To the Graduate Council:

I am submitting herewith a dissertation written by Martha Lynn Rowe entitled "Pyrimidine Nucleoside Phosphorylation in Developing Seeds and Germinating Seedlings of Wheat." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Otto J. Schwarz, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

PYRIMIDINE NUCLEOSIDE PHOSPHORYLATION IN DEVELOPING SEEDS AND GERMINATING SEEDLINGS OF WHEAT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Martha Lynn Rowe

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ABSTRACT

Uridine- and thymidine-phosphorylating enzymes were measured in developing and germinating seeds of Triticum aestivum v. Arthur and T. aestivum v. Lemhi.

Because crude extracts were to be used in the developmental study, characteristics of unpurified nucleoside phosphotransferase (NPTase) were examined. Crude NPTase was found to be relatively heat and cold stable with a broad pH optimum of between 7.5 and 8.0. It did not require sulfhydryl protection and could not be separated from an associated AMP phosphohydrolase activity by ammonium sulfate fractionation.

In the developmental study with two varieties of wheat, NPTase activity was found to be very low in all of the true seed tissues during seed maturation. Uridine-phosphorylating activity was due primarily to uridine kinase. Thymidine phosphorylation was very low in all tissues throughout seed maturation, with a brief appearance by thymidine kinase in the developing embryo. In germinating seeds, uridine-phosphorylating activity was present from earliest stages of germination but showed a decrease in activity followed by a recovery after 48 hours imbibition. Experiments using [α-^{32}P]ATP and [γ-^{32}P]ATP indicated that uridine kinase was present during early germination but had disappeared by 96 hours. Uridine phosphorylation at later stages of germination was accomplished by NTPase. Thymidine

phosphorylation did not begin until after 36 hours of germination and was the result of NPTase activity.

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AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
dTMP	deoxythymidine 5'-monophosphate
Bis-Tris Propane	1,3-bis[tris(hydroxymethyl)methylamino] propane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid
NPTase	nucleoside phosphotransferase
P_i	inorganic phosphate
POPOP	phenyloxazolylphenyloxazolyl-phenyl
PPO	2,5-diphenyloxazole
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
Tris	tris(hydroxymethyl)aminomethane
UMP	uridine 5'-monophosphate

CHAPTER I

INTRODUCTION

The synthesis of nucleotides is a necessity in any developmentally active part of a plant. Sites of cell division and differentiation, such as meristems and developing and germinating seeds, require nucleotides for nucleic acid synthesis, energy conservation and transfer, as intermediates in the biosynthesis of certain carbohydrates and lipids, and as links in the chains of action of various hormones. The present work examines those enzymes in developing and germinating seeds that phosphorylate nucleosides to yield monophosphoryl nucleotides. The focus in this study is on pyrimidine nucleoside phosphorylation, in particular the phosphorylation of deoxythymidine and uridine.

Nucleotides may be synthesized de novo in a cell, or they may be reconstructed by phosphorylation of nucleosides derived from the breakdown of nucleic acids and nucleotides.

The major de novo pathway of pyrimidine nucleotide synthesis proceeds via orotic acid to uridine monophosphate (UMP). Uridine monophosphate may be converted to deoxyuridine 5'-monophosphate (dUMP) and then methylated to form deoxythymidine 5'-monophosphate (dTMP) (56). Uridine and thymidine are not found as intermediates in this pathway.

Direct phosphorylation of these nucleosides may occur by the so-called "salvage" pathway. Two types of "salvage" enzymes responsible for nucleoside phosphorylation are nucleoside kinase and nucleoside phosphotransferase. Both, strictly speaking, are phosphotransferases, since they catalyze the transfer of a phosphoryl group from a donor to the alcohol group of a nucleoside (35, 65). The major difference between the two is the type of phosphoryl group donor. Nucleoside kinases such as thymidine kinase (ATP:thymidine 5'-phosphotransferase; E.C. 2.7.1.75) and uridine kinase (ATP:uridine 5'-phosphototransferase; E.C. 2.7.1.48) catalyze the transfer of the γ-phosphoryl group from a nucleoside triphosphate (usually ATP) to a nucleoside acceptor. In both of the kinases mentioned above, phosphoryl transfer is to the 5'-hydroxyl of the acceptor nucleoside (35, 65).

$$\text{Nucleoside}_1 + \text{nucleoside}_2\text{-P-P-P*} \longrightarrow \text{nucleoside}_1\text{-P*} + \text{nucleoside}_2\text{-P-P}$$

The kinases have an absolute requirement for a divalent metal cation such as Mg^{2+}, and both exhibit end-product inhibition--thymidine kinase by its distal product deoxythymidine triphosphate (dTTP) (16, 51) and uridine kinase by uridine triphosphate (UTP) (2, 71). Nucleoside phosphotransferases (Nucleotide:3'-deoxynucleoside 5'-phosphotransferase; E.C. 2.7.1.77) catalyze the transfer of a phosphoryl group from a nucleoside monophosphate to an acceptor nucleoside (35).

$$\text{Nucleoside}_1 + \text{nucleoside}_2\text{-P} \rightleftharpoons \text{nucleoside}_1\text{-P} + \text{nucleoside}_2$$

These phosphotransferases are generally non-specific and phosphorylate a wide range of nucleosides (13). Despite the systematic name of the enzyme adopted by the enzyme commission, phosphoryl donors other than nucleoside monophosphates, e.g., phenylphosphate (12, 13), may be used as substrate. Phosphoryl transfer to the 5'-hydroxyl of the acceptor nucleoside is not absolute in all cases, although it is the most common position phosphorylated, and both ribo- and deoxyribonucleosides may function as phosphoryl group acceptors (17). Nucleoside phosphotransferases do not require Mg^{2+}, and they are not inhibited by the triphosphate derivatives of the acceptor nucleosides.

A BRIEF HISTORY OF PYRIMIDINE SALVAGE ENZYME RESEARCH IN PLANTS

In 1948, Axelrod (7) reported that certain phosphatases which caused "low energy" phosphate hydrolysis could also effect phosphate transfer to an alcohol acceptor, including an alcohol group on a nucleoside. These particular phosphatases with transfer capability produced low yields of nucleotides. Brawerman and Chargaff (12) were the first to report a nucleoside phosphotransferase in plant material, from an ammonium sulfate fraction of malt diastase. They subsequently found much higher activity in germinating wheat (13), and in this source even low concentrations of nucleoside were phosphorylated. Nucleoside phosphotransferase has since been reported

in a wide range of organisms--bacteria (14, 26), protozoa (4, 14), animals (14, 59, 83), and plants (11, 14, 33, 43).

Thymidine kinase activity in E. coli was described by Kornberg et al. (55), and it has been reported in a wide variety of organisms (10, 16, 32, 46, 70, 82, 98), as has uridine kinase (3, 24, 71, 75, 96, 97).

In the case of plant tissues, it has not always been clear which nucleoside phosphorylating enzymes are present. In particular, reports concerning thymidine phosphorylation in plants have been confusing, and misinformation about this type of activity persists even in current reviews. Crude extracts of many plant tissues contain phosphatases that are capable of dephosphorylating ATP to AMP. Thus, when ATP is used as phosphoryl donor in a phosphotransferase assay with plant crude extracts as enzyme source, it may be unclear whether ATP, ADP, or AMP is serving as the actual phosphoryl donor. As a specific example, consider a common technique for measuring thymidine kinase activity: ^{14}C-deoxythymidine and Mg^{2+}/ATP are combined in an assay mixture, and crude extract is added as enzyme source. After a length of time the reaction is terminated, the products are separated by chromatography, and the amount of ^{14}C label in dTMP is measured. In such an assay, the source of the phosphoryl group transferred to thymidine cannot be determined, and therefore it is unclear whether the enzyme activity is an ATP:phosphotransferase (kinase) or an AMP:phosphotransferase, assuming that both might be present.

In the early to mid 1950's, Brawerman and Chargaff demonstrated AMP:nucleoside phosphotransferase (NPTase) activity in

plant tissues (12, 13, 14), but noted that considerable phosphatase activity was also present in crude extracts. Although Tunis and Chargaff (91, 92, 93, 94) effected separation of the NPTase in carrot from most of the phosphatase activity, some residual hydrolytic activity persisted in the NPTase fraction. In 1963, Hotta and Stern (46, 47) reported that thymidine kinase activity appeared in Lilium and Trillium microspores just prior to DNA synthesis and that this rise in activity was due to de novo synthesis of the enzyme. In 1965, Hotta and Stern (48) showed that 48 hour-old wheat embryos double their thymidine kinase activity within 24 hours. When these 48 hour-old embryos were excised and grown in nutrient medium, no increase was observed in thymidine kinase activity unless exogenous thymidine was added to the nutrient medium. Wanka et al. (98) reported that thymidine kinase from the roots of germinating corn and wheat could be dissociated by ammonium sulfate fractionation into two components. Each component alone showed very little conversion of ^{14}C-thymidine to dTMP; when the components were combined, they showed activity 15 times higher than the sum of their separate activities. Wanka and Walboomers (97) looked at the pattern of development of the two thymidine kinase components and also uridine kinase in germinating corn. They found that thymidine kinase activity was almost zero during the first 36 hours of germination. Component P (permanent), measured as thymidine phosphorylation upon addition of component T (transient) to the embryo extract, was present from the beginning of germination and remained constant during the first

48 hours, after which it increased somewhat. Component T was not found until after 36 hours, at which time it increased in activity through 96 hours. Uridine kinase showed a different pattern of development. At the beginning of germination, significant uridine kinase activity was present, but this activity decreased over the next 48 hours and then began to increase again. Schwarz and Fites (80, 81) measured apparent thymidine kinase and uridine kinase activities in peanut hypocotyls. They too found that, as in wheat, thymidine phosphorylation did not begin until after 36 hours germination. Uridine phosphorylation preceded thymidine phosphorylation in peanut hypocotyls, but was not present from the beginning of imbibition. Thymidine kinase activity has also been reported in segments of Vicia faba roots showing mitotic activity (58).

In 1971, Arima et al. (4) suggested a reinterpretation of the results of Wanka et al. (98) and Wanka and Walboomers (97). Arima et al. found thymidine kinase-like activity both in Tetrahymena and in germinating potatoes; the latter activity could be separated into two components. Their suggestion was that these two components represented, not separated parts of a single kinase, but two totally separate enzyme activities. The activity corresponding to component P was an ATP-hydrolyzing activity, while that corresponding to component T was a nucleoside phosphotransferase. Thus, when ATP was used as phosphoryl donor in what was supposedly an assay for kinase activity, the ATP was actually hydrolyzed to AMP by the phosphohydrolase, with the AMP then functioning as the true phosphoryl

donor for the phosphotransferase. Neither component alone could effect both ATP hydrolysis and phosphoryl transfer, hence the apparent lack of activity when the two components were separated. Deng and Ives (33) showed this interpretation to be true for nucleoside phosphorylating activity in germinating barley roots. They used [^{32}P]ATP, labelled in either the α or γ phosphoryl position, as phosphoryl donor in assays of thymidine phosphorylating activity in crude extracts of barley root. Only the α-phosphoryl group appeared in the dTMP product, never the γ-phosphoryl group. Brunori et al. (19) later suggested that perhaps not enough care was taken by Deng and Ives in preparation of the crude extract to ensure stability of a thymidine kinase enzyme. Such kinases generally require the presence of a sulfhydryl reagent in order to maintain activity. Brunori et al. examined a number of plant tissues and concluded that thymidine kinase did indeed exist in most of these tissues. Their conclusions were based on the relative efficiency of ATP and AMP as phosphate donors and on the ability of dTTP to inhibit the thymidine phosphorylation reaction (dTTP inhibits thymidine kinase strongly, but has little effect on NPTase). They did not provide direct evidence of γ-phosphoryl transfer from ATP by performing the [α-/γ-^{32}P]ATP experiment. This experiment has been done by Mullin and Fites (68) with peanut hypocotyls and by Hofman and Schwarz (45) with germinating wheat, and in both cases no evidence of thymidine kinase activity was found; the sole thymidine phosphorylating activity was a nucleoside phosphotransferase.

Hofman and Schwarz did attempt to stabilize a possible thymidine kinase by addition of a sulfhydryl reagent to the extraction and assay media, but still could not detect thymidine kinase activity. Various reports of thymidine kinase activity in plant tissues have been made since the mid 1970's (8, 41, 64) and also some claiming a lack of thymidine kinase (11, 43), but none of these reports offer completely convincing evidence in support of their claims.

Less work has been done with uridine phosphorylating activity in plant tissues, but the problems are the same as with thymidine phosphorylation. Wanka and Walboomers (97) studied uridine kinase-like activity in germinating corn and found that activity was high during the first 36 hours of germination (in contrast to thymidine phosphorylating activity), declined over the next 12 hours, and then increased again after 48 hours. These same workers also reported that the first appearing activity was not dissociable, but that the second activity could be separated into two components, much like the thymidine phosphorylating activity they had studied. They suggested that perhaps two different uridine kinases existed, one early appearing and one late. Wanka and Bauer (95) subsequently reported that the late appearing uridine phosphorylating activity not only phosphorylated uridine, but also deoxythymidine, cytidine, deoxycytidine, and deoxyuridine; they also reported an early appearing cytidine kinase. Schwarz and Fites (81) reported results similar to those of Wanka et al. (98) in peanuts. Deng and Ives (33) suggested that the early appearing uridine phosphorylating activity

was probably a true uridine/cytidine kinase and that the later activity was most likely the same nucleoside phosphotransferase responsible for thymidine phosphorylation as well. These same workers later purified the early uridine kinase from ungerminated corn seed (34).

A summary of contributions in the area of pyrimidine nucleoside phosphorylation in plants is given in Table 1-1. Most of the work to date has been done with actively dividing parts of vegetative tissues, e.g., root tips, or with germinating seeds. Yet, although the salvaging of deoxythymidine and uridine should have value during the active cell division and growth that accompanies the development of a seed from fertilization to full maturation, nucleoside phosphorylation in developing seeds has not been explored. Therefore, the major portion of this work examines the patterns of pyrimidine nucleoside phosphorylating activity in both developing and germinating seeds of wheat.

Table 1-1. Important events in the understanding of pyrimidine nucleoside phosphorylation in higher plants.

Date	Contribution
1953	Brawerman & Chargaff report nucleoside phosphotransferase activity of plant origin. (12)
1955	Brawerman & Chargaff report wide distribution of NPTase among organisms. (14)
1956	Tunis & Chargaff achieve separation of NPTase activity from strictly hydrolytic phosphatase activity in carrot. (91)
1963	Hotta & Stern report presence and induction of thymidine kinase in *Lilium* microspores. (46)
1964	Wanka *et al.* report dissociability of thymidine kinase from germinating corn and wheat into two components. (98)
1965	Hotta & Stern demonstrate inducibility of thymidine kinase in germinating wheat embryos. (48)
1966	Wanka & Walboomers report pattern of development of components P and T of thymidine kinase in corn seedlings; they also report two different uridine kinase activities in the same organism. (97)
1971	Arima *et al.* suggest that component P is really an ATP-phosphohydrolase activity and component T is NPTase. (4)
1972	Deng & Ives demonstrate with [^{32}P]ATP experiment that NPTase is only thymidine phosphorylating enzyme in barley seedlings; they also report a true uridine kinase early in corn germination, later replaced by NPTase. (33)
1974	Brunori *et al.* suggest that improper treatment of extracts results in deceptively low thymidine kinase levels in plant tissues. (19)
1978	Hofman & Schwarz and Mullin & Fites both demonstrate absence of thymidine kinase in wheat and peanut seedlings, respectively. (45, 68)
1979	Richard *et al.* demonstrate formation of a phosphoryl-enzyme intermediate in NPTase reaction. (77)
1982	Prasher *et al.* suggest a modified ping-pong pathway for NPTase, involving phosphate transfer to a nucleoside or to water. (72)

CHAPTER II

AMP:NUCLEOSIDE PHOSPHOTRANSFERASE IN CRUDE EXTRACTS

INTRODUCTION

An enzyme activity in crude extract must compete with and be influenced by countless other factors in that extract during assay. For this reason, crude and purified activities may differ in optimal assay conditions. Because the work with developing seeds and seedlings in Chapter III is done using crude extracts, the properties of unpurified AMP:nucleoside phosphotransferase (NPTase) are examined in this chapter. NPTases from carrot (17, 18, 78) and from barley seedlings (72) have been purified and well-characterized. It is likely that the wheat NPTase is similar in character to these two enzymes, especially the barley NPTase.

There was no intention to slight the pyrimidine nucleoside kinases, since they too are included in the developmental study. One type of assay for comparison of both uridine and thymidine phosphorylation was all that could be handled easily. Since thymidine kinase has yet to be found in wheat, it was not possible to optimize its assay. Uridine kinase does exist in wheat, but is specific for uridine phosphorylation, while NPTase will phosphorylate both thymidine and uridine.

METHODS AND RESULTS

All of the experiments described in this section used crude extracts of four-day-old whole wheat seedlings (Triticum aestivum v. Lemhi) as enzyme source. The Lemhi wheat seeds were purchased in 1974 and stored in a cold room at 8 C. Despite their advanced years, the Lemhi seeds have consistently shown a high percentage germination, about 98%, under clean laboratory conditions in petri dishes.

The wheat seeds, which had been treated with fungicide, were washed and surface sterilized for 15 minutes with a solution containing 0.5% Tween 20 and 5.0% commercial hypochlorite (0.25% NaOCl). After this initial wash, the seeds were rinsed three times in sterile deionized water, once for 3-5 seconds in 0.1 N HCl, and three times again in the sterile water. Seeds were planted in rectangular pyrex casserole dishes which had been autoclaved along with triple layers of Whatman No. 1 chromatography paper. Approximately 90 ml of sterile distilled water were added to each dish prior to seeding. Seeds were placed ventral furrow down on the paper, with even spacing such that seedling contact and contamination would be minimized during the four day growth period. Seed number per dish was 70 (7 x 10). All seed sowing was carried out in a laminar flow hood and dishes were covered with transparent plastic food wrap before being placed in a growth chamber. The growth chamber in which the seeds and young seedlings were kept was set on a 16-hour light:8-hour dark cycle,

with a "day" temperature of 22 C and a "night" temperature of 18 C. The four day growth period was selected because the enzyme activity of interest was known to be present in good quantity at this stage of development (45) and because the wheat tissues at this stage were still tender enough to be disrupted easily.

At four days after sowing, whole wheat seedlings were separated from remaining endosperm and seed coat, weighed, and chilled briefly in ice water prior to being ground with buffer, any additives, and a pinch of sand in a chilled mortar. The standard buffer for tissue extraction was 50mM HEPES-KOH, pH 7.2, previously shown to be effective in this procedure (45). Approximately 3 ml of buffer were used per gram fresh weight of tissue. The tissue homogenate was filtered through eight layers of chilled, buffer-dampened cheesecloth, then centrifuged for 20 minutes at 15,000 rpm in the small head of a Beckman high speed refrigerated centrifuge at 2 C. The supernatant was used as crude enzyme source.

Enzyme Assays

The reaction catalyzed by NPTase is shown below. In this reaction a phosphoryl group is transferred from a nucleoside

$$\text{NUCLEOSIDE}_1 + \text{N}_2\text{MP} \rightleftharpoons \text{N}_1\text{MP} + \text{NUCLEOSIDE}_2$$

$$(^{14}\text{C-thymidine} + \text{AMP} \rightleftharpoons {}^{14}\text{C-dTMP} + \text{adenosine})$$

monophosphate donor to a nucleoside acceptor. Products are a new nucleoside and nucleoside monophosphate.

