION AT 30°C

The assay conditions were as described in Methods except $^3$H-Urd (10 mCi/m mole), 0.5 mM was used. 2.2 µg of Fraction V protein was added to the reaction mixture and incubated at 30°C for the time period indicated.
Fig. 8. CONCENTRATIONS OF ATP AND Mg\(^{++}\) OPTIMUM FOR URIDINE KINASE ACTIVITY

Conditions were as described in Methods, except that the concentrations of ATP and MgCl\(_2\) were varied as indicated. 2.2 \(\mu\)g of Fraction V enzyme was used.

A. ATP and MgCl\(_2\) were varied independently. ATP optimum with MgCl\(_2\) fixed at 10 mM, •—•; MgCl\(_2\) optimum with ATP fixed at 10 mM, o—o.

B. ATP and MgCl\(_2\) varied together in a 1:1 ratio.
this concentration, the enzyme activity decreased gradually (Fig. 8B).

d) pH optimum

The effect of pH on the enzyme activity was studied over a pH range of 5.0 to 10.25 using a number of buffers, limiting each one to the range in which it has a strong buffering capacity. All buffers were made up at constant ionic strength, based upon data obtained by calculation or from a table (97). The pH curve obtained (Fig. 9) was strongly biphasic, with distinct pH optima at 7.5 and 9.0 at 30°C. This was different from what had been reported for uridine kinase in other organisms, since pH optima vary widely with enzymes from different sources (22, 23, 24). However, a similar biphasic pH curve has been demonstrated for the deoxycytidine kinase from Lactobacillus acidophilus where the presence of two molecular forms or separated species of the enzyme has been suggested (78).

e) Nucleoside specificity

Uridine kinase from corn seed showed very high specificity toward its nucleoside. The strict stereospecific demands of the enzyme for the phosphate acceptor were reflected by its inability to phosphorylate either 2'-deoxyuridine or 2'-deoxycytidine at all, while uridine and cytidine could be readily phosphorylated (Table 17). Uridine and cytidine were the only two nucleosides that could be phosphorylated among all nucleosides tested. While the uridine and cytidine phosphory-
Fig. 9. EFFECT OF VARIATION OF THE pH ON THE RATE OF URIDINE PHOSPHORYLATION

All buffers were made up at constant ionic strength. 2.2 μg of Fraction V enzyme was assayed as described in Methods, except the buffer was as indicated (final ionic strength = 0.04). The total assay volume was 100 μl, and after incubation and heating 200 μl H₂O was added; pH's were measured at 30°C in the final assay mixture after 20 min. incubation.

- - - o, Na-acetate buffer; o — o, imidazole-HCl; A — A, phosphate buffer; a — a, Tris-HCl; u — u, glycine-NaOH; Δ — Δ, lycine-NaOH.
TABLE 17

PHOSPHATE ACCEPTOR SPECIFICITY OF URIDINE KINASE FROM CORN SEED

Conditions were as described in Methods, except that tritiated nucleosides (0.02 mM, 60 - 250 mCi/m mole) were substituted for uridine as shown, and 4.5 µg of Fraction V enzyme was used in a final reaction mixture of 100 µl.

<table>
<thead>
<tr>
<th>Acceptor Nucleosides</th>
<th>Nucleotides formed (nmoles/mg protein /30 min,)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urd</td>
<td>174</td>
</tr>
<tr>
<td>Cyd</td>
<td>37.5</td>
</tr>
<tr>
<td>dUrd</td>
<td>0</td>
</tr>
<tr>
<td>dCyd</td>
<td>0</td>
</tr>
<tr>
<td>Ado</td>
<td>0</td>
</tr>
<tr>
<td>dAdo</td>
<td>0</td>
</tr>
<tr>
<td>Guo</td>
<td>0</td>
</tr>
<tr>
<td>dThd</td>
<td>0</td>
</tr>
<tr>
<td>dGuo</td>
<td>0</td>
</tr>
<tr>
<td>araCyd</td>
<td>0</td>
</tr>
</tbody>
</table>
lation ratio varied from 2 to 1 in Novikoff ascites tumor (23) and Tetrahymena (24), the phosphorylation ratio for uridine and cytidine of corn seed uridine kinase was much larger, around 5 at sub-saturating nucleoside concentrations. Indications that the phosphorylation ratio remained constant throughout the whole purification (Table 18) and that cytidine and uridine were mutually competitive (Fig. 10) seemed to suggest that only one enzyme was involved in the phosphorylation of the two nucleosides.

f) Metal ion requirement

Uridine kinase showed an absolute requirement for divalent metal ion for activity. Magnesium ion supported the highest uridine kinase activity. The enzyme also able to utilize some other divalent cations in partial replacement of \( \text{Mg}^{++} \) (Table 19). Similar partial replacement of the \( \text{Mg}^{++} \) requirement by \( \text{Mn}^{++} \) and \( \text{Fe}^{++} \) has been demonstrated for the uridine kinase of Ehrlich ascites tumor cells (22) and Tetrahymena (24).

g) Phosphate donor specificity

All of the naturally occurring nucleoside triphosphates and ADP and AMP were tested for their capacity to phosphorylate uridine. Among those tested, GTP, dGTP, dATP and ATP gave the highest phosphorylation activities, while the end products UTP and CTP showed very poor or no ability at all to serve as phosphate donors (Table 20). Similar
TABLE 18

NUCLEOSIDE ACCEPTOR SPECIFICITY OF URIDINE KINASE DURING PURIFICATION

Conditions were as described in Methods, except 0.1 M phosphate buffer pH 7.0 replaced 50 mM Tris-HCl (pH 8.0 at 4°C) when assaying the enzyme activity in the crude extract fraction.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity&lt;sup&gt;a&lt;/sup&gt; (units/mg protein)</th>
<th>Phosphorylation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urd</td>
<td>Cyd</td>
</tr>
<tr>
<td>Crude extract</td>
<td>2.40</td>
<td>0.58</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>20.20</td>
<td>4.30</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>73.0</td>
<td>15.53</td>
</tr>
<tr>
<td>Sepharose 4B-200</td>
<td>171.2</td>
<td>36.77</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1 unit of the enzyme is defined as the quantity of enzyme that catalyzes the formation of 1 nmole of UMP or CMP in 30 min. at 30°C.
Fig. 10. EFFECT OF URIDINE CONCENTRATION ON URIDINE PHOSPHORYLATION; INHIBITION BY CYTIDINE.

The reaction conditions are as described in Methods except that uridine concentrations are varied as shown and cytidine is added as detailed below.

○—○, control; ●—●, 0.25 mM Cyd; ▲—▲, 0.50 mM Cyd.
TABLE 19
DIVALENT CATION SPECIFICITY OF URIDINE KINASE FROM CORN SEED

Standard assay conditions were described in Methods, except 10 mM final concentrations of ATP and Mg\(^{2+}\) (or other divalent cations as shown) were present. 2.2 \(\mu\)g of Fraction V enzyme was used.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl(_2)</td>
<td>100(^a)</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>64.4</td>
</tr>
<tr>
<td>FeSO(_4)</td>
<td>52.0</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>13.3</td>
</tr>
<tr>
<td>Co(NO(_3))(_2)</td>
<td>8.1</td>
</tr>
<tr>
<td>BaCl(_2)</td>
<td>0.4</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>0</td>
</tr>
<tr>
<td>CuSO(_4)</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.20</td>
</tr>
<tr>
<td>None + 10 mM EDTA</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)174 nmoles UMP formed/\(\mu\)g protein/30 min. at 30 °C
<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>0.3 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>140</td>
<td>272</td>
</tr>
<tr>
<td>dATP</td>
<td>157</td>
<td>290</td>
</tr>
<tr>
<td>ADP</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>AMP</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>GTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dGTP</td>
<td>2</td>
<td>5.6</td>
</tr>
<tr>
<td>GTP</td>
<td>256</td>
<td>262</td>
</tr>
<tr>
<td>dGTP</td>
<td>243</td>
<td>248</td>
</tr>
<tr>
<td>dATP</td>
<td>1</td>
<td>4.8</td>
</tr>
<tr>
<td>UTP</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>dUTP</td>
<td>0</td>
<td>2.2</td>
</tr>
</tbody>
</table>
results concerning the phosphate donor specificity have been reported (22, 23, 24) although the order of effectiveness varied from one source to another. The small amount of UMP formed when utilizing ADP and AMP as phosphate donors might be from traces of ATP in commercial ADP and AMP (personal communication with Dr. R. Raznik).

h) Sulfhydryl dependence

The uridine kinase from corn is quite sensitive to sulfhydryl group inhibitors (Table 21). Inhibition could be prevented by the addition of thiol reagents. Uridine kinase after passing through a P-6 column in the absence of β-ME lost about 20% of its activity compared to the column containing β-ME. It also lost most of its activity at 4°C in three days in the absence of β-ME, but most of its activity could be regained by incubating the enzyme with 50 mM DTT. Because of these effects, uridine kinase was usually preincubated at room temperature with 50 mM DTT before doing kinetic assays.

i) Effect of "protective agents" on uridine kinase activity at elevated temperature

Uridine kinase from corn seed is fairly labile at elevated temperatures. At 40°C it has a half-life of 35 min. and at 45°C only 10 min. if in the presence of Tris-HCl (pH 8.0 at 4°C) only.