The assay for determining the activity of NPTase made use of a ^{14}C-labelled nucleoside, [^{14}C]thymidine, as acceptor, and an unlabelled nucleoside monophosphate, AMP, as phosphoryl donor. Of the two pyrimidine nucleosides under consideration in this report (thymidine and uridine), thymidine is the better phosphoryl acceptor. AMP was used as phosphoryl donor in order to be consistent with later studies comparing efficiency of phosphoryl transfer from AMP and ATP. A typical assay mixture contained in final concentrations: 0.02 mM [2-^{14}C] thymidine (0.01 µCi/assay), 5 mM AMP, 50 mM HEPES-KOH, pH 7.0, and enough crude extract to give less than 8% net thymidine phosphorylation (see below), all in a total volume of 100 µl. Standard reaction time was five minutes at 35 C, with timing begun upon addition of crude extract to the reaction mixture. The reaction was terminated by heating the mixture in a boiling water bath for two minutes, followed by transfer of the assay tubes to an ice bath. Tubes were centrifuged at 5,000 rpm for five minutes in order to return all condensate to the bulk mixture and to precipitate denatured protein. Fifty µl of each reaction mixture was then spotted on sheets of Whatman 3 mm chromatography paper and allowed to dry thoroughly. Chromatograms were developed by ascending chromatography using water-saturated butanol as solvent. This system effected the separation of nucleosides (upper spots) from nucleotides (at origin), but did not resolve the various nucleotides. Nucleoside and nucleotide spots were located under far UV light, cut out, placed in separate scintillation vials, each with 10 ml of cocktail containing 4.2 g PPO and

0.053 g POPOP per liter of toluene, and counted in a liquid scintillation spectrometer. NPTase activity was determined by converting net percent phosphorylation of the thymidine [(cpm nucleotide spot/(cpm nucleotide spot + cpm nucleoside spot)) x 100] to net nmoles of dTMP produced. It should be noted that the nucleotide spot probably included dTMP + dTDP + dTTP, as there was the possibility that nucleotide phosphorylating enzymes had further phosphorylated some of the dTMP product initially produced by action of NPTase. The variability of this assay for a single tissue extraction is ± 5%. For successive tissue extractions, variability is less than ± 9%.

In addition to the NPTase assay, in some experiments AMP phosphohydrolase activity was measured. The assay mixtures for this assay were identical to those for the NPTase measurement, with the exception that water was substituted for the [^{14}C]thymidine. Activity was measured as nmoles of inorganic phosphate released per minute. Inorganic phosphate was determined by the method of Itaya and Ui (50), which is valid within the range of 0.5-20 μg P_i per ml.

The purity of the ^{14}C-labelled nucleosides was checked periodically by chromatography on thin layers of polyethyleneimene-cellulose (PEI-cellulose), developed first in distilled water, followed by drying and redevelopment in 1 M LiCl (73, 74).

Liquid scintillation counting was performed in a Beckman LS 200 spectrometer which had a counting efficiency of 94% for ^{14}C. The amount of quench due to the solid paper support and presence of nucleoside was approximately 25%, varying only slightly within the

range of nucleoside/nucleotide levels encountered within an assay. Because the results were expressed as a ratio of nucleotide: nucleotide + nucleoside, quench could be virtually ignored, especially since differences in paper size within the range used did not appear to affect the amount of quench. Protein was determined by the Lowry method (57).

Measurement of NPTase in Crude Extracts

The presence of reactions that compete with the forward NPTase reaction must be considered when measurement of NPTase activity in crude extracts is undertaken. Preliminary experiments with crude extracts from four day old wheat seedlings have shown that degradation of thymidine over the usual assay reaction time is negligible. Further phosporylation of the dTMP product also is not a problem because separation of thymidine nucleotides is unnecessary in the assay procedure described earlier. While higher phosporylation of the AMP phosphoryl donor is a possibility, this reaction can also be regarded as negligible when compared with the hydrolysis of AMP that takes place in crude extracts. AMP phosphohydrolase activity is considerable and can be detected not only by measurement of P_i release, but also visually on the developed paper chromatograms of assay reaction mixtures, where an unlabelled spot corresponding to the position of adenosine is consistently found. In preliminary experiments it was found that a 5 mM final concentration of AMP in the assay reaction mixture was sufficient for this substrate not

to become limiting during the course of the assay; that is, after a five minute reaction time, the addition of more AMP did not cause an increase in NPTase activity. The hydrolysis of dTMP product in the reaction is also a variable to consider. However, since the low concentration of thymidine used in the assay also means a relatively low level of dTMP produced compared to the amount of AMP still available in the reaction mixture, it is doubtful that significant hydrolysis of dTMP takes place over the course of the reaction. This suggestion presumes that the phosphohydrolyzing activity present in crude extracts is not specific for any one nucleoside alone. In fact, this is the case for the phosphohydrolyzing activity found to be associated with purified NPTase enzyme from carrot (18) and barley (72); presumably, this activity is also associated with wheat NPTase (see later experiments). Whether or not there exists a specific dTMP phosphohydrolase in wheat extracts is unknown. In addition to the competing reactions already mentioned, the back reaction using adenosine as phosporyl acceptor and dTMP as phosporyl donor is also a possible factor limiting the measurable activity of NPTase.

Whatever competing reactions may be affecting the apparent activity of the NPTase in crude extracts, it is the case that one rarely sees above 30% net thymidine phosphorylation under the assay conditions described; the same is true using uridine as phosphoryl acceptor. By contrast, the uridine kinase activity found in 12-hour imbibed wheat embryos (see Chapter III) produces as high as 98% net

uridine phosphorylation, with assay conditions the same as for the NPTase-catalyzed reaction, excepting the use of ATP rather than AMP as the phosphoryl donor. Increasing the thymidine substrate concentration by three-fold does not increase the percent phosphorylation (Figure 2-1b), although it increases the absolute amount of dTMP produced (Figure 2-1a). The amount of thymidine substrate utilized in the NPTase assays reported here was kept small in order to conserve label while still having a reasonable ratio of labelled to unlabelled nucleoside. So that enzyme concentration would correspond to enzyme activity in a linear fashion, it was determined that the crude extract would have to be diluted so as to give less than 8% net thymidine phosphorylation. Figure 2-2 shows the results of one experiment in which an extract using 3 ml of buffer per gram fresh weight of seedling tissue was subjected to serial dilutions prior to assays. Activity was linear with respect to enzyme concentration up until a phosphorylation level of about 7%, after which point it began to level off. Estimations of relative enzyme amount or activity would probably be underestimated at phosphorylation levels much above 8%. Figure 2-3 shows the desired linear relationship between enzyme concentration and activity in an experiment that began with an initially dilute crude extract (less than 8% thymidine phosphorylation) and further reduced the enzyme concentration.

Storage of Crude Extract in Refrigerator and Freezer

Stability of the NPTase activity during refrigerator and freezer storage of crude extract was examined. Equal volumes of

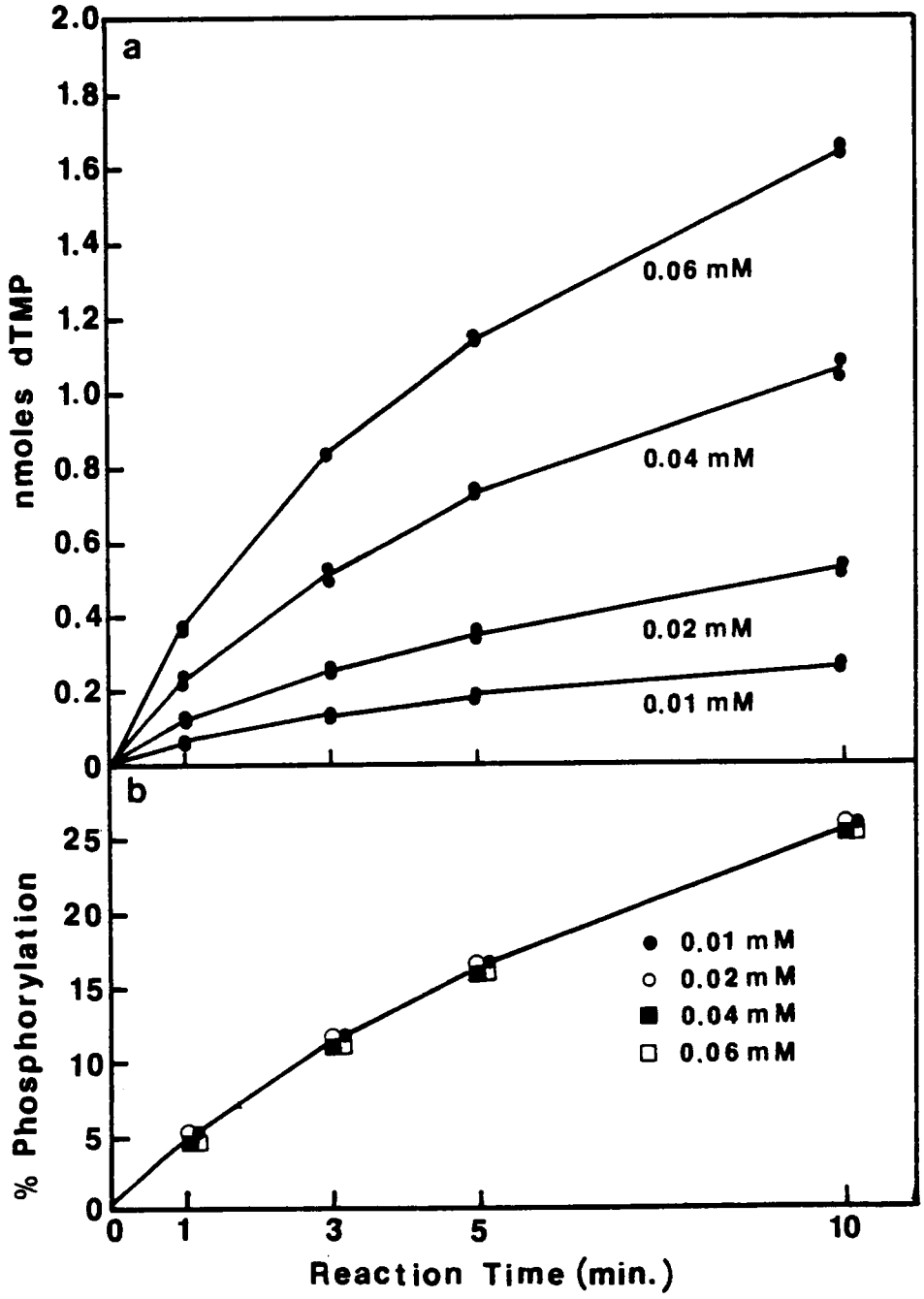

Figure 2-1. NPTase activity at different thymidine substrate concentrations, expressed as (a) nmoles of product and (b) percent phosphorylation of substrate.

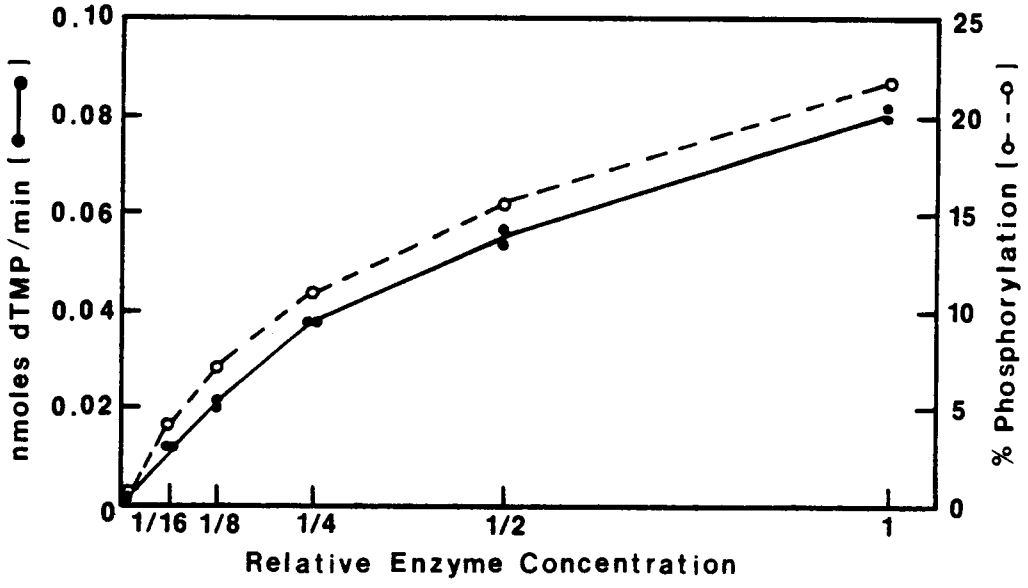

Figure 2-2. Relationship between NPTase activity and relative enzyme concentration in crude extracts.

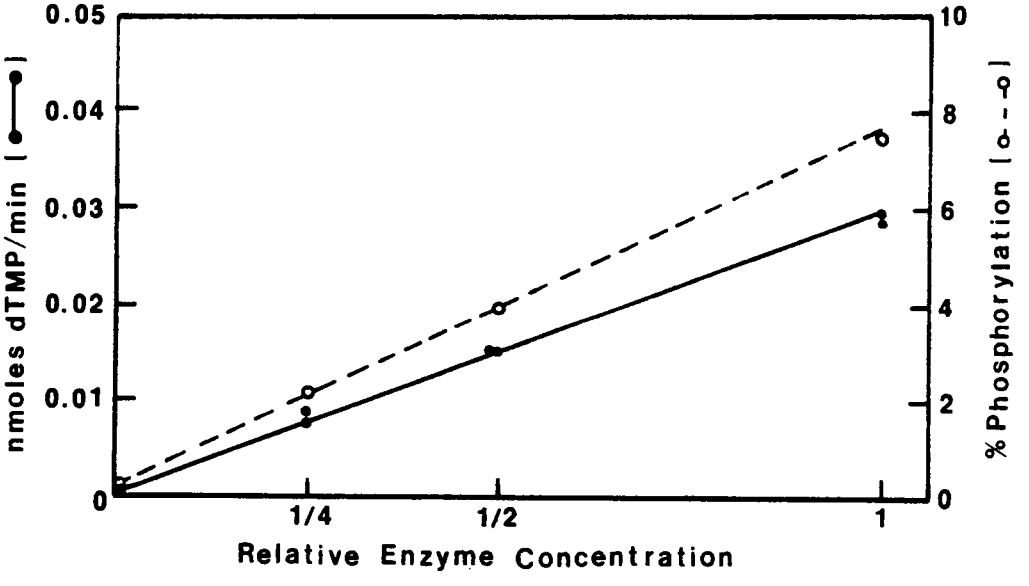

Figure 2-3. Relationship between NPTase activity and relative enzyme concentration in dilute crude extracts.

crude extract were placed in a lab refrigerator (4 C) or freezer (-18 C) for various lengths of time (see Figures 2-4 and 2-5). Prior to assay, extracts were centrifuged at 10,000 rpm for ten minutes and the supernatant brought to assay temperature. Figure 2-4 shows specific activity of NPTase over a two-week period in the refrigerator. While there is a slight decrease in specific activity over the first several days, by the end of a week there has actually been an increase in specific activity over the initial value. Examination of protein values reveals that there is little change in these levels over the first several days, but the protein values for days 7 and 14 have decreased by about 30%. These decreases in protein in the stored extract reflect removal of less stable proteins and account for the increase in specific activity, since total activity does not change noticeably over the storage period. Figure 2-5 shows specific activity of NPTase over an eight week period in the freezer. Specific activity shows a steady decrease over the first five weeks, and then a sudden increase by the eighth week. The increase in specific activity in this case cannot be attributed to a sudden decrease in protein of the sample; rather the activity of the sample increases. One possible explanation of this phenomenon is that some inhibitor of NPTase has become inactive during the lengthy storage; however, no further studies were undertaken to clarify the matter.

Tissue extracts are sometimes stored in solution with compounds such as ammonium sulfate or glycerol, since these have been shown to stabilize some enzyme activities. Although no long term storage

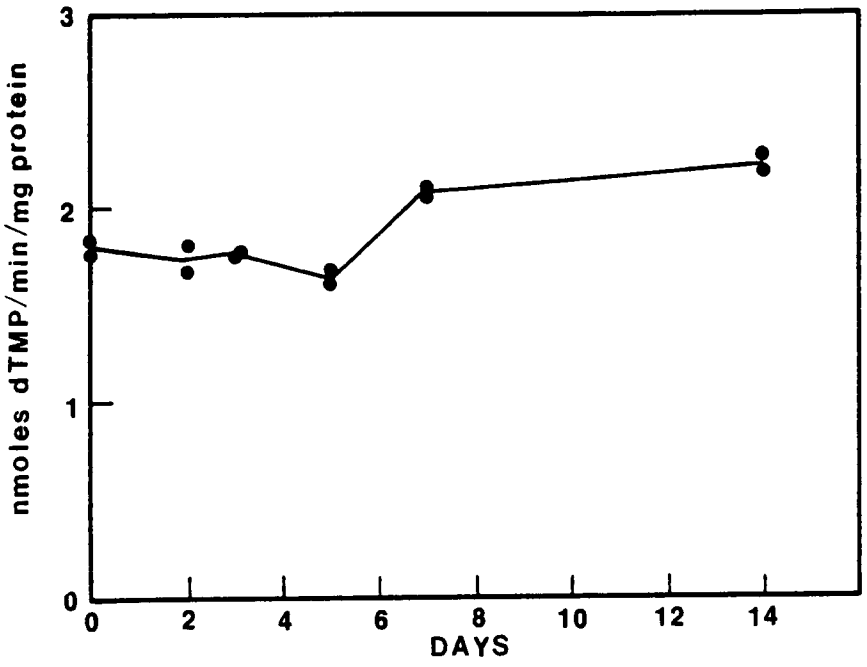

Figure 2-4. Stability of NPTase during refrigerator storage.

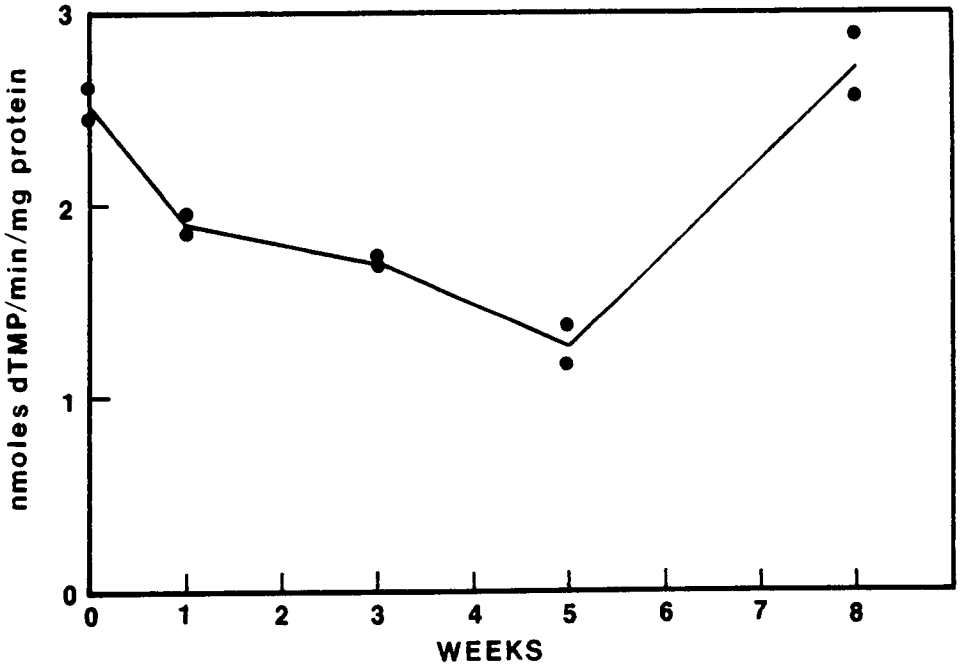

Figure 2-5. Stability of NPTase during freezer storage.

experiments were performed using either substance, it may be worthwhile to note that overnight contact with 50% glycerol caused a 30% decrease in NPTase activity. Overnight storage with 50% ammonium sulfate caused a 60% increase in specific activity, attributable to the precipitation of non-NPTase protein; most NPTase activity is precipitated at 60-80% ammonium sulfate saturation (see p. 32). Whether the ammonium sulfate actually stabilizes the NPTase activity to any significant degree over longer storage times is unknown.