A series of supposed "protective agents" were added and preincu-
TABLE 21

EFFECT OF SULFHYDRL SPECIFIC REAGENTS ON URIDINE KINASE ACTIVITY

Conditions were as described in Methods, except that DTT was omitted and the reagents shown were added as indicated. The enzyme used was 1.6 μg of Fraction V protein passed through a P 6 column preequilibrated with 20 % glycerol and 50 mM Tris-HCl (pH 8.0 at 4°C) buffer just before assay.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>% inhibition</th>
<th>% activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0(^a)</td>
<td>0(^a)</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>1 x 10(^{-5}) M</td>
<td>83.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 x 10(^{-4}) M</td>
<td>89.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 x 10(^{-3}) M</td>
<td>31.4</td>
<td></td>
</tr>
<tr>
<td>NEM</td>
<td>1 x 10(^{-2}) M</td>
<td>65.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 x 10(^{-6}) M</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>PMB</td>
<td>1 x 10(^{-5}) M</td>
<td>51.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 x 10(^{-5}) M</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>PMB + DTT</td>
<td>40 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None + DTT</td>
<td>40 mM</td>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) 120 nmoles UMP formed / 30 min. / mg protein
bated with the enzyme at 45°C for various time periods before assaying its residual enzyme activity. A series of enzyme decay curves were obtained by comparing the enzyme activities at each time point with that at the zero time point in the presence of the same protecting agents in the preincubation tubes. As shown in Fig. 11, the order of effectiveness of the protective agents are DTT-ATP-Mg²⁺ > DTT > 20% glycerol > GTP > Tris-SO₄ > Tris-HCl > BSA > GTP. DTT, glycerol, ATP-Mg²⁺ and GTP, the enzyme activator, all gave significant positive protection effects at this temperature. Tris-SO₄, which has been found to be a better protective agent against denaturation of many proteins (99) showed little protective effect here. BSA, which has been found to stabilize many labile enzymes such as thymidine kinase and deoxycytidine kinase (5, 29) on the contrary, showed a minimal protective effect here. Possibly the wrong concentration of BSA might have been selected; as has been demonstrated in the case of thymidine kinase, the effect of BSA toward enzyme activity was very concentration dependent (5). The end product inhibitor, GTP, also did not protect uridine kinase against heat inactivation. In fact, GTP speeded up the decay of activity to some extent. This is again contrary to what has been reported in other enzyme systems; for example, the deoxycytidine kinase from calf thymus was found to be substantially stabilized by its inhibitory product, dGTP (100).
Fig. 11. HEAT INACTIVATION OF URIDINE KINASE IN THE PRESENCE OF "PROTECTIVE AGENTS".

Experimental conditions and assay procedures were as described in the Text and Methods. Each enzyme activity in the presence of certain "protective agent" at zero time point was taken as 100% activity and used for comparing the enzyme activity after inactivation in the presence of the same agents for various lengths of time. The enzyme used was 2.2 μg of Fraction V protein.

- ••••, 40 mM DTT-10 mM ATP-Mg²⁺;
- ○○○○, 40 mM DTT;
- △△△△, 20% glycerol;
- ▲▲▲▲, 0.29 mM GTP;
- ▼▼▼▼, 50 mM Tris-SC₂O₄;
- □□□□, 50 mM;
- ▼▼▼▼, 0.23 mg/ml BSA;
- ▼▼▼▼, 0.29 mM CTP.

(All incubations were carried out in 50 mM Tris-HCl(pH 7.4 at 23°C) with the exception of ▲▲▲▲, where Tris-SC₂O₄ was used instead).
5. Kinetics of Uridine Kinase

a) Effect of variable nucleoside concentration on the reaction rate, with a saturating ATP concentration.

When the uridine concentration was varied over a fairly wide range (5 - 200 μM) under saturating concentrations of ATP, the double reciprocal plot appears as a straight line (Fig. 10, control line) and normal Michaelis-Menten behavior was observed. However, when the uridine concentration was varied over a higher concentration range to greater than 200 μM, a substrate inhibition type of double reciprocal plot was observed (Fig. 12). In order to eliminate the possibility that such phenomena might be due to the trace amount of contaminants in the uridine samples used for this study, uridine samples from different chemical companies were checked chromatographically and used to repeat this experiment. The data were all quite consistent with that illustrated (Fig. 12).

With cytidine as the variable substrate, the double reciprocal plot was also linear over the concentration range of 5 - 200 μM (Fig. 13), however, the kinetic behavior of this substrate has not been investigated at concentrations greater than 200 μM. The $K_m$'s for uridine and cytidine were $5.3 \times 10^{-5}$M and $1.25 \times 10^{-4}$M respectively. Cytidine was a competitive inhibitor of uridine phosphorylation with $K_i$ of $2.15 \times 10^{-4}$M (from Fig 10). The kinetic constants for uridine and cytidine indicated that in the presence of both substrates of equal concentration, uridine would be preferentially utilized.
Fig. 12. EFFECT OF VARYING Urd CONCENTRATION ON THE REACTION RATE, WITH ATP-Mg\(^{2-}\) SATURATING.