Effect of PVPP on Extraction of NPTase Activity

Polyvinylpyrrolidone (PVP) is often used in extraction of plant tissues because of its ability to adsorb phenolics which may interfere with enzyme activities. Although PVP as purchased commercially (Polyclar AT; GAF Corporation) may be somewhat effective, it has been reported that the highly cross-linked form, polyvinylpolypyrrolidone (PVPP), formed by boiling PVP in 10% HCl followed by extensive water washing to remove Cl-, is more effective (1). Also, hydration of PVPP in extraction buffer prior to use is recommended. Table 2-1 shows the results of three wheat seedling extractions using (a) unwashed, but hydrated PVP, (b) unhydrated, but washed PVPP, and (c) hydrated, washed PVPP. The effect on extractable NPTase activity is not large, but what difference there is appears to indicate that the hydration step is more important than the pre-washing.

In order to determine what effect addition of PVPP to the extraction buffer had on NPTase activity in crude extracts, the

Table 2-1. Effect of PVP pre-treatment on extraction of NPTase activity.

PVP Addition	nmoles dTMP/min/mg protein	
PVP - hydrated	1.95 2.11	(2.03)
PVPP - dry	1.92 1.87	(1.90)
PVPP - hydrated	2.26 2.26	(2.26)

following amounts of hydrated PVPP were included in the extraction procedure: 0, 0.01, 0.05, 0.1, 0.2, and 0.5 g PVPP per g fresh weight of tissue. Figure 2-6 shows that amounts of PVPP, at least up to 0.2 g/g fr. wt. of tissue, can increase extractable NPTase activity, but amounts of PVPP as high as 0.5 g/g fr. wt. of tissue can have an inhibitory effect. Even with PVPP, the increase in NPTase activity was not much more than 10%.

Effect of KCl on Extraction of NPTase

In some preliminary experiments it was noted that a certain percentage (about 20%) of the total NPTase activity remained in the

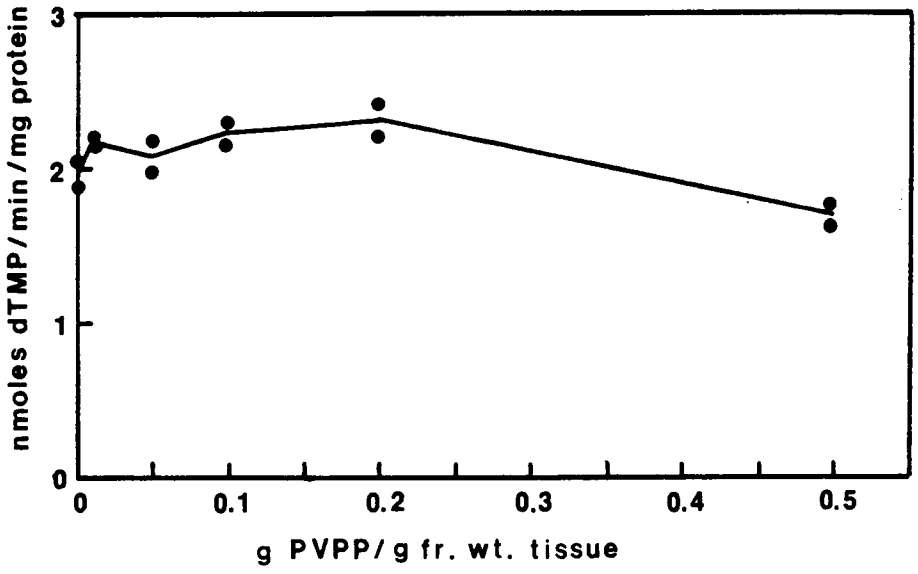

Figure 2-6. Effect of PVPP on extraction of NPTase.

pellet fraction of the wheat seedling homogenate. While some of this activity could have been the result of entrapment within unruptured organelles, it was also possible that a certain amount of NPTase remained loosely bound to membranes. The addition of KCl to extraction buffers can help to release such loosely bound enzymes by increasing the ionic strength of the surrounding medium, thereby disrupting ionic attractions between the enzyme and other membrane components. Therefore, 0.1 M KCl was added to the extraction buffer and the supernatant was assayed immediately or after overnight dialysis against 50 mM HEPES-KOH, pH 7.2, in order to remove the KCl. It was not known whether the presence of salt in the assay reaction mixture would interfere with the reaction itself or with the subsequent chromatography. The results, shown in Table 2-2,

Table 2-2. Effect of KCl addition on extraction of NPTase activity.

Salt Treatment	nmoles dTMP/min/mg protein	
-0.1 M KCl (no dialysis)	3.15 2.99	(3.07)[a]
+0.1 M KCl (no dialysis)	2.90 2.96	(2.93)
-0.1 M KCl (with dialysis)	2.66 2.46	(2.56)
+0.1 M KCl (with dialysis)	2.49 2.41	(2.45)

[a]Average of the two individual figures.

indicate that the presence of salt did not interfere markedly with the assay, although spots on the developed chromatogram were more diffuse. On the other hand, the addition of KCl to the extraction buffer did not enhance the recovery of NPTase activity.

Sulfhydryl Reagents in Extraction and Assay Buffers

Some enzymes in solution require the presence of reagents that will keep sulfhydryl groups at critical sites on the enzyme in a reduced and protected state. For example, a uridine kinase found in corn has been reported to require the presence of sulfhydryl

reagents in order for activity to be measured (33). It has also been suggested (19) that other nucleoside kinases require some sulfhydryl protection. Apparently, since some NPTase activity can be measured in the absence of any sulfhydryl reagents, such an additive is not absolutely essential. However, in order to test whether the presence of sulfhydryl reagent gave higher enzyme activity, four commonly used sulfhydryl reagents were added singly to the extraction and assay buffers of crude extracts. Three different concentrations (see Table 2-3) were tested for each of the following reagents: 2-mercaptoethanol, dithiothreitol, ascorbic acid, and glutathione. The results, shown in Table 2-3, indicate that none of the four sulfhydryl reagents gave improved activities over that obtained with HEPES buffer alone. In fact, the presence of any of the four gives lower activity, down to almost 50% of the control activity with ascorbic acid and glutathione. Normally, sulfhydryl reagents were omitted from the extraction and assay buffers for measuring NPTase activity, the exception being the use of dithiorthreitol in the extraction buffer used for the developmental studies reported in Chapter III (see Methods).

Buffer and pH for Assay of NPTase Activity

Six buffers with overlapping pH ranges of effectiveness were used as assay buffer to measure NPTase activity. The buffers, all at a final concentration of 0.1 M, are shown in Figure 2-7, along with the pH range used for each. Figures 2-8a and 2-8b show the

Table 2-3. Effect of sulfhydryl reagents on extraction and assay of NPTase activity.

Sulfhydryl Reagent	Final Conc. (M)	nmoles dTMP/min/mg protein	
HEPES Buffer only	——	2.55 2.43	(2.49)[a]
2-Mercaptoethanol	10^{-4}	1.90 1.86	(1.88)
	10^{-3}	2.07 2.02	(2.05)
	5×10^{-3}	2.19 1.79	(1.99)
Dithiothreitol	5×10^{-5}	2.05 1.95	(2.00)
	10^{-4}	1.95 1.93	(1.94)
	10^{-3}	1.84 1.89	(1.87)
Ascorbic Acid	10^{-4}	1.56 1.50	(1.53)
	10^{-3}	1.29 1.18	(1.24)
	5×10^{-3}	1.35 1.23	(1.29)
Glutathione	10^{-4}	1.31 1.22	(1.27)
	10^{-3}	1.22 1.29	(1.26)
	5×10^{-3}	1.42 1.31	(1.37)

[a]Average of the two individual figures.

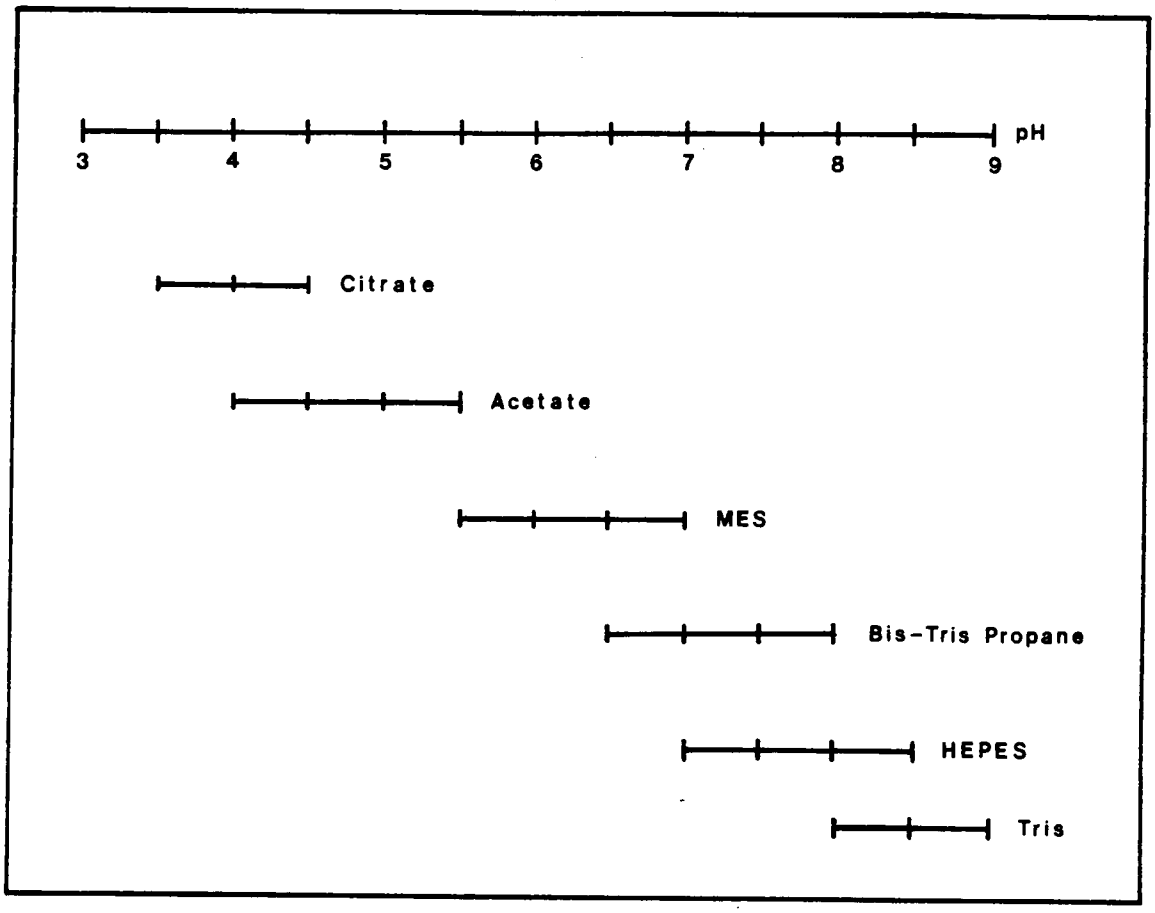

Figure 2-7. Buffers and pH ranges used to determine pH optimum of NPTase in crude extracts.

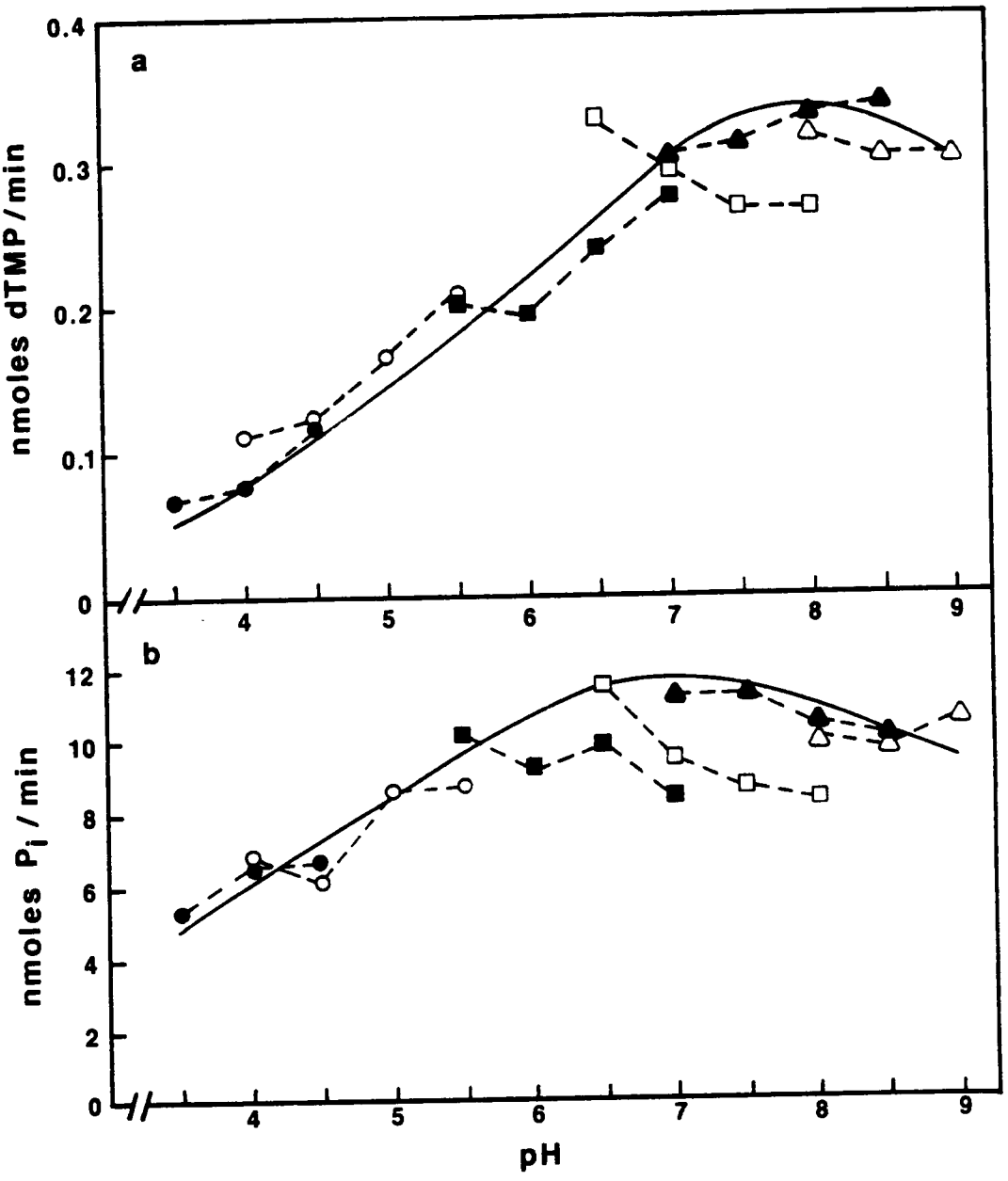

Figure 2-8. Effect of buffer and pH on (a) NPTase and (b) AMP phosphohydrolase activities. Buffers: (●) Citrate; (o) Acetate; (■) MES; (□) Bis-Tris Propane; (▲) HEPES; (△) Tris.

effects of different buffers and pH's on both NPTase activity and AMP phosphohydrolase activity in crude extracts. If one can look through the buffer effects, it appears that the NPTase activity shows a somewhat broad optimum between pH 7.0 and 8.5; the AMP phosphohydrolase activity also has a fairly broad optimum, apparently between pH 6.5 and 7.5.

Effect of Temperature on the Stability of NPTase

A good temperature at which to measure NPTase activity is 35 C (45). Because some assays must be run over extended lengths of time (e.g., in the case of very low enzyme activity), it was necessary to find out how well NPTase activity held up at 35 C over such an extended period. Equal volumes of a crude extract were incubated at 35 C for various lengths of time, up to two hours. At the end of each pre-incubation time, the samples were chilled, centrifuged at 10,000 rpm for 10 minutes, and then assayed at 35 C as usual. Figure 2-9 shows that NPTase activity is indeed stable over at least a two-hour period at 35 C.

A test was also made to determine if 35 C was indeed a suitable temperature at which to assay NPTase activity. Equal volumes of wheat crude extract were pre-incubated for 20 minutes at the following temperatures: 20, 25, 30, 35, 40, 45, 50, 60, and 70 C. At the end of each incubation period, the sample of crude extract was chilled in an ice bath, centrifuged at 10,000 rpm for ten minutes, and then used as enzyme source for the NPTase and AMP

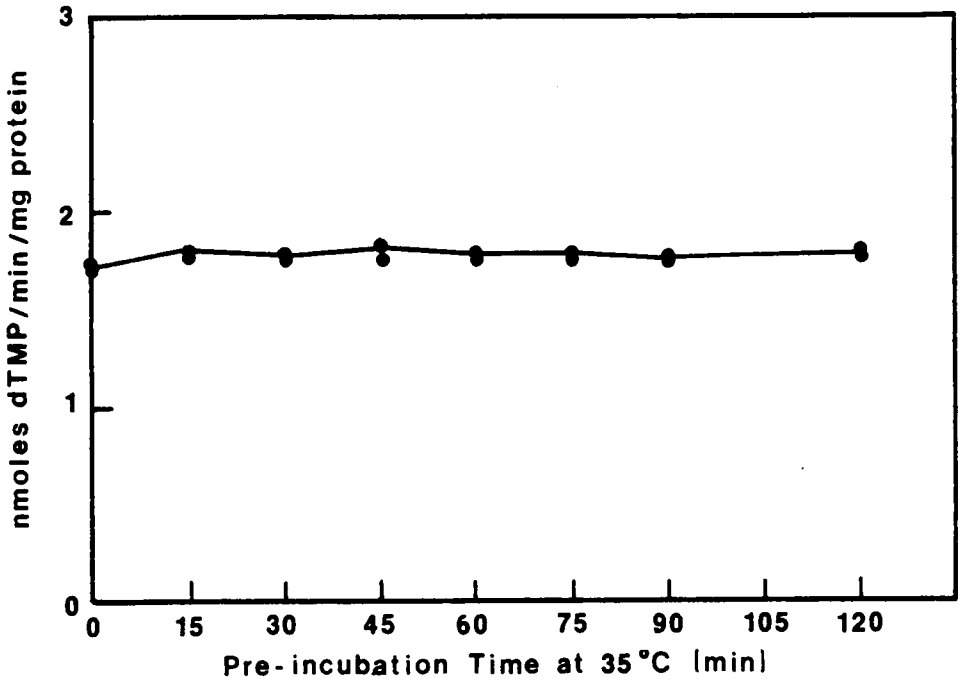

Figure 2-9. NPTase stability over time at 35 C.

phosphohydrolase assays. The results, plotted in Figure 2-10, show that NPTase activity is fairly stable up to 50 C, beyond which temperature it rapidly drops off. At no point is there a sudden increase in specific activity for NPTase, as is sometimes the case when other more heat-labile proteins denature at lower temperatures. AMP phosphohydrolase activity shows a small increase in specific activity at 50 C, and then rapidly drops off at higher temperatures. There is a slight drop in NPTase activity above 30 C, but for a 5 minute assay reaction time, 35 C is an acceptable assay temperature.