Conditions were as described in Methods, except that the Urd concentration was varied as indicated. The enzyme used was 2.2 \(\mu\)g of Fraction V protein.
Fig. 13. EFFECT OF VARYING Cyd CONCENTRATION ON THE REACTION RATE, WITH ATP-Mg$^{2+}$ SATURATING.

Conditions were as described in Methods for the cytidine kinase assay, except that the Cyd concentration was varied as indicated. The enzyme used was 2.2 μg of Fraction V protein.
The effect of UTP toward uridine phosphorylation over a wide range of variable uridine concentrations was studied. Fig. 14 illustrated that UTP inhibition appeared to be noncompetitive with respect to the phosphate acceptor, uridine. UTP has a $K_i$ of 0.79 mM as revealed by the secondary plot of the slope against the inhibitor, UTP, concentrations from Fig. 14 (101).

b) The effect of variable phosphate donor, ATP-Mg$^{2+}$ concentration on the reaction rate, with a subsaturating concentration of uridine.

ATP-Mg$^{2+}$ complexes have been found to be the actual substrates in a great number of kinase enzymes which require both ATP and Mg$^{2+}$ for activity (96, 102-104). Uridine kinase from corn showed absolute requirements of ATP and Mg$^{2+}$. Also, from the observation that Mg$^{2+}$ and ATP each displayed distinct concentration optima when varied against fixed concentrations of the other (Fig. 8), and that these optima occurred at a 1:1 ATP:Mg$^{2+}$ ratio, the ATP-Mg$^{2+}$ complex was therefore assumed to be the active substrate form of ATP in the uridine kinase reaction. Therefore, to avoid both the complicated procedures for calculating the true ATP-Mg$^{2+}$ complex concentration from dissociation constants, or having to worry about the effect of a large excess of Mg$^{2+}$, the MgCl$_2$ concentration was always maintained at 25% molar excess over the total concentration of all nucleoside triphosphates present in the reaction mixture. For all practical purposes, this ensured that all nucleoside triphosphates added were fully complexed.
Fig. 14. EFFECT OF VARYING Urn CONCENTRATION ON THE REACTION RATE, WITH ATP-Mg²⁺ SATURATING: INHIBITION BY UTP.

Conditions were as described in Methods, except that the Urn concentration was varied as indicated, and UTP was added as detailed below. The enzyme was 2.2 µg of Fraction V protein. ○—○, control; •—•, 0.5 mM UTP; ▲—▲, 1.0 mM UTP.
When ATP was varied over a wide concentration range with a fixed concentration of uridine, the double reciprocal plot was linear and showed normal Michaelis-Menten kinetics (Fig. 15, control line). Its apparent $K_m$ was 0.588 mM. The addition of either UTP or CTP increased the apparent $K_m$ of ATP-Mg$^{2+}$, but without affecting the $V_{max}$. Thus, both UTP and CTP are competitive toward the phosphate donor and with $K_i$'s of 0.12 mM and 0.13 mM respectively (Fig. 15, Fig. 16). This is similar to the uridine kinase of Novikoff ascites tumor (23), and the Tetrahymena system (24). The effect of CTP concentration on uridine phosphorylation at fixed concentrations of ATP-Mg$^{2+}$ (5 mM) and uridine (20 μM) was also studied. Fig. 17 shows that the amount of uridine phosphorylated decreased with the increase of CTP concentration, and when CTP increased to around 10 mM, uridine phosphorylation was completely inhibited. This was probably because under such conditions the phosphate donor site of the enzyme was completely occupied by CTP which could not serve as a phosphate donor.

In contrast to UTP and CTP which inhibited uridine kinase, GTP and dGTP activated this enzyme. The activation was more effective in the less purified enzyme fractions. With the most highly-purified fraction, the activation was most marked with a subsaturating concentration of ATP-Mg$^{2+}$. When the effect of GTP toward uridine phosphory-
Fig. 15. EFFECT OF VARYING ATP-\(\text{Mg}^{2+}\) CONCENTRATION ON THE REACTION RATE, WITH THE Urd CONCENTRATION NONSATURATING; INHIBITION BY UTP.

The Urd concentration was fixed at 20 \(\mu\text{M} \ (125 \text{ mCi/m mole})\), \(\text{ATP-}\text{Mg}^{2+}\) was varied as shown, UTP was added as indicated below. Other conditions were as described in Methods. The enzyme used was 2.2 \(\mu\text{g}\) of Fraction V protein.

\(\circ\cdots\circ\), control; \(\bullet\cdots\bullet\), 0.5 \(\text{mM}\) UTP; \(\Delta\cdots\Delta\), 1.0 \(\text{mM}\) UTP.
Fig. 16. Effect of varying ATP-Mg\(^{2+}\) concentration on the reaction rate, with the Urd concentration nonsaturating; inhibition by CTP.

The conditions were the same as described in the text of Fig. 15, except CTP replaced UTP as detailed below. The enzyme was 2.2 μg of Fraction V protein.

○—○, control; •—•, 0.25 mM CTP; □—□, 0.5 mM CTP.
Fig. 17. The effect of CTP on uridine phosphorylation.

Conditions and assay procedures were as described in Methods, except CTP was added at the concentrations shown. 2 µg of Fraction V enzyme was used. Under these conditions, 13% of H^3-Urd was phosphorylated in the control tube and was taken as 100% activity.
Urd PHOSPHORYLATED, %

CTP, mM

50 100

0 2 4 6 8 10
Fig. 18. Effect of varying ATP-Mg$^{2-}$ concentration on the reaction rate of uridine phosphorylation; activation by GTP; inhibition by CTP and reversal of CTP inhibition by GTP.

The Urd concentration was fixed at 20 μM (125 mCi/m mole). ATP-Mg$^{2-}$ was varied as shown, GTP and CTP were added as detailed below. Other conditions were described in Methods. The enzyme used was 2 μg of Fraction V protein.

○—○, control; •—•, 0.15 mM GTP; ■—■, 0.5 mM CTP; ▲—▲, 0.15 mM GTP + 0.5 mM CTP.
Fig. 19. Effect of varying GTP-Mg$^{2-}$ on the reaction rate of uridine phosphorylation.

Conditions were as described in Methods, except GTP-Mg$^{2-}$ was added as indicated and ATP-Mg$^{2-}$ was omitted. 2.0 µg of Fraction V protein was used here.
lation was studied with varying concentrations of ATP-Mg\(^{2-}\), the
double reciprocal plot was linear and intersected with that of the
control line on the 1/v axis (Fig. 18). Therefore, GTP probably
activated the enzyme by competing with ATP for the phosphate donor site
because it is a better phosphate donor. This was confirmed when the
effect of varying concentrations of GTP toward uridine phosphorylation
was studied with a fixed amount of uridine and in the absence of any
other phosphate donor. Fig. 19 shows that GTP itself is indeed a very
good phosphate donor for uridine phosphorylation with an apparent \(K_m\)
of \(6.1 \times 10^{-5} \text{ M}\), about 10 times less than that of ATP. From the fact
that both CTP inhibition and GTP activation were due to the competition
for the phosphate donor site, it seems clear that reversal of CTP
inhibition by GTP (Fig. 18) can be explained by a very efficient
phosphate donor, GTP, displacing a very poor donor, CTP, from the
phosphate-donor site. The curved line in Fig. 18 obtained when both
GTP and CTP were added is probably due to the transition in the utiliza-
tion of the phosphate donor from the one with a higher \(K_m\), ATP-Mg\(^{2-}\), to
the one with a lower \(K_m\), GTP-Mg\(^{2-}\). However, such curvature did not
appear on the CTP activation line (in Fig. 18). This is probably
because the slope of that line is too slight to reveal any deviation
from linearity.

The kinetic constants of the uridine kinase from corn seed are
summarized in Table 22.
TABLE 22

SEVERAL KINETIC CONSTANTS OF URIDINE KINASE FROM CORN SEED

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km</th>
<th>K_i</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cyd</td>
<td>UTP</td>
<td>GTP</td>
</tr>
<tr>
<td>Urd</td>
<td>$5.3 \times 10^{-5}$ M</td>
<td>$2.1 \times 10^{-4}$ M</td>
<td>$7.9 \times 10^{-4}$ M</td>
<td></td>
</tr>
<tr>
<td>Cyd</td>
<td>$1.25 \times 10^{-4}$ M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP-Mg$^{2-}$</td>
<td>$5.88 \times 10^{-4}$ M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP-Mg$^{2-}$</td>
<td>$6.1 \times 10^{-5}$ M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) $K_{mapp}$

c) The reaction mechanism of uridine kinase from corn seed

Both uridine and ATP-Mg$^{2-}$ concentrations were varied over a fairly wide range to study the effect of changing substrate concentrations on the velocity of uridine phosphorylation. As shown in Fig. 20 and Fig. 21, changes of the concentration of one substrate did not affect the apparent affinity constant of the other substrate. However, the $V_{max}$ for one substrate was increased with increasing concentrations of the other substrate. Therefore, uridine kinase from corn seed appeared to have one of the sequential mechanisms. This is different from what has been suggested for the same enzyme from Novikoff ascites tumor where a ping-pong reaction mechanism was proposed (23). However, Orengo's conclusion was based on
Fig. 20. Effect of varying uridine and ATP-Mg$^{2-}$ concentrations on the reaction rate of uridine kinase.