Ammonium Sulfate Fractionation of the Crude Extract

Although no thorough purification of NPTase from wheat was attempted, an ammonium sulfate fractionation was performed on the

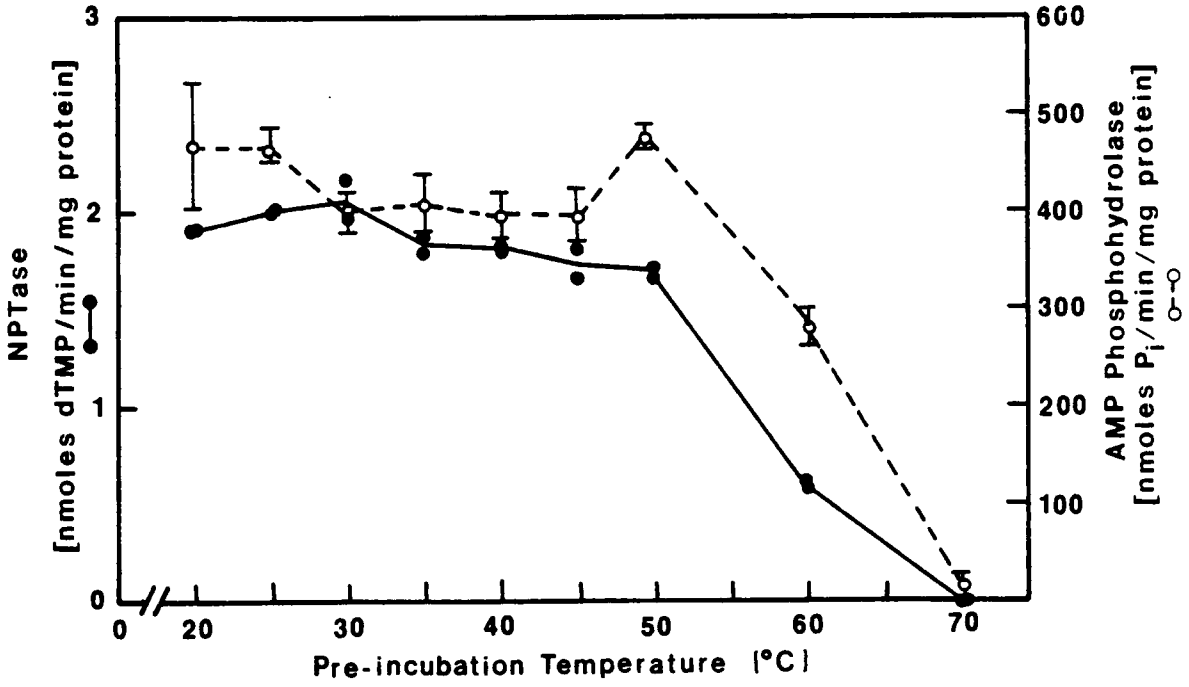

Figure 2-10. NPTase and AMP phosphohydrolase activities after 20 minutes pre-incubation at different temperatures.

crude extract in order to see if the AMP phosphohydrolase activity could be separated from NPTase activity in one step. After tissue homogenization and centrifugation, the crude supernatant was brought to 10% saturation with ammonium sulfate and centrifuged at 15,000 rpm for 10 minutes. The pellet was resuspended in a small volume of 50 mM HEPES buffer, pH 7.2, placed in dialysis tubing, and dialyzed overnight against the same buffer, with at least one change of buffer. After dialysis, the volume of the solution in the dialysis tubing was determined and the contents assayed for NPTase and AMP

phosphohydrolase activities. Meanwhile, the supernatant from the previous 10% cut was brought to 20% ammonium sulfate saturation, and the procedure described above repeated. Salt cuts were made at 10% saturation increments through 90% salt saturation.

Figure 2-11 shows a typical ammonium sulfate fractionation of the crude extract, with NPTase specific activity and total activity plotted in Figure 2-11a and AMP phosphohydrolase specific and total activity plotted in Figure 2-11b. Also included in each graph is the total protein per fraction. Both NPTase and AMP phosphohydrolase show peak specific activities in the 70% salt saturation fraction. Also, in both cases, most of the total activity is collected between 50% and 70% salt saturation of the crude extract. In addition, AMP phosphohydrolase shows a fairly high specific activity in the 10% salt cut; whether or not this early precipitating activity represents a different protein is unknown.

DISCUSSION

It appears that NPTase in crude extracts of four-day-old wheat seedlings is a fairly heat stable activity that can also hold up during refrigerator and freezer storage. The activity was found to have a pH optimum of roughly 7.5-8.0. Prasher *et al.* (72) report a somewhat lower pH optimum for the purified NPTase from barley, about pH 6.5. Slightly higher AMP phosphohydrolase activity at around pH 7.0, and perhaps the activities of other unmeasured enzymes that compete with the phosphotransferase reaction at a somewhat lower

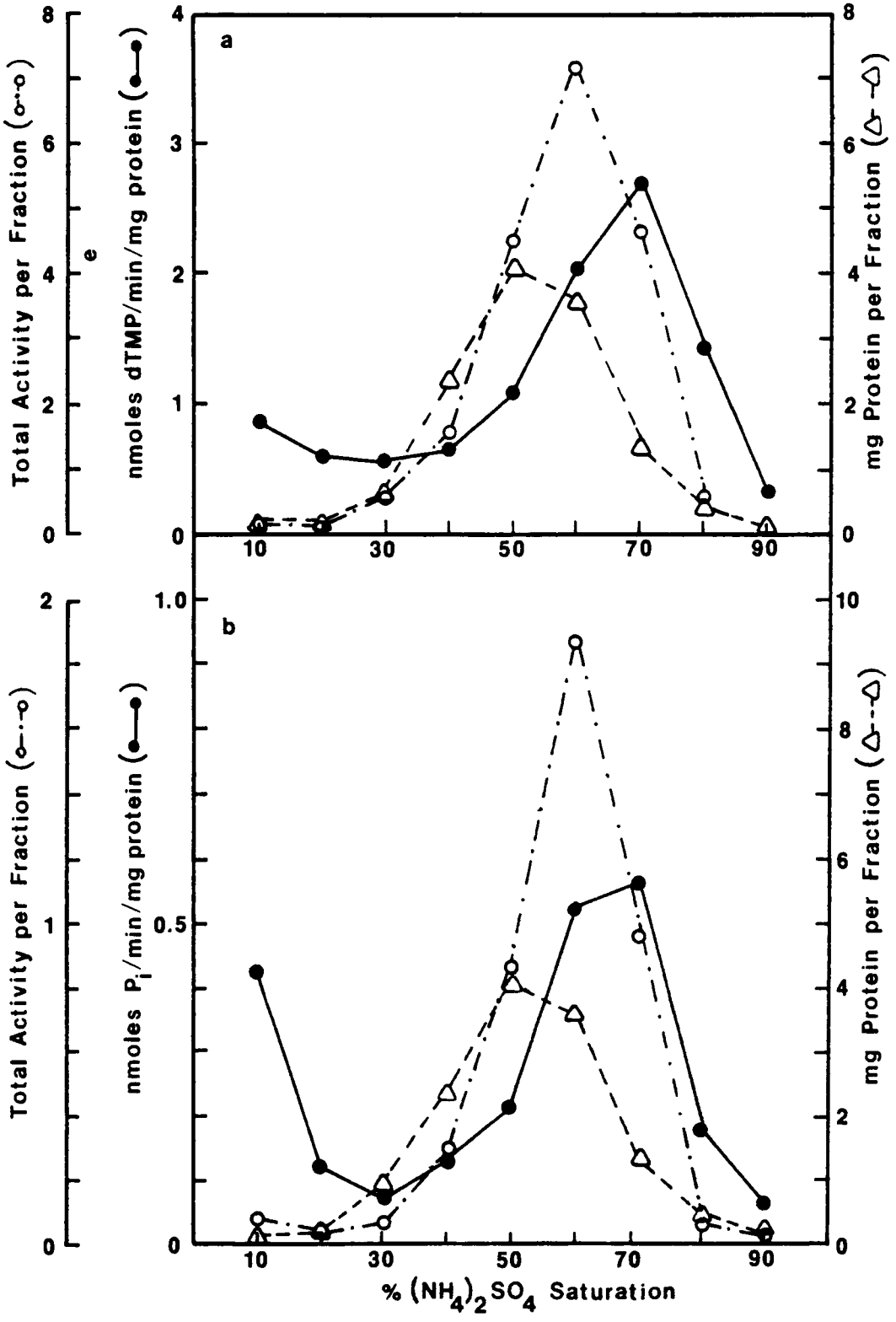

Figure 2-11. $(NH_4)_2SO_4$ fractionation of wheat crude extract: (a) NPTase; and (b) AMP phosphohydrolase.

pH, could account for a seemingly higher pH optimum for NPTase in crude extracts as opposed to NPTase in a more purified state. NPTase activity does not seem to require the presence of sulfhydryl reagents, as is the case for most nucleoside kinases. NPTase from carrot (18) and barley (72) likewise did not require sulfhydryl protection. One influence on NPTase activity that was not investigated in this work was the possible requirement for a divalent metal cation such as Mg^{2+}, which is required by all kinases. However, previous work by Hofman & Schwarz (45) has shown that NPTase in crude wheat extracts does not require such a divalent metal cation. In the developmental work reported in Chapter III, where both AMP and ATP were used as phosphoryl donors, Mg^{2+} was included whenever ATP was used as a phosphoryl donor, but omitted when AMP was used. The addition of KCl to the extraction medium did not enhance NPTase activity. It may be that the activity associated with the pellet fraction is due to NPTase compartmentalized within unruptured organelles, or perhaps some of the enzyme is more intimately associated with a membrane, such that simply increasing the ionic strength of the extracting medium would not be sufficient to release it. If this were the case, the addition of a detergent during the extraction procedure might release such a bound activity. The idea that some NPTase could be associated with a membrane surface is not unreasonable, given the probable role of this activity in rephosphorylating nucleosides that have been transported from one cell to another or from one intracellular compartment to another. Rapid attachment of a

phosphoryl group to an incoming nucleoside would not only prepare it for further use, but would also prevent its loss from that particular compartment by making it a charged molecule. However, the fact that about 80% of the total NPTase from wheat seedlings is readily released in soluble form argues against most of the activity having any strong association with membranes. The addition of PVPP to the extraction medium helped to increase the recovery of NPTase to a small degree.

It has been found that the purified NPTase proteins from carrot (18) and from barley (72) can exhibit not only phosphotransferase activity, in which a phosphoryl group is transferred from a nucleotide monophosphate to the 5' position of another nucleoside, but also a phosphohydrolyzing activity in which the phosphoryl group is transferred from the nucleoside monophosphate to water. These two functions are believed to involve different sites on the enzyme. It is not surprising, therefore, that most of the AMP phosphohydrolase activity measured in crude wheat extracts shows similar heat stability and ammonium sulfate precipitation properties as the NPTase activity. It is probable that both the NPTase and AMP phosphohydrolase activities from wheat could be co-purified and shown to exist as separate functions of a single active protein. This does not necessarily mean, however, that there are not some other distinct AMP phosphohydrolyzing activities present in wheat.

CHAPTER III

DEVELOPMENTAL PROFILE OF PYRIMIDINE NUCLEOSIDE PHOSPHORYLATION

INTRODUCTION

During the development of a wheat embryo and during subsequent germination, pyrimidine nucleotides are required for RNA and DNA synthesis and also for the formation of nucleotide-sugars used in cell wall biosynthesis and in starch synthesis. It is reasonable to expect that, in addition to the probable de novo pathway of pyrimidine nucleotide biosynthesis, enzymes responsible for regenerating pyrimidine nucleotides from nucleosides would be present in the developing and germinating embryo. The purpose of the following experiments is to determine what kinds of pyrimidine nucleoside phosphorylating enzymes are present at different stages in the development of the wheat embryo.

The first set of experiments examines the developmental period from anthesis through seed maturation. Although the main tissue of interest is the embryo, other tissues of the developing caryopsis are also examined for the sake of comparison. The second set of experiments looks at the germinating embryo and early stages of seedling development. Thymidine phosphorylation in four day old wheat seedlings has already been examined (45), but uridine phosphorylation has not. In addition to measuring general nucleoside

phosphorylation, an attempt is also made to distinguish between different kinds of nucleoside phosphorylating enzymes, specifically between nucleoside phosphotransferase and nucleoside kinases.

METHODS

Two varieties of wheat were used for comparison in the developmental studies described below: Triticum aestivum L. v. Lemhi, a soft, white winter wheat, the age and storage conditions of which are given in Chapter II; and T. aestivum L. v. Arthur, a soft, red winter wheat obtained from Dr. Vernon Reich of the Plant and Soil Science Department at The University of Tennessee School of Agriculture. At time of sowing, the Arthur seed was fresh, having been harvested in the summer of 1982.

Developing Seed Study

For the study following enzyme activities from anthesis to seed maturity, the two varieties of wheat were planted at the Plant Science Field Research Laboratory in October of 1982. Arthur is well adapted to the east Tennessee climate and is grown commercially in the area. Lemhi is no longer grown commercially and is better suited to a drier climate, since it is susceptible to leaf rusts and powdery mildew. The development of the young wheat seedlings was followed throughout the early spring of 1983. The Lemhi seedlings showed faster early growth while the Arthur seedlings remained quite small. Unfortunately, the Lemhi seedlings suffered from some late frosts and from attacks of leaf rust. The Arthur seedlings, although

slower to start their spring growth, actually came to anthesis about one and a half weeks before the Lemhi seedlings. It should also be noted that while the Arthur seedlings appeared quite robust and healthy throughout the growth period, the Lemhi seedlings were in poor condition, due to their early cold injury and disease attack, and also to decreased vigor from long seed storage. The Lemhi plants did follow normal development eventually, but at harvest it was apparent that Lemhi seeds were less well filled than the Arthur seeds. Because the Arthur heads reached anthesis at an earlier point in the spring when it was cooler, the time period from anthesis to harvest was longer than for Lemhi heads which reached anthesis later and developed somewhat more rapidly, probably due to the warmer temperatures during this time. The entire sampling period for Arthur was 6 weeks; the sampling period for Lemhi was 5 weeks.

In wheat, anthesis (shedding of pollen) occurs while the florets are still closed. Within a few hours after anthesis, the florets open somewhat and the stamens lengthen so that they become visible to an observer. For simplicity's sake, anthesis was dated from the day on which stamens first became externally visible in a head of wheat. Heads were tagged and dated on the day the first florets reached anthesis and were harvested at two- to three-day intervals thereafter until seeds were dehydrated to such a point that embryos could not easily be removed intact.

Whole heads of wheat were harvested, placed on ice while still in the field, and transported to the laboratory within 20 minutes

of harvest. Heads were measured for length, weighed, and then the primary florets from five spikelets in the middle of each of five heads were removed. There were two primary florets per spikelet so that tissues from 50 individual flowers were dissected out of the five heads within a sample. In wheat, the spikelets in the middle of the head generally develop earliest and, since it was from the earliest indication of anthesis in the head that dating commenced, it was these most mature spikelets that were collected. In most spikelets of these two varieties, only the two primary florets develop seeds. In the earliest samples (roughly days 0-4 post-anthesis), the entire pistil from each primary floret was collected, the pistil consisting mostly of ovary. The 50 ovaries collected were then ground in a chilled mortar with 1 ml of 50 mM HEPES-KOH, pH 7.0, containing 2.5 mM dithiothreitol (DTT). The DTT was included in case any of the uridine or thymidine phosphorylating activities were kinases that require such sulfhydryl protection, even though the presence of DTT could cause some reduction in NPTase activity. By day 6 post-anthesis the green ovules could be separated easily from the surrounding ovary tissue. Each type of tissue was ground separately in the extraction buffer given above. On day 13 post-anthesis for Arthur and day 11 for Lemhi the following tissues could be easily discerned under a dissecting scope and separated from one another: the ovary wall, now thinner and less succulent than at first; the green "ovule integuments," possibly also including the developing aleurone layer when dissected from

the rest of the endosperm; the endosperm, now somewhat solid and beginning to fill out a little; and the embryo, visible as a darker, somewhat translucent area at the pointed end of the fruit on the side opposite the ventral furrow. Each of these tissues was ground separately in extraction buffer. It should be noted that no thorough microscopic examination of tissue sections was performed in order to characterize the above mentioned developing wheat seed parts; therefore, the tissue designations given above are acknowledged to be somewhat rough, especially the layers surrounding the developing starchy endosperm and embryo. For example, the aleurone layer, technically a part to the endosperm tissue, is thought to have been removed along with the integuments surrounding the endosperm and embryo. On one day only in each variety, day 26 post-anthesis in Arthur and day 24 post-anthesis in Lemhi, was the thin membranous layer presumed to be the aleurone separated from the ovule integuments and ground with the rest of the starchy endosperm. In both Arthur and Lemhi, as the seeds matured, the ovary wall became very thin and transparent; the ovule integuments became much thinner and paler green; the endosperm became gooey and whitish as it filled with starch, with a consistency much like bread dough; and the embryo became well-defined and somewhat lenticular in shape. By day 33 in Arthur and day 30 in Lemhi, the seed kernels were mostly brown and starting to look somewhat dehydrated. At this point and until the end of the sampling period, the old ovary layer, ovule integuments (+ aleurone), and starchy endosperm (now much harder and more

powdery) could no longer be separated easily and were therefore left as one unit for grinding. The embryo was removed from the seed as an intact unit and ground separately. The sample period ended for each variety of wheat when the heads were brown and dry, the seeds also brown with hard endosperm, and the embryo somewhat shrunken in size and dehydrated. The seed moisture content at this time was about 17.5%. Seed moisture content was followed throughout the entire developmental period.

After each tissue sample was ground with buffer in the chilled mortar, the grindate was centrifuged at 15,000 rpm for 20 minutes in a refrigerated centrifuge at 2-4 C; the supernatant was used as the source of enzyme activities. A white lipid layer was generally present on top of the embryo supernatant after centrifugation; the aqueous layer beneath the lipid was gently sucked off with a pasteur pipet. The assay reaction mixtures were as described in Chapter II. Either 0.02 mM [2-^{14}C] uridine or [2-^{14}C] thymidine was used as phosphoryl acceptor, and 5 mM AMP or 5 mM Mg^{2+}/ATP used as phosphoryl donor. Chromatography, liquid scintillation counting, and calculation of activities were also as described in Chapter II.

Early Seedling Study

Uridine and thymidine phosphorylation were also followed in Lemhi and Arthur embryos and seedlings from early stages of seed germination through one week after seed sowing. Surface sterilization and seed planting in pyrex dishes were as described in Chapter II.

Embryos or young whole seedlings were harvested from 12 hours after sowing through 96 hours (4 days) at 12-hour intervals, and from 96 through 168 hours (7 days) at 24-hour intervals. Embryos or seedlings were excised, weighed, chilled, and ground in 50 mM HEPES-KOH, pH 7.0, containing 2.5 mM DTT. The grindate was then centrifuged at 15,000 rpm for 20 minutes at 2-4 C and the supernatant used as enzyme source. Assay conditions were as described above, as were treatments of the final reaction mixture and data work-up.

[^{32}P]ATP Study

In order to distinguish between a nucleoside kinase and the less specific nucleoside phosphotransferase (e.g., uridine kinase and NPTase), the assay conditions were changed so that unlabelled nucleoside was used along with [^{32}P]ATP, labelled in either the α- or γ-phosphoryl position. In this way, the transfer of a specific phosphoryl group to the nucleoside acceptor could be followed. The [α-^{32}P]ATP was synthesized by the method of Symons (90) and the [γ-^{32}P]ATP by the method of Glynn and Chappell (40). Assay conditions were as described above with the exception that 1.0 mM unlabelled uridine was used as phosphoryl acceptor and 5 mM Mg^{2+}/[α-^{32}P]ATP or Mg^{2+}/[γ-^{32}P]ATP used as phosphoryl donor (approximately 1 µCi/assay). After the 5 minute reaction time, tubes were boiled for 2 minutes, transferred to an ice bath, then centrifuged at 5,000 rpm for 5 minutes in order to return condensate to the bulk reaction mixture. The entire volume of reaction mixture was then spotted on Whatman 3 mm chromatography paper to which had already been applied 0.5 µmoles

of unlabelled carrier UMP. Chromatograms were developed for 10 hours by descending chromatography using 1 M ammonium acetate:70% ethanol (3:7, v/v) as solvent mixture (45) in order to separate the UMP product from remaining ^{32}P-ATP and its degradation products, and from inorganic phosphate. After the chromatograms were removed from the tank and dried, the spot corresponding to UMP was cut out and the nucleotide eluted with 2 ml of 0.15 M ammonium formate. This chromatogram eluate was then applied to a 0.85 X 4.0 cm column of Dowex 1-X8 anion exchange resin (formate form). The sample was washed into the column with 20 ml of water and the column then developed with a linear gradient of 0-0.4 M ammonium formate (50 ml of water or ammonium formate solution in each chamber of the gradient maker). The column flow rate was approximately 0.4 ml per minute and 2.5 ml fractions were collected. Elution of nucleotide was followed by absorbance at 260 nm of each fraction. Radioactivity in each fraction was determined by counting 1 ml of a fraction in 10 ml of Fisher Scintiverse II cocktail mixture for aqueous samples. Scintillation samples were counted in a Beckman LS 3800 liquid scintillation counter using a window of 600-1000 for ^{32}P.

RESULTS

Developing Seed Study

During the course of development, water content of the seeds was monitored. Dehydration at later stages is known to be linked to certain metabolic changes necessary for proper seed maturation.

As can be seen from Figure 3-1, there was a steady decline in moisture content of seeds throughout the sampling period in both Arthur and Lemhi varieties. The total drop in percent water per seed was about the same for both varieties, but because of the compressed time period for Lemhi development, the decrease was somewhat more precipitous in this variety, especially toward the end of the sampling period.

Uridine phosphorylating activity using AMP or MG^{2+}/ATP as phosphoryl donor was measured in the four tissue groups as described under Methods. Figures 3-2 through 3-9 show uridine phosphorylation in Arthur and Lemhi. Groups of conjoined points indicate stages of seed development that had the same degrees of tissue dissection. For example, in Figure 3-2, ovary tissues from the first three sample points for variety Arthur could not be dissected one from another, thus necessitating the grinding of the entire ovary. The ground tissues naturally included the ovary wall, and so the combined activities were included in the graph depicting uridine phosphorylating activity in the ovary wall. By day 6 post-anthesis, the ovary wall could be separated from the other tissues and so appears by itself from day 6 through day 29. By day 33, the ovary wall could no longer be separated from the rest of the seed minus the embryo; therefore, the last three sample points include ovary wall tissue plus ovule integuments and endosperm. The other graphs were drawn similarly in an effort to provide continuity from the beginning of sampling to the end.

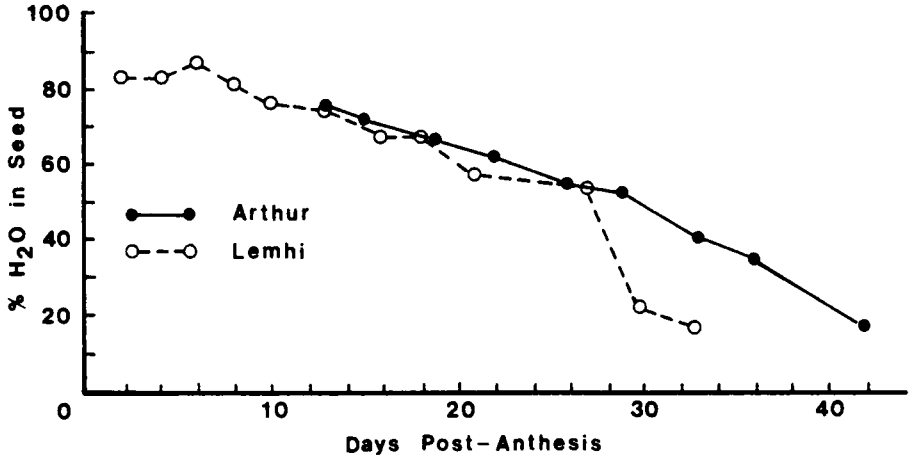

Figure 3-1. Water content of developing Arthur and Lemhi seeds.

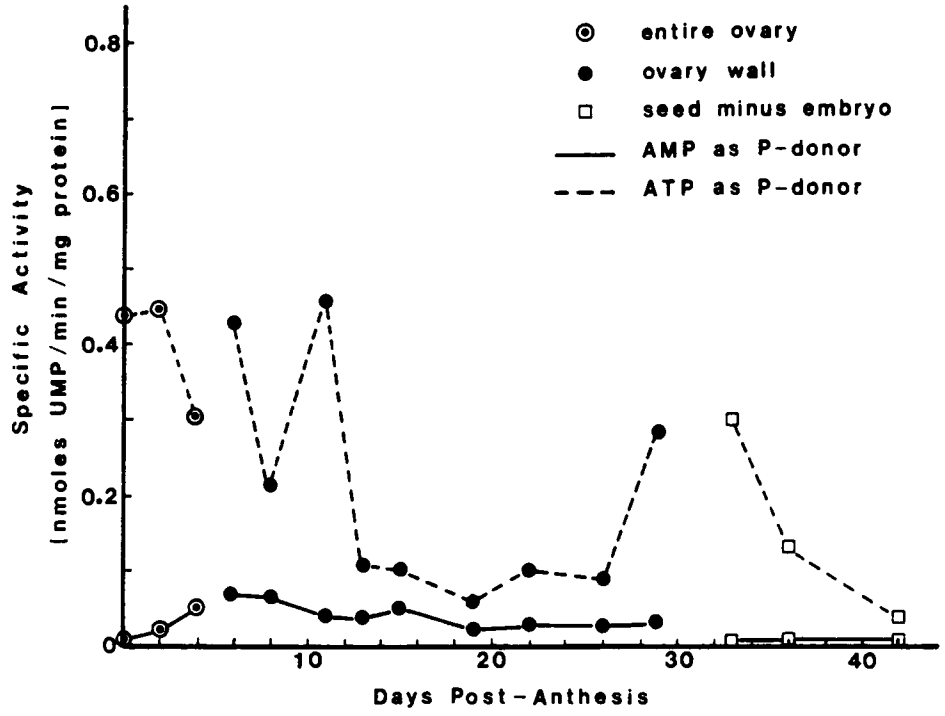

Figure 3-2. Uridine-phosphorylating activity in the ovary wall of Arthur.

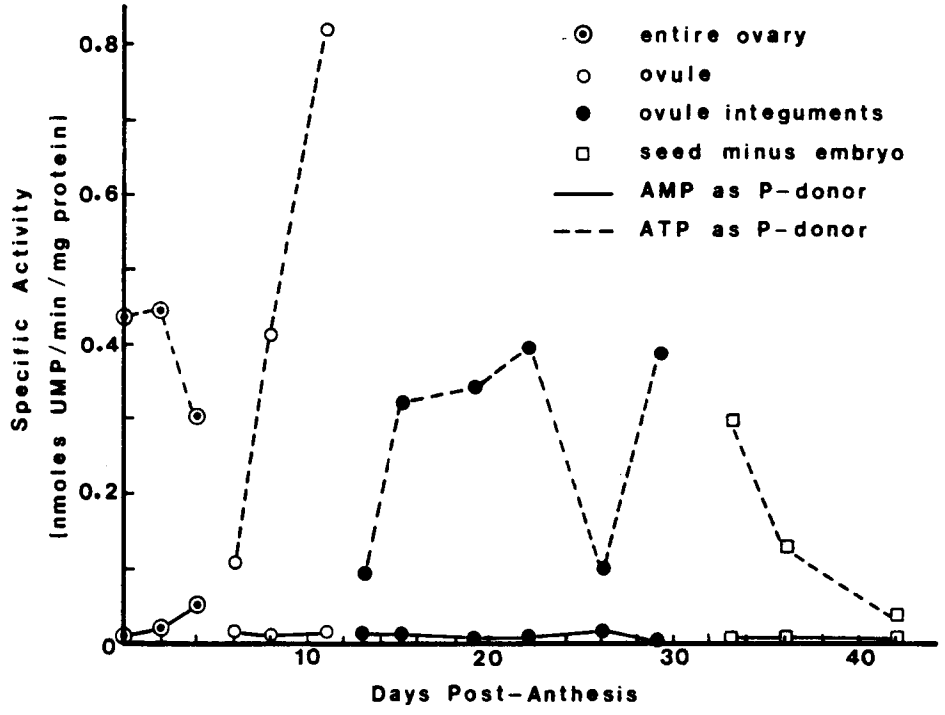

Figure 3-3. Uridine-phosphorylating activity in the ovule integuments of Arthur.

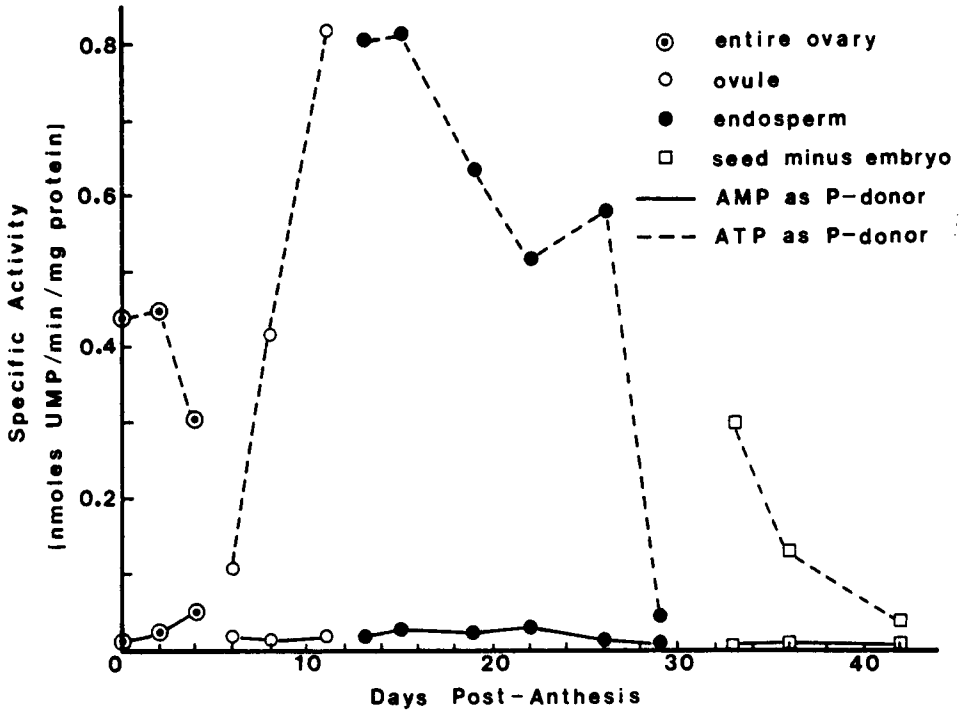

Figure 3-4. Uridine-phosphorylating activity in the endosperm of Arthur.

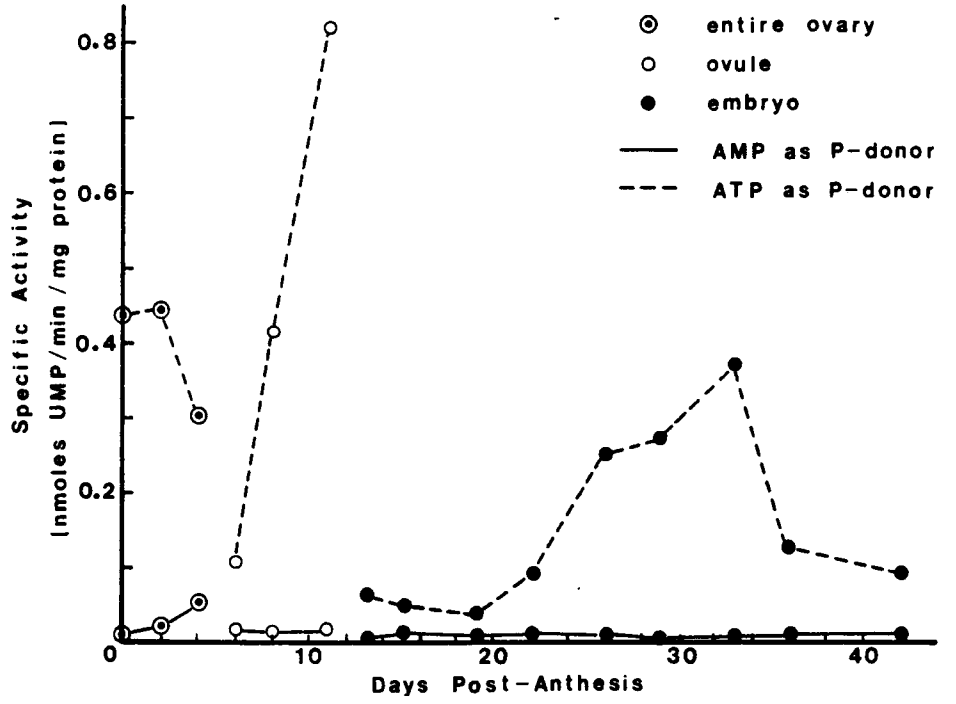

Figure 3-5. Uridine-phosphorylating activity in the embryo of Arthur.

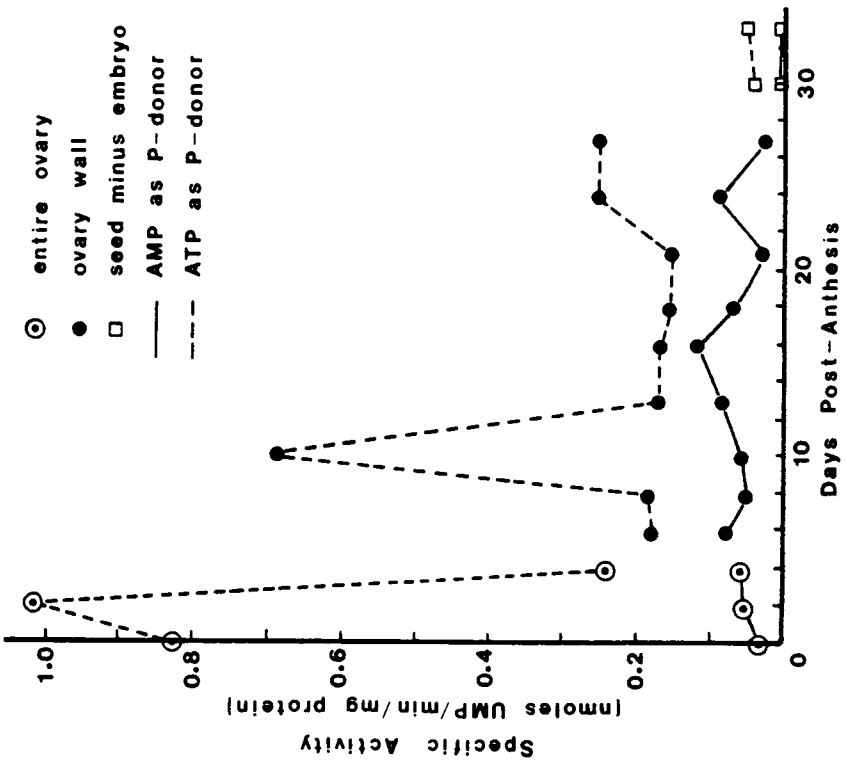

Figure 3-6. Uridine-phosphorylating activity in the ovary wall of Lemhi.

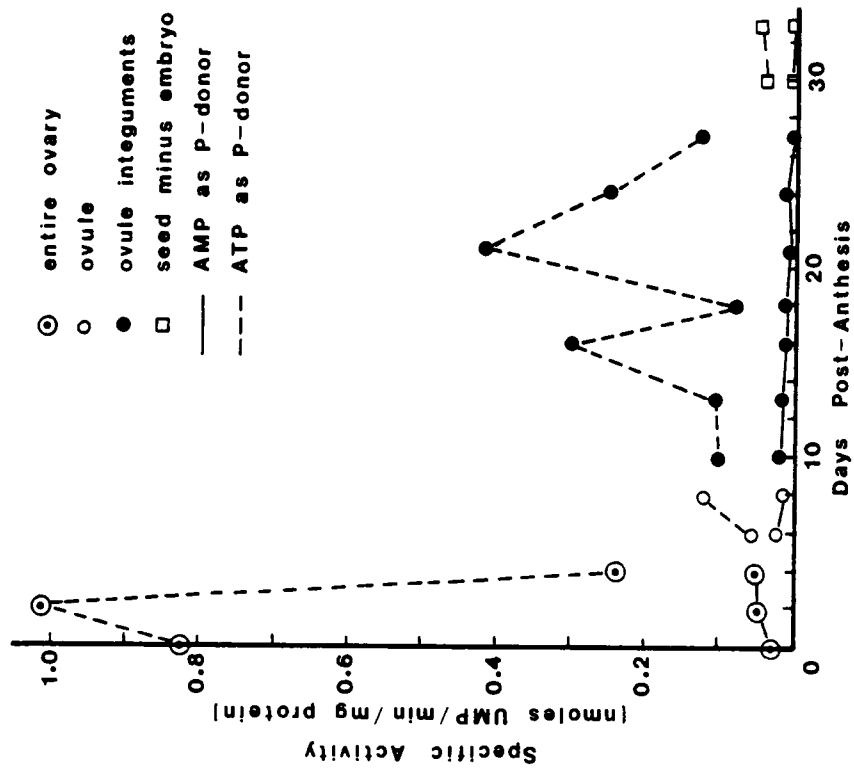

Figure 3-7. Uridine-phosphorylating activity in the ovule integuments of Lemhi.