Conditions were as described in Methods, except that the Urd concentration was varied as indicated on the x-axis and ATP-Mg$^{2-}$ concentration was varied as detailed below. The enzyme was 2.2 µg of Fraction V protein.

- - - , 0.3125 mM; - - - , 0.625 mM; - - - , 0.833 mM; - - - , 1.25 mM; - - - , 2.50 mM.
Fig. 21. EFFECT OF VARYING ATP-Mg\(^{2+}\) AND Urd CONCENTRATIONS ON THE REACTION RATE.

Conditions were as described in Methods, except that the ATP-Mg\(^{2+}\) concentration was varied as indicated on the x-axis and uridine concentration was varied as detailed below. The enzyme was 2.2 µg of Fraction V protein.

- •—•, 5 µM Urd; •—•, 7.5 µM Urd; •—•, 10.0 µM Urd;
- •—•, 20 µM Urd; •—•, 50 µM Urd.
relatively few data points and he might easily have misinterpreted the data, particularly if the variable concentration range was not appropriately chosen. In addition, many kinases have been found to have one of the sequential mechanisms (105).

In order to distinguish between ordered sequential and random sequential mechanisms, the product inhibition patterns have been studied. As revealed by Fig. 22, Fig. 23, Fig. 24, and Fig. 25, at nonsaturating levels of the nonvaried substrates, UMP is noncompetitive with both substrates, Urd and ATP-Mg\(^{2-}\) while ADP is noncompetitive with Urd but is competitive with ATP-Mg\(^{2-}\). Therefore, by applying "Cleland's Rules" (101), corn seed seems to have an ordered Bi Bi reaction mechanism with ATP-Mg\(^{2-}\) and ADP as the leading or obligatory reactants.
Fig. 22. EFFECT OF VARYING ATP-Mg²⁺ CONCENTRATION ON THE REACTION RATE, WITH Urd CONCENTRATION NONSATURATING; INHIBITION BY UMP.

The Urd concentration was fixed at 20 μM (125 mCi/mMole). ATP-Mg²⁺ concentration was varied as shown, UMP was added as indicated below. Other conditions were as described in Methods. The enzyme was 1.8 μg of Fraction V protein.  
- control; ▲-▲, 1 mM UMP; ●-●, 2 mM UMP.
Fig. 23. **EFFECT OF VARYING Urd CONCENTRATION ON THE REACTION RATE, WITH ATP-Mg$^{2+}$ CONCENTRATION NONSATURATING; INHIBITION BY UMP**.

The ATP-Mg$^{2+}$ concentration was fixed at 2.5 mM. Urd concentration was varied as shown. UMP was added as detailed below. The conditions were as described in Methods. The enzyme was 1.8 µg of Fraction V protein.

- •—•, control; ▲—▲, 1 mM UMP; ●—●, 2 mM UMP.
Fig. 24. EFFECT OF VARYING ATP-\(Mg^2+\) CONCENTRATION ON THE REACTION RATE, WITH Urd CONCENTRATION NONSATURATING; INHIBITION BY ADP.

The conditions were the same as in Fig. 22, except that ADP instead of UMP was added as detailed below.

\(\circ\)——\(\circ\), control; \(\bullet\)——\(\bullet\), 2.5 mM ADP; \(\Delta\)——\(\Delta\), 5.0 mM ADP.
Fig. 25. EFFECT OF VARYING Urd CONCENTRATION ON THE REACTION RATE, WITH ATP-Mg\(^{2+}\) CONCENTRATION NONSATURATING; INHIBITION BY ADP.

The conditions were the same as in Fig. 23, except that ADP instead of UMP was added as detailed below.

- ○—○, control; ○—●, 2.5 mM ADP; ▲—▲, 5.0 mM ADP.
DISCUSSION

It appears that a nonspecific nucleoside phosphotransferase is widely distributed throughout the plant and plays an active role in nucleoside phosphorylation. Many plant extracts have demonstrated the ability to synthesize thymidylate from thymidine using AMP as the phosphate donor. However, up to now, not a single instance of a regulatory thymidine kinase has been reported. Thus, it seems probable that the enzyme activity identified as "thymidine kinase" in such plants as grains (16, 35), peanuts (17, 20), and Vicia faba beans (18), is actually a nonspecific nucleoside phosphotransferase. The "thymidine kinase" in corn and peanut was found to be able to phosphorylate uridine, deoxyuridine, cytidine, and deoxycytidine nonspecifically, in addition to thymidine (17, 106), thereby clearly showing a characteristic of phosphotransferase rather than of a kinase. The peanut enzyme was reported to require ATP and Mg$^{2+}$ (20), but this could be explained by the Mg$^{2+}$ stimulation of ATP-hydrolyzing activity which degrades ATP to AMP, providing the phosphate donor for the phosphotransferase activity. Indeed, a very active ATP degradation activity was noted in that system. As for the bean root enzyme (18), although the author did assay the enzyme activity in the presence of an ATP-regenerating system and if a check was made of the effectiveness of ATP-regeneration it was not mentioned. When their experimental conditions were repeated
(data not shown), ATP was quickly broken down to AMP and adenosine using the enzyme from the crude plant root extracts. In addition, AMP is superior to ATP as a phosphate donor for nucleoside phosphorylation by both bean and peanut extracts (Table 2), a property of phosphotransferase rather than a kinase.

Several pieces of experimental evidence fit together to suggest a reinterpretation of the recombination experiment with two (NH$_4$)$_2$SO$_4$ fractions carried out by Wanka, et al (16). Their experiment was repeated with extracts of barley seedlings, and the true phosphate donor was found to be AMP. This was shown both by direct addition of AMP to the reaction and by the isotope experiment of specifically labeling the phosphate on ATP to identify the particular phosphate moiety transferred. Component P was able to degrade ATP to AMP, and component T was able to phosphorylate thymidine using AMP as the phosphate donor. The combined fractions from corn were also later shown to be quite nonspecific with regard to the nucleoside phosphorylated (106). In crude extracts of corn seedlings, AMP could replace ATP as the phosphate donor, and dTTP did not inhibit phosphorylation of thymidine. All the above evidence seems to suggest that the superadditive effect on thymidine phosphorylation of the two (NH$_4$)$_2$SO$_4$ components was due to the combined actions of an ATP hydrolyzing enzyme and nucleoside phosphotransferase activity, rather than the recombination of two subunits of one enzyme, thymidine kinase. After this part of the experi-
mental work had been completed, such a mechanism was shown to operate in the cases of Tetrahymena pyriformis and germinating potato by Arima et al. In both instances, dTTP failed to inhibit thymidine phosphorylation, and AMP could serve as a phosphate donor.

Although thymidine kinase, an important salvage enzyme for reutilizing thymidine in many other organisms, is apparently missing from plants, the barley root extracts were able to form dTTP from thymidine. The roots were also able to incorporate thymidine into DNA. This suggests that nucleoside phosphotransferase can serve as a salvage enzyme in barley seedlings.

In contrast to the nonspecific phosphorylation of thymidine and other nucleosides in seedlings of corn and peanuts two or three days after onset of germination, there is a more specific and well-regulated phosphorylation mechanism operating in the initial germination stage. In both cases, the appearance of uridine or cytidine phosphorylation activity precedes the appearance of thymidine phosphorylation activity. Several in vivo isotope incorporation studies demonstrate that RNA synthesis precedes DNA synthesis. Uridine was incorporated into the RNA soon after the germination of corn. Peanuts incorporate $^{32}$P-orthophosphate into cytoplasmic RNA but not DNA at 24 hr. In wheat grains, rapid incorporation of $^{14}$C-uridine to RNA precedes $^{14}$C-leucine, and $^{14}$C-thymidine incorporation into protein
and DNA respectively (110). All this evidence indicates that RNA synthesis may be initiated immediately, being catalyzed by enzyme systems apparently present in dormant seeds. The uridine phosphorylation enzyme in the initial germinating stage or in dry seed has been examined extensively and shows some characteristics of true uridine kinase (Table 12, Table 13, and Table 14). This activity falls off rapidly after 36 hrs. and presumably after this time in the life of plant, another type of uridine phosphorylation enzyme, i.e. nonspecific nucleoside phosphotransferase, takes over the role of uridine phosphorylation. How such a transition takes place is not clear. However, the falling off of uridine kinase activity could be due either to the turnover of the enzyme or to the rapidly increasing amount of ATPase activity following the germination process. This ATPase ultimately degrades ATP so rapidly that it obscures uridine kinase activity. The evidence that uridine kinase increases steadily while phosphatase activity declines rapidly during peanut fruit ontogeny (109) seems to favor the latter possibility. Nevertheless, the presence of uridine kinase activity in dry corn seed certainly suggests that this enzyme may play a physiological role during seed development and maturation. In addition to uridine kinase, some other enzymes related to RNA synthesis, such as RNA polymerase (111), some enzymes concerned with purine nucleotide synthesis (112), and pyrimidine metabolism (113) have been demonstrated in dormant seeds.
Uridine kinase from corn seed has been purified about seventy fold through several purification steps, including streptomycin sulfate, (NH₄)₂SO₄ precipitation, DEAE cellulose column chromatography and Sepharose 4B-200 gel chromatography. However, the molecular weight has not yet been determined. The enzyme was excluded from Bio Gel P 300 and Sepharose 6B 100 gel, and the molecular weight seems therefore to be unusually large for an enzyme. The reason for this is not known. However, it might be due to asymmetry in the shape of the protein molecule or to the association of the enzyme with RNA as reflected by the positive orcinol test and the somewhat low 280/260 nm UV absorption ratio for a protein in the final preparation of enzyme solution. The incubation of the purified enzyme with RNase B and RNase T₁ at physiological pH and room temperature for 30 min, neither increases the 280/260 ratio nor lowers the molecular weight of the enzyme. In addition, uridine kinase loses its activity rapidly in high salt solution. It loses more than 85% of activity even when assayed in the presence of 0.25 M (NH₄)₂SO₄, 0.5 M NaCl, or 0.25 M guanidine-HCl. The method of using high salt concentrations to dissociate nucleic acid from protein (116) therefore fails to work here. However, the high molecular weight may not necessarily be due to the small amount of RNA contamination. From the observation that the enzyme activity lasts for years in dry seed stored at room temperature, certain special storage forms of the enzyme or a storage protein complex of high molecular weight
for stabilizing the enzyme activity in the dry seed might complicate the molecular weight determinations.