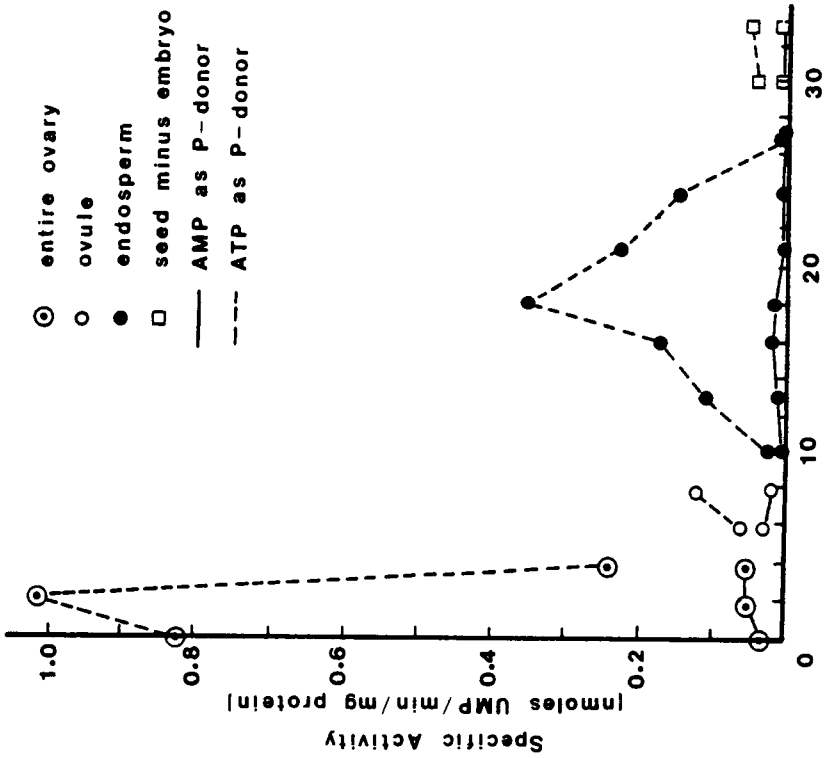

Figure 3-8. Uridine-phosphorylating activity in the endosperm of Lemhi.

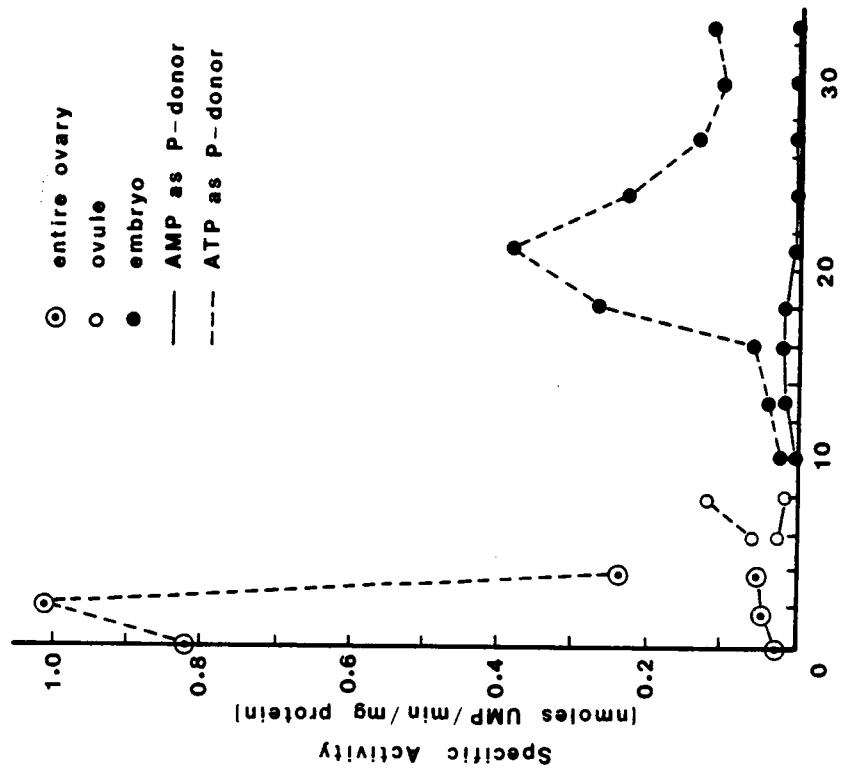

Figure 3-9. Uridine-phosphorylating activity in the embryo of Lemhi.

In whole ovaries of Arthur and Lemhi soon after anthesis, it can be seen that there is considerable uridine phosphorylating activity when ATP is used as phosphoryl donor, indicating the probable presence of a uridine kinase. There also appears to be some NPTase present as evidenced by the activity measured when using AMP as phosphoryl donor; however, the amount of uridine NPTase activity measured would not account for much of the uridine phosphorylation when ATP is used as phosphoryl donor, even if an ATPase were present. When the ovary wall is separated from the rest of the developing seed (Figures 3-2 and 3-6), the apparent uridine kinase activity fluctuates initially in this tissue, then falls, remains low for a time, and eventually rises again just before the seed becomes too dehydrated to permit separation of the wall from the adjacent tissues. During this middle period of seed development, NPTase activity remains low in the ovary wall. By day 33 in Arthur and day 30 in Lemhi, when only combined ovary wall, ovule integuments, and endosperm could be measured, uridine NPTase activity was practically non-existent. Apparent uridine kinase activity at this time was still fairly high in Arthur, falling to a low level at the end of sampling, but already fallen to a low level in Lemhi by the time combined tissues were examined. If one looks at the levels of apparent uridine kinase activity in the three non-embryo tissues just before their final combination toward the end of sampling, it can be seen that the ovary wall and ovule integuments probably contribute most to the activity seen in the combined tissues, since

the endosperm tissue (Figures 3-4 and 3-8) shows little to no apparent uridine kinase activity just prior to the time when it could no longer be separated from adjacent tissues.

In whole ovules, after removal of the ovary wall but before separation of embryo and endosperm from ovule integuments (e.g., Figures 3-3 and 3-7), apparent uridine kinase shows a strong increase in activity in Arthur, but only a slight increase in Lemhi. In Arthur, most of this apparent uridine kinase activity would appear to be due to activity contributed by the endosperm, since this is the only one of the three tissues comprising the ovule that shows a high level of apparent uridine kinase activity after its separation from the other tissues (Figure 3-4). In Lemhi, where the intact ovule does not show very high uridine kinase activity, none of the subsequently separated tissues show especially high levels of activity either (Figures 3-7, 3-8, and 3-9). The apparent uridine kinase activity in the ovule integuments alone (Figures 3-3 and 3-7) fluctuates considerably in both Arthur and Lemhi.

In Arthur, in the endosperm alone (Figure 3-4), apparent uridine kinase activity starts off at a high level, falls somewhat, then rises again before falling to a very low level. In Lemhi, endosperm uridine kinase levels are low at first, then peak and fall to a very low level (Figure 3-8). In both varieties, the final peak in activity before decline in the endosperm occurs shortly before the peak in apparent uridine kinase activity in the embryos (Figures 3-5 and 3-9). In both ovule integuments and endosperm, uridine NPTase activity is very low throughout.

In examining pyrimidine nucleoside phosphorylation in the developing wheat seeds, it is primarily the embryo that is of interest to this study, since this is the tissue that gives rise to the young seedlings whose development from germination is examined in the next section. Both Arthur and Lemhi show similar patterns of uridine phosphorylating activity in the embryo (Figures 3-5 and 3-9). At no time is there much in the way of uridine NPTase activity. Apparent uridine kinase activity, on the other hand, is initially low and then rises to a peak at a time when the moisture content of the seed is about 40% for Arthur and 50% for Lemhi. After this point, the activity level declines, although not to as low a level as initially present in this tissue.

Thymidine phosphorylating activity (Figures 3-10 through 3-17) in the developing seeds shows quite a different pattern from that of uridine phosphorylating activity. When one compares similar tissues in both wheat varieties, the patterns of thymidine phosphorylating activity are very similar. In both the ovule integuments (Figures 3-11 and 3-15) and endosperm (Figures 3-12 and 3-16), activity is very low no matter what the phosphoryl donor. In the ovary wall (Figures 3-10 and 3-14), thymidine phosphorylating activity is somewhat higher, the pattern of rising and falling levels being similar in both varieties and with both phosphoryl donors, with the exception of the last point at which the ovary wall alone was examined in Lemhi. The fact that thymidine NPTase activity and apparent thymidine kinase activity follow such similar patterns in the ovary wall during seed

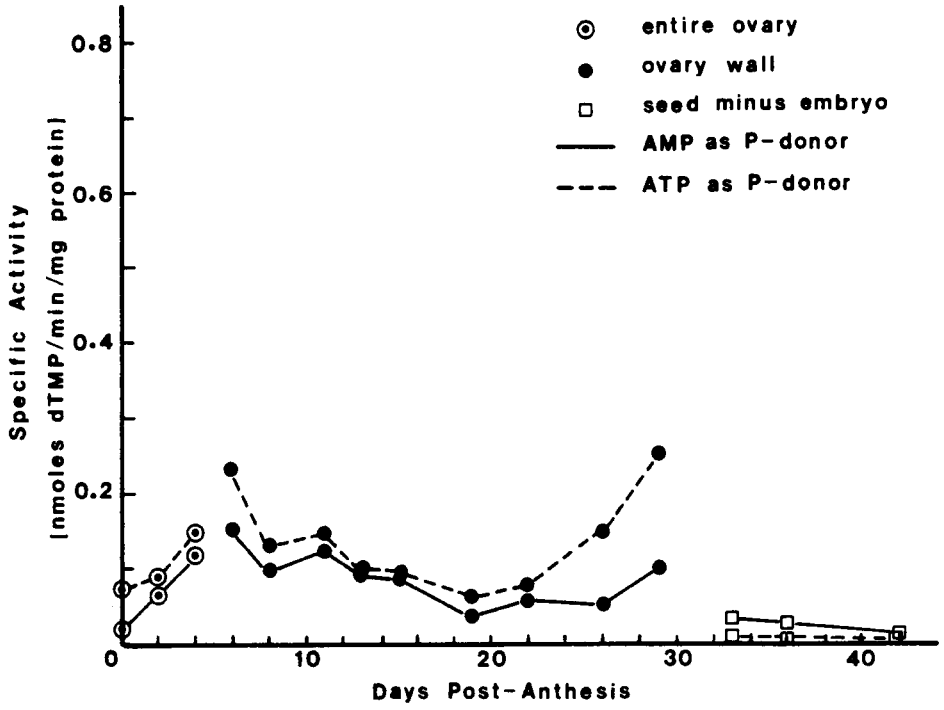

Figure 3-10. Thymidine-phosphorylating activity in the ovary wall of Arthur.

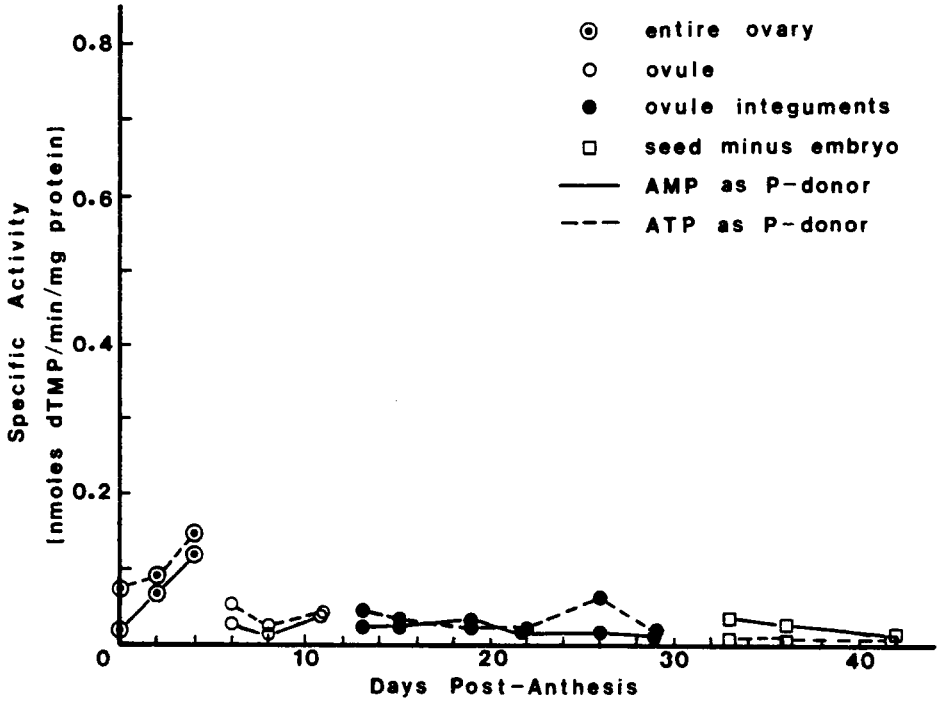

Figure 3-11. Thymidine-phosphorylating activity in the ovule integuments of Arthur.

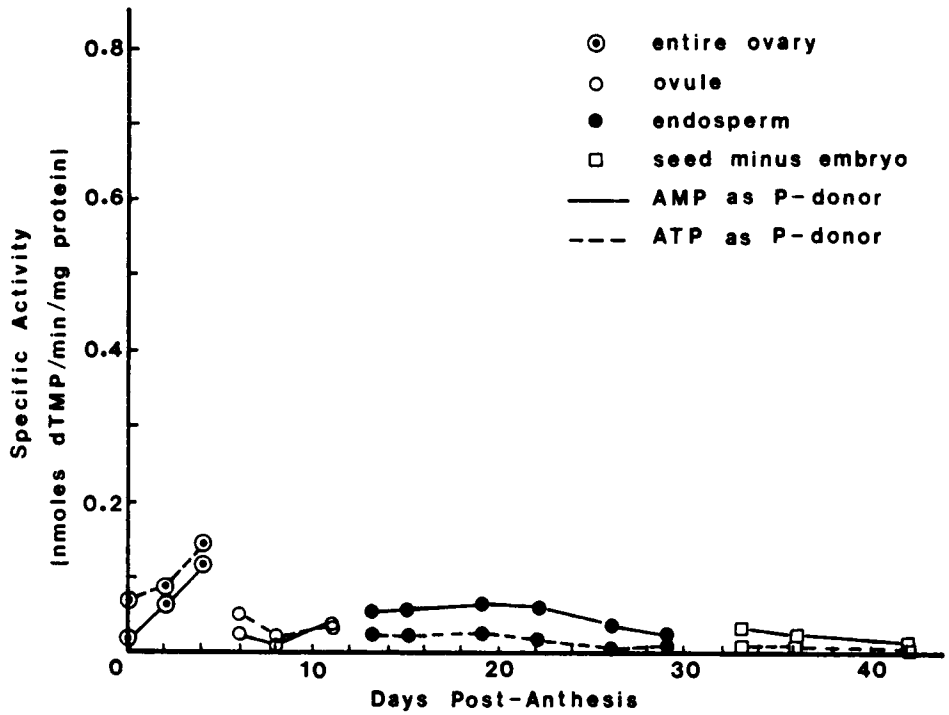

Figure 3-12. Thymidine-phosphorylating activity in the endosperm of Arthur.

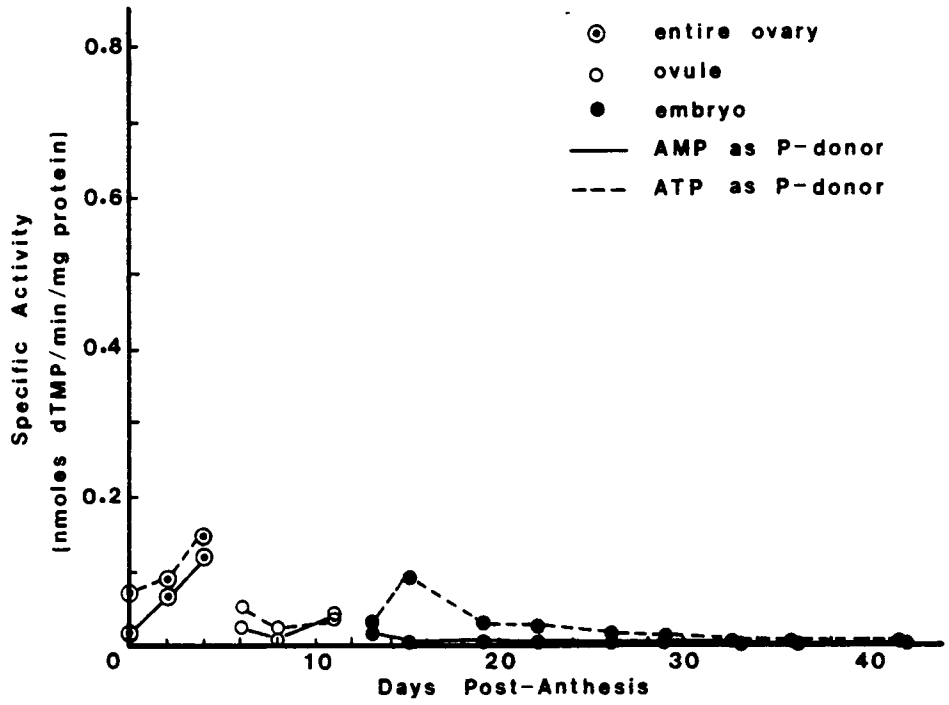

Figure 3-13. Thymidine-phosphorylating activity in the embryo of Arthur.

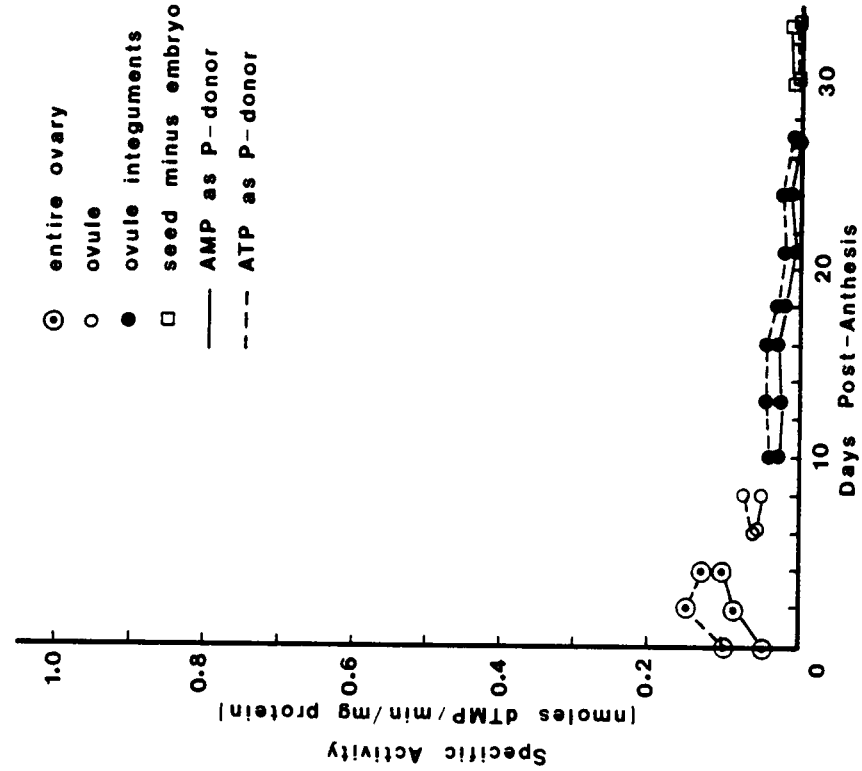

Figure 3-15. Thymidine-phosphorylating activity in the ovule integuments of Lemhi.