Nevertheless, the final enzyme preparation is free of all enzymes which might interfere with the assay for uridine kinase activity, including uridine phosphorylase, nucleotide kinase, ATPase, cytidine deaminase, and adenylate kinase.

The enzyme shows a high degree of specificity toward the nucleoside acceptor. Thus, neither deoxynucleosides nor purine compounds are phosphorylated. With respect to the pyrimidine nucleosides, enzyme activity was limited to the ribonucleosides of uracil and cytosine; even the β-arabinoside of cytosine is not phosphorylated. The ratio of the phosphorylation rate of uridine to cytidine remains relatively constant throughout purification and the mutual competitive inhibition that exists between the two nucleosides indicates that the same enzyme site is involved with both nucleosides. The enzyme has a higher affinity for uridine than for cytidine, as reflected in the respective Michaelis-Menten constants of $5.3 \times 10^{-5}$ M and $1.25 \times 10^{-4}$ M.

Inhibition of the enzyme by HgCl$_2$, NEM, and pMB was demonstrated. The inhibition by pMB can be prevented by DTT. The inhibition is probably due to the interaction of the inhibitors with the active SH groups of the enzyme (115).
The kinetic data reveal that a simple regulatory mechanism is operating for this enzyme. The formation of UMP is inhibited by UTP and CTP, the end products of pyrimidine ribonucleotide synthesis. The specific structural requirements for inhibitory action are reflected by the results that neither dUTP nor dCTP shows any inhibitory action on UMP formation. Similar inhibition of uridine kinase activity has been reported in many other organisms (21-24). The inhibitions of uridine kinase by CTP and UTP are competitive with the phosphate donor and noncompetitive with the phosphate acceptor. In addition to the negative feedback control of the enzyme activity by end products, uridine kinase is activated by GTP and dGTP. The activation by GTP is competitive with the phosphate donor, ATP. dGTP is probably activating the enzyme in a similar way. These data, together with the phosphate donor specificity data (Table 19), reveal the simple inhibition-activation model operating for this enzyme. CTP and UTP are very poor phosphate donors and simply inhibit the enzyme by competing for the phosphate donor site; GTP and dGTP are much better phosphate donors than ATP and therefore activate the enzyme by competing the phosphate donor site and serving as more effective donors for uridine phosphorylation. In other words, GTP and dGTP counteract the inhibitions of UTP and CTP simply by competing with the inhibitor for the phosphate donor binding site on the enzyme molecule.

On the basis of such a regulatory mechanism, the maintenance of the level of the ribopyrimidine nucleotides in corn can be controlled
in the following way. The first enzyme of the salvage pathway of the ribopyrimidine nucleosides is subject to two competing regulations. When the ribonucleotide triphosphates, UTP or CTP accumulate as a consequence of a decreased rate of RNA and DNA synthesis, the uridine-cytidine kinase is blocked and the entire pathway will be shut down. However, the end products of the purine biosynthesis pathway, GTP, and dGTP (also possibly ATP and dATP) are able to reverse the inhibition of the ribopyrimidine nucleoside triphosphates. Therefore, a balance between the purine and pyrimidine nucleoside triphosphates may be maintained, and the cell may be prevented from being deficient in the intermediates such as UDP and CDP which are important precursors for the deoxypyrimidine nucleotides and therefore for DNA synthesis.
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