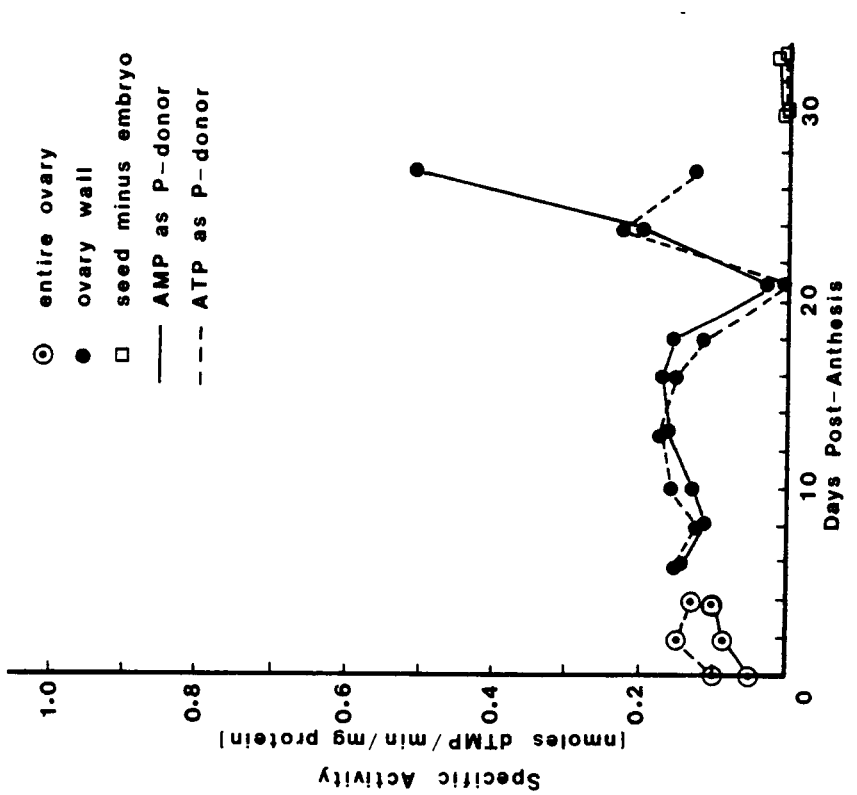

Figure 3-14. Thymidine-phosphorylating activity in the ovary wall of Lemhi.

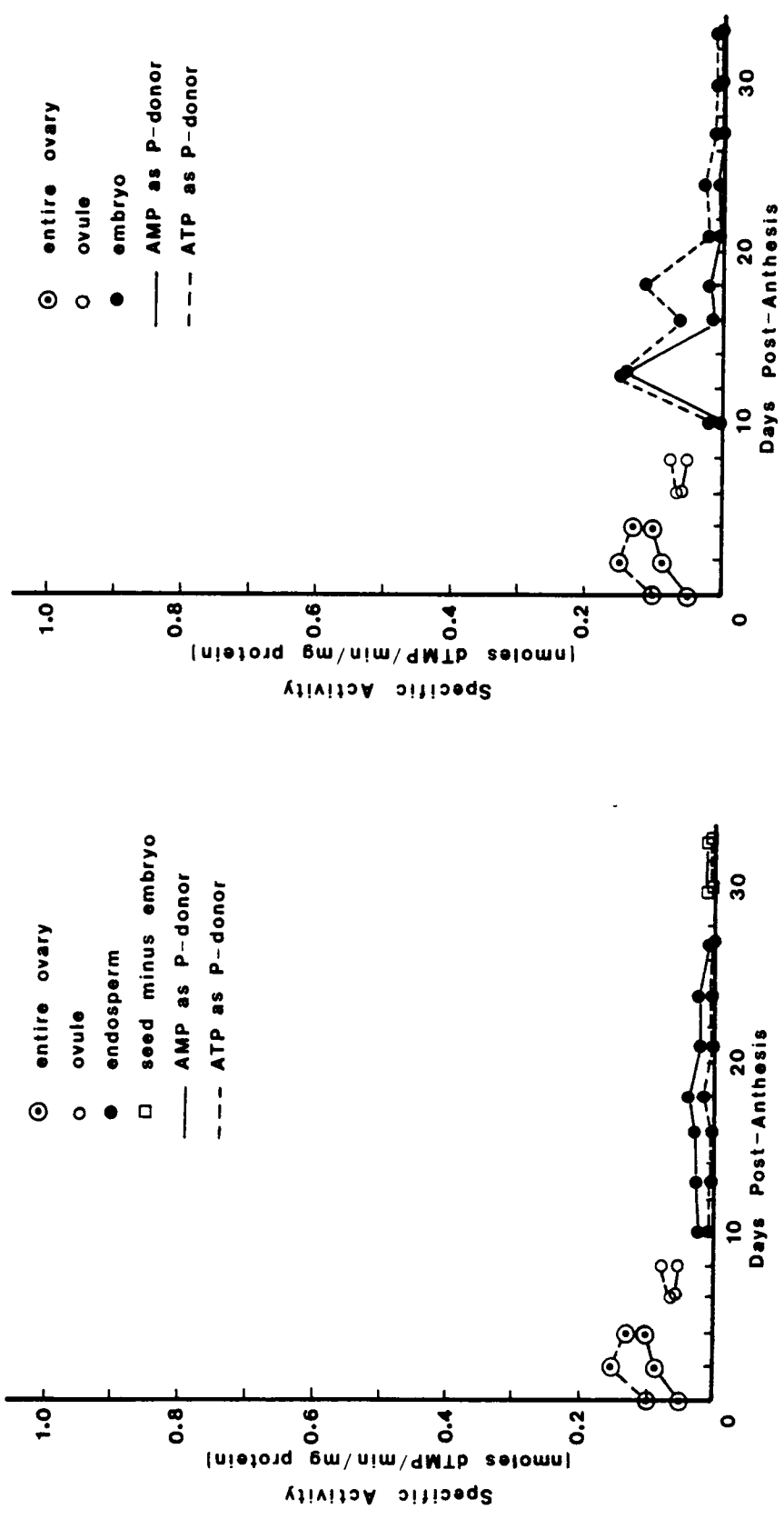

Figure 3-16. Thymidine-phosphorylating activity in the endosperm of Lemhi.

Figure 3-17. Thymidine-phosphorylating activity in the embryo of Lemhi.

development suggests that both activities may be due to the same enzyme. The apparent thymidine kinase activity in this case could be due to combined activities of ATP phosphohydrolase and NPTase. This is a reasonable suggestion since thymidine kinase has never been shown to be present in wheat parental tissues, and the ovary wall is leftover parental tissue now giving rise to the developing seed.

In the developing embryo in both Arthur and Lemhi (Figures 3-13 and 3-17), there is a transient increase in thymidine phosphorylating activity with ATP as phosphoryl donor about two to two-and-a-half weeks post-anthesis. This could indicate the presence of a thymidine kinase, given that the NPTase activity is very low at this point.

The patterns of uridine NPTase activity and thymidine NPTase activity within each variety are fairly similar. The major difference between the two nucleoside substrates, when AMP is used as phosphoryl donor, is that the level of activity with uridine is generally lower than that with thymidine. This is not surprising if one accepts that the NPTase phosphorylating uridine is the same enzyme that also phosphorylates thymidine, since it has been reported that uridine is a poorer substrate for NPTase than is thymidine (72).

The patterns of AMP phosphohydrolase activity in the various tissues of the two wheat varieties (Figures 3-18 through 3-21) do not bear a striking resemblance to those of NPTase activity using either thymidine or uridine as substrate. However, in tissue such

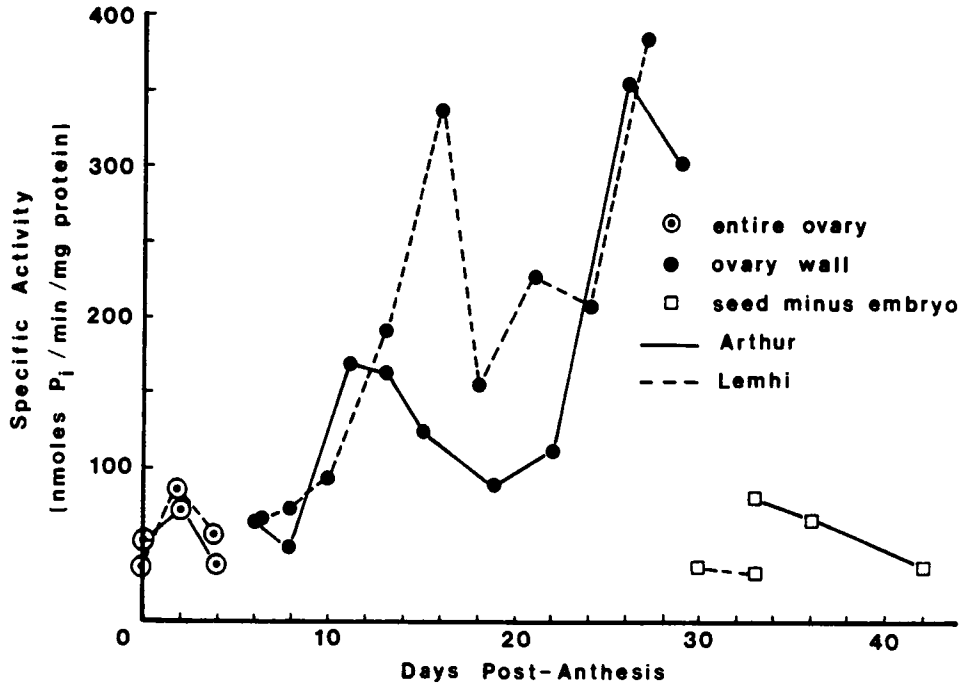

Figure 3-18. AMP Phosphohydrolase activity in the ovary wall of Arthur and Lemhi.

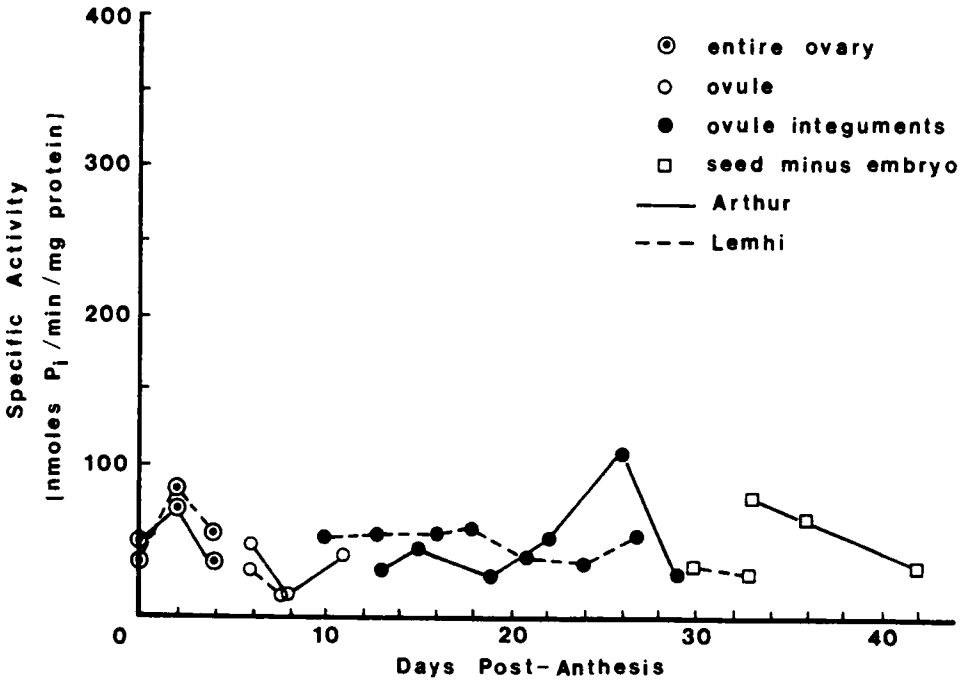

Figure 3-19. AMP Phosphohydrolase activity in the ovule integuments of Arthur and Lemhi.

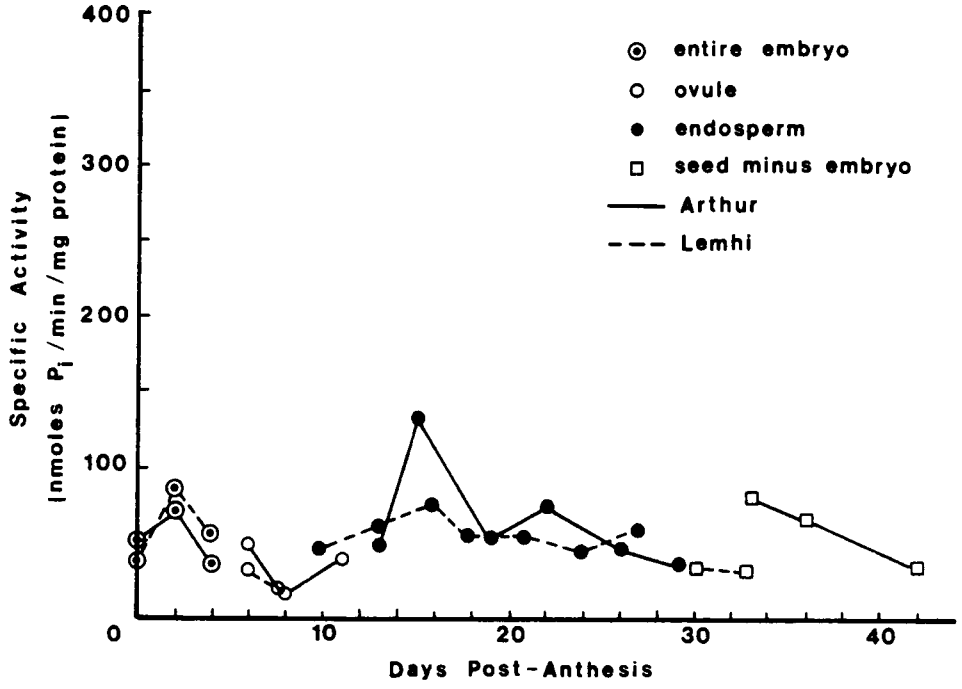

Figure 3-20. AMP Phosphohydrolase activity in the endosperm of Arthur and Lemhi.

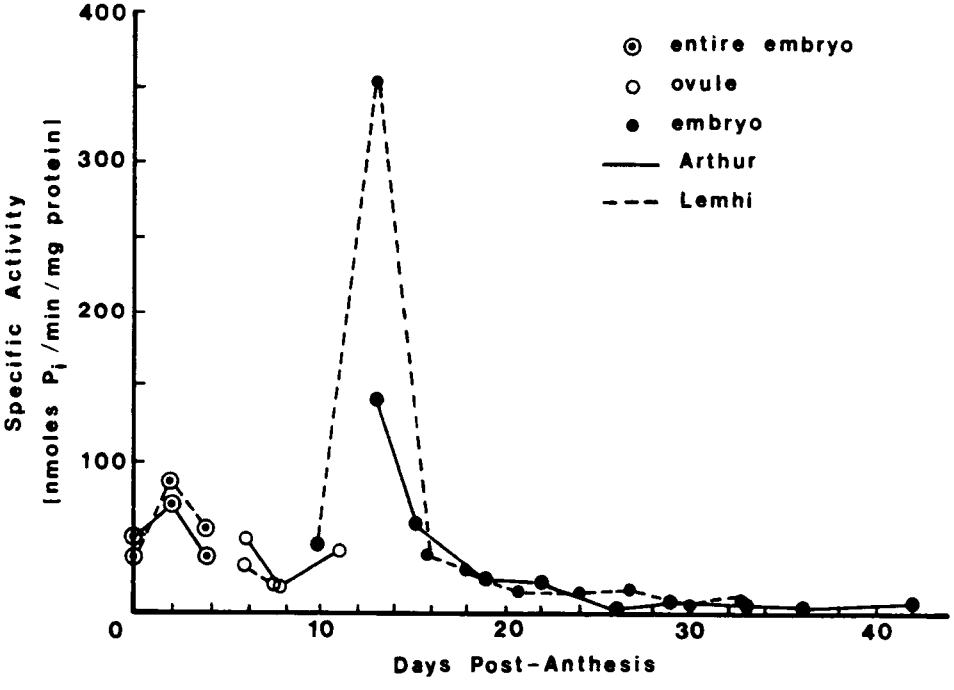

Figure 3-21. AMP Phosphohydrolase activity in the embryo of Arthur and Lemhi.

as the ovary wall, where NPTase is fairly active, AMP phosphohydrolase activity is also higher (Figure 3-18), even though the peaks and troughs of these two activities do not coincide. In the three other groups of tissues, where NPTase activity is relatively low, AMP phosphohydrolase activity is also relatively low. One might expect overall levels of these two activities to be similar if, as is probable, the NPTase enzyme contains a nucleotide hydrolyzing function as well as the phosphotransferase function. This is not to say that other AMP phosphohydrolyzing activities are not also present, but in these experiments such activities were not distinguished from that associated with NPTase.

Figures 3-22 through 3-25 show total protein per floret for the four groups of tissue in Arthur and Lemhi. Total protein appears gradually to decrease in the ovary wall (Figure 3-22). In the ovule integuments protein appears to increase, except for a sudden drop on day 26 in Arthur and on day 24 in Lemhi (Figure 3-23). Total protein in the endosperm also increases over time, with a rather dramatic increase on day 26 in Arthur and a smaller increase on day 24 in Lemhi (Figure 3-24). These sudden changes in total protein on days 26 and 24 in Arthur and Lemhi, respectively, can probably be accounted for by the slight alteration in tissue dissection, mentioned earlier, for these two tissue groups; that is, whereas the thin membranous structure judged to be the aleurone normally was included with the ovule integuments, on day 26 in Arthur and day 24 in Lemhi this membranous layer was instead included with the

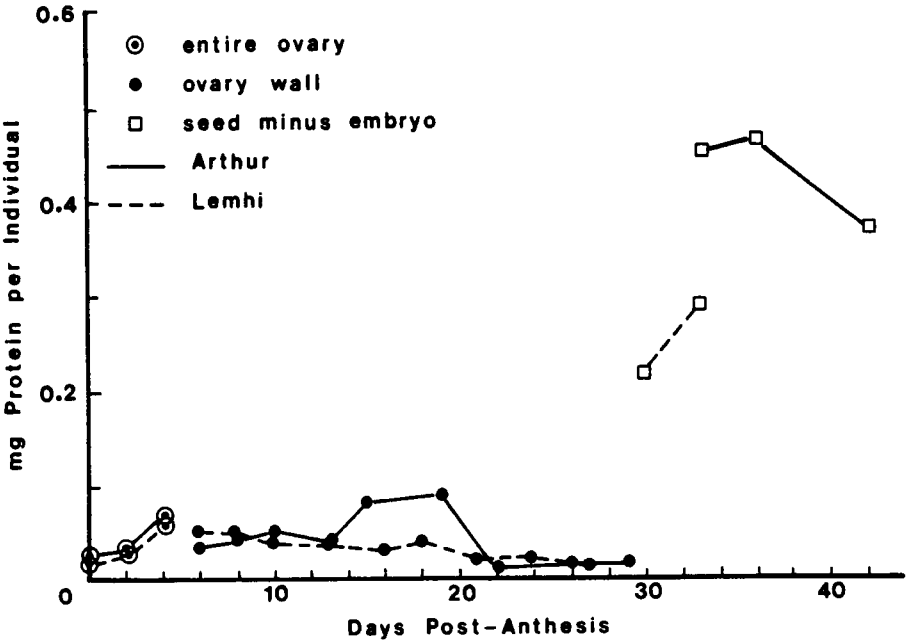

Figure 3-22. Total protein per floret in the ovary wall of Arthur and Lemhi.

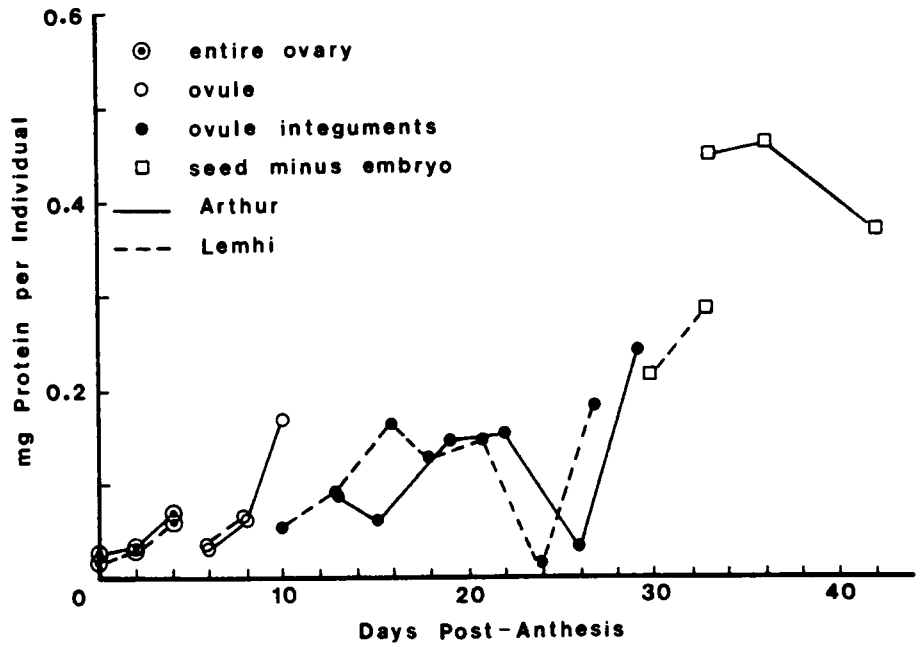

Figure 3-23. Total protein per floret in the ovule integuments of Arthur and Lemhi.

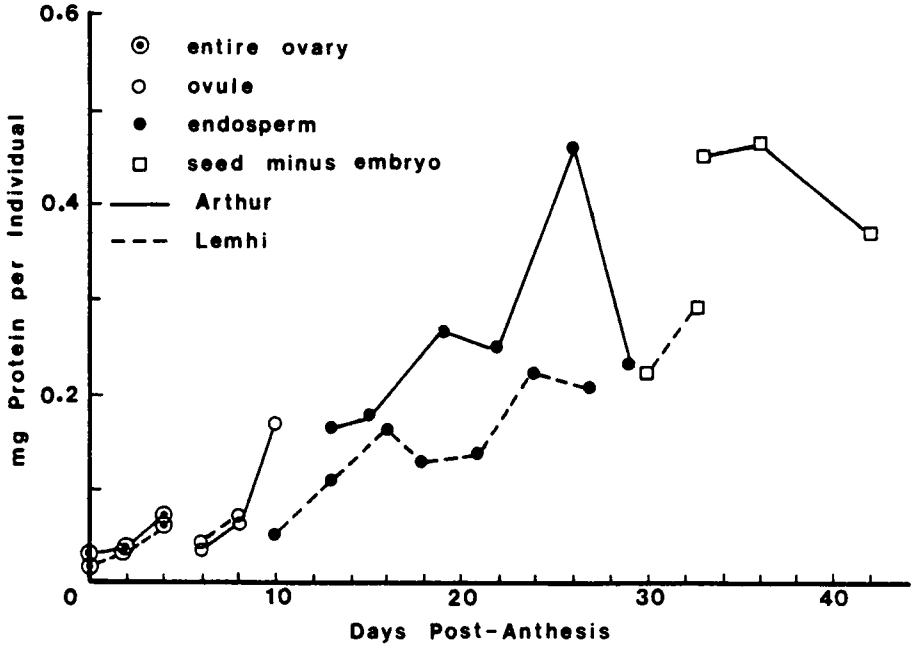

Figure 3-24. Total protein per floret in the endosperm of Arthur and Lemhi.

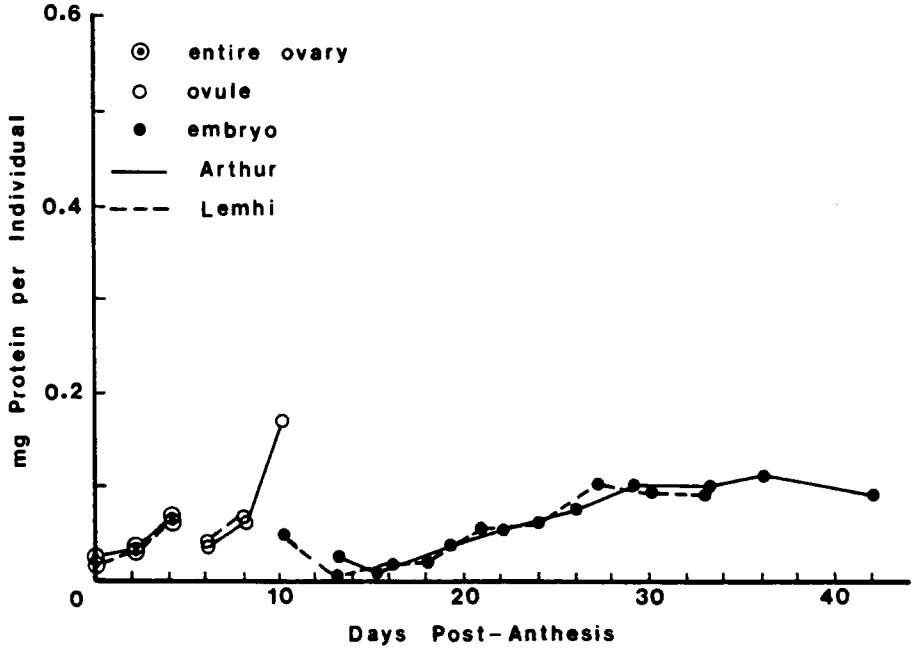

Figure 3-25. Total protein per floret in the embryo of Arthur and Lemhi.

starchy endosperm. Because the aleurone is metabolically very active and has a high protein content, its inclusion with a particular tissue group could cause an apparent increase in total protein within that group.

Total protein in the embryo (Figure 3-25) gradually increases and then levels off toward the end of seed development.

Early Seedling Study

As seen in the seed development study, the mature embryos contain a uridine kinase, but practically no NPTase nor thymidine kinase. Arthur and Lemhi varieties were further examined to determine if these same activities could be found in germinating embryos and early seedlings.

In the case of uridine phosphorylating activities (Figures 3-26 and 3-27), it is not surprising to find that there is an apparent uridine kinase present in the embryos of both varieties during the early germination stages, at least through 24 hours after seed sowing. One can say with a fair amount of certainty that the activity using ATP as phosphoryl donor for the phosphorylation of uridine at this stage is a true uridine kinase because there is no NPTase activity (i.e., using AMP as phosphoryl donor) at this time. Therefore, the combination of an ATP phosphohydrolase and NPTase to produce a false appearance of uridine kinase activity is not a possibility. Unfortunately, after 36 hours it becomes less clear which types of uridine-phosphorylating activity are actually present, since NPTase

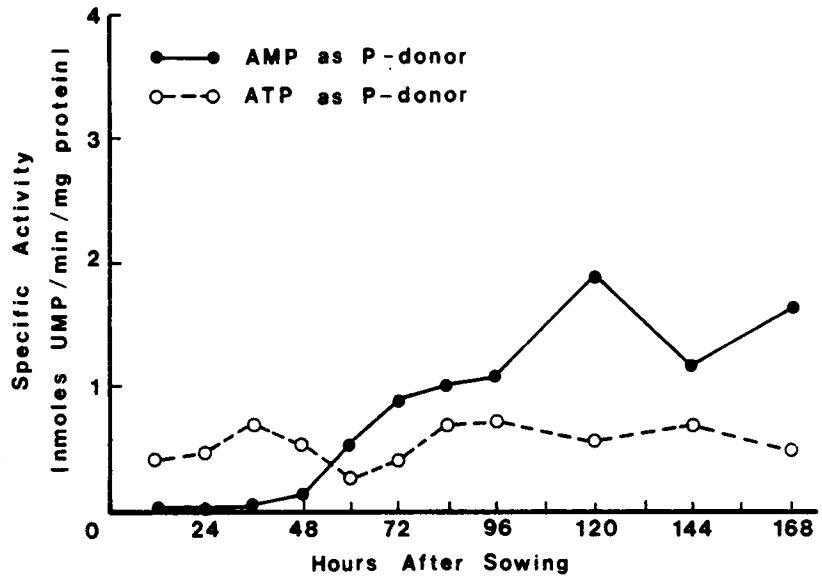

Figure 3-26. Uridine-phosphorylating activity in germinating seeds and young seedlings of Arthur.

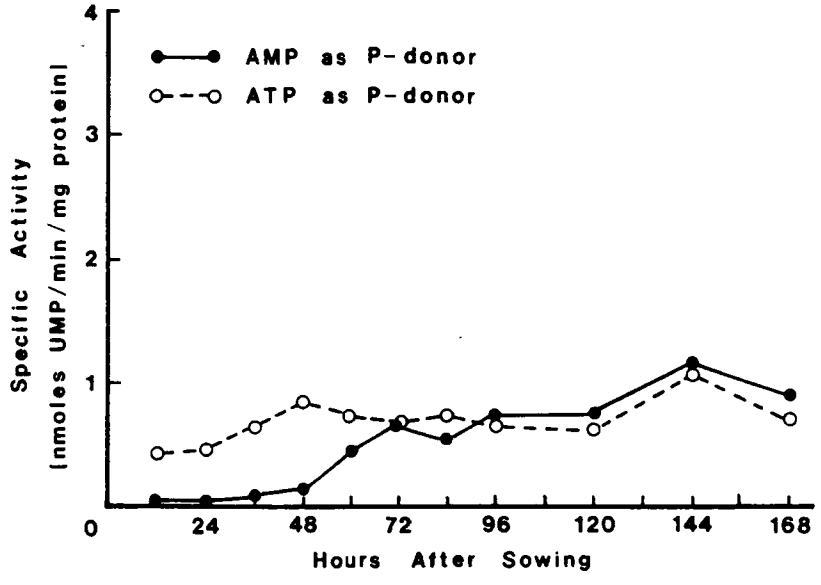

Figure 3-27. Uridine-phosphorylating activity in germinating seeds and young seedlings of Lemhi.

now begins to appear in the embryos and continues to increase in the young seedlings. Although ATP phosphohydrolase activity was not quantified in this study, evidence of its activity was present in the degradation products of ATP found on the chromatograms of assay mixtures from every stage of development in this particular study. Therefore, although uridine phosphorylation using an ATP phosphoryl donor continues throughout the sampling period, it is no longer possible to say whether this activity is the result of combined uridine kinase and NPTase/ATP phosphohydrolase or the NPTase/ATP phosphohydrolase alone. It is likely that the slight increase in uridine phosphorylating activity with ATP at 36 h in Arthur (Figure 3-26) and at 48 h in Lemhi (Figure 3-27) is due to combined activities of the existing uridine kinase and the now appearing NPTase (again, ATP phosphohydrolase being present throughout). Whether or not uridine kinase continues to be present at later stages of development cannot be determined from these data, obtained by following the phosphorylation of ^{14}C-labelled uridine.

Thymidine phosphorylating activity in germinating embryos and young seedlings is shown in Figures 3-28 and 3-29. Note that during the earliest stages of germination there is essentially no thymidine phosphorylation, no matter which phosphoryl donor is made available. By 36 h after sowing in both varieties, NPTase has begun to appear and rapidly increases over the next 48 hours. At the same time, an apparent ATP:thymidine phosphorylating activity also begins to appear, but does not reach the level of activity of the NPTase.

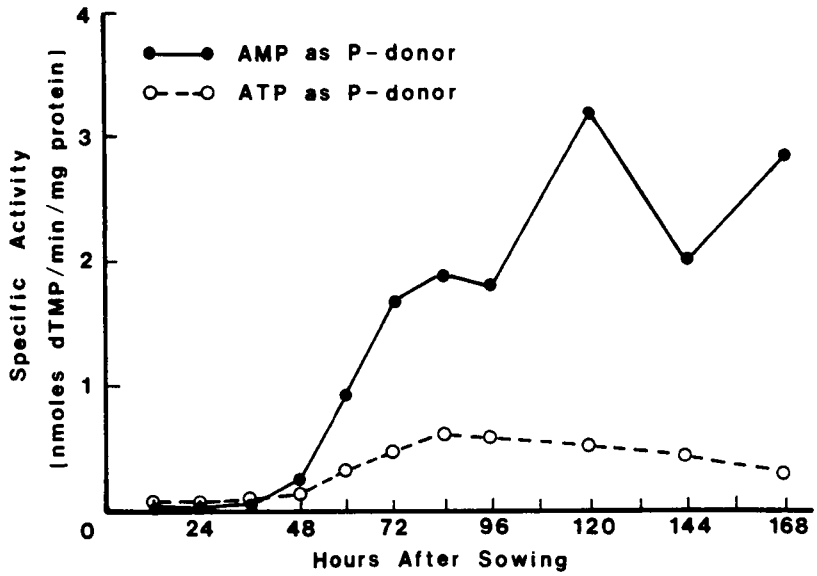

Figure 3-28. Thymidine-phosphorylating activity in germinating seeds and young seedlings of Arthur.

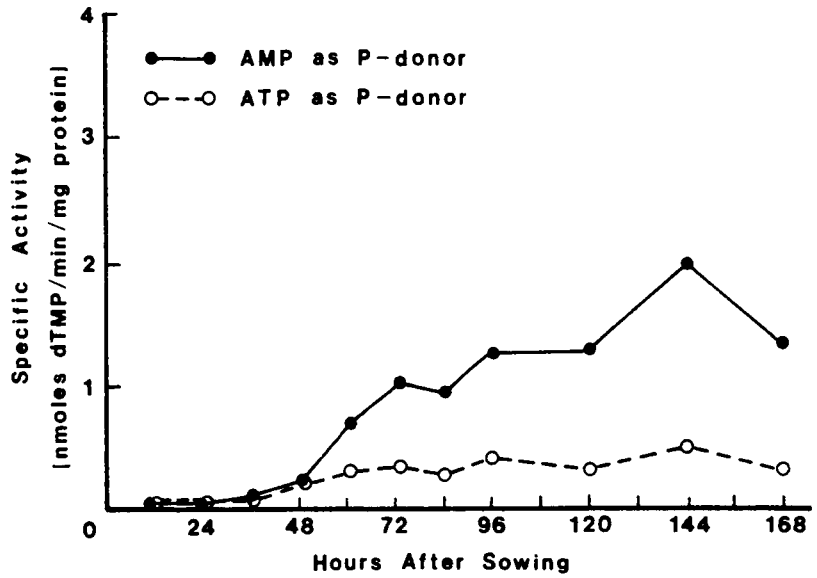

Figure 3-29. Thymidine-phosphorylating activity in germinating seeds and young seedlings of Lemhi.

In earlier work by Hofman and Schwarz (45) it was shown that 4 day old (96 h) Lemhi wheat seedlings contained no thymidine kinase and that the activity utilizing ATP as phosphoryl donor at this time was in fact due to the combined activities of NPTase and ATP phosphohydrolase. This is probably the case in both Arthur and Lemhi seedlings throughout the seven day sampling period.

Figures 3-30 and 3-31 show AMP phosphohydrolase activity in Arthur and Lemhi, respectively. Note that the pattern of activity during germination and early seedling development is quite similar to that of NPTase. This is not surprising since plant NPTases have been shown to contain an NMP hydrolytic function as well as the phosphotransferase function, as discussed in Chapter II.

[^{32}P]ATP Study

It has been pointed out that when unlabelled ATP is used as phosphoryl donor and [^{14}C]uridine is used as acceptor, and when both uridine kinase and NPTase may be present simultaneously, it becomes impossible to distinguish the two types of activity. Because this possibility existed in older wheat seedlings (past 48 hours after sowing), it was necessary to change the assay conditions in order to identify the true phosphoryl donors: AMP for NPTase activity and ATP for uridine kinase activity. It was hoped that the use of [^{32}P]ATP, labelled in either the α- or γ-phosphoryl position, would allow this distinction to be made. The results shown in Figures 3-32 through 3-34 are somewhat disappointing because the separations of UMP from inorganic phosphate on the Dowex columns are incomplete.

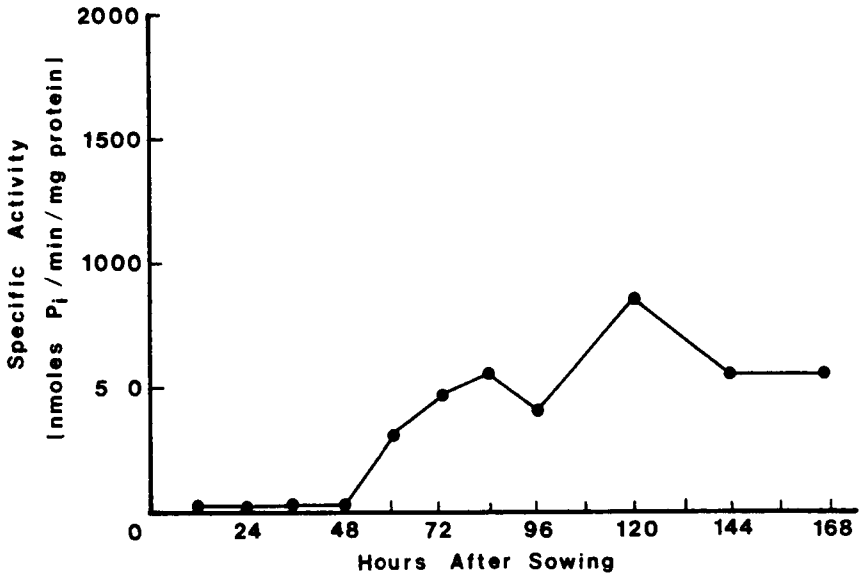

Figure 3-30. AMP Phosphohydrolase activity in germinating seeds and young seedlings of Arthur.

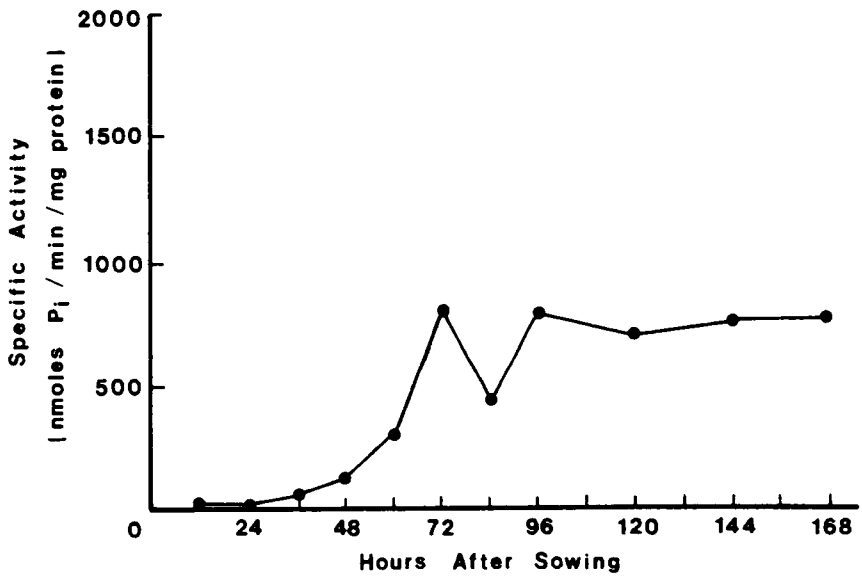

Figure 3-31. AMP Phosphohydrolase activity in germinating seeds and young seedlings of Lemhi.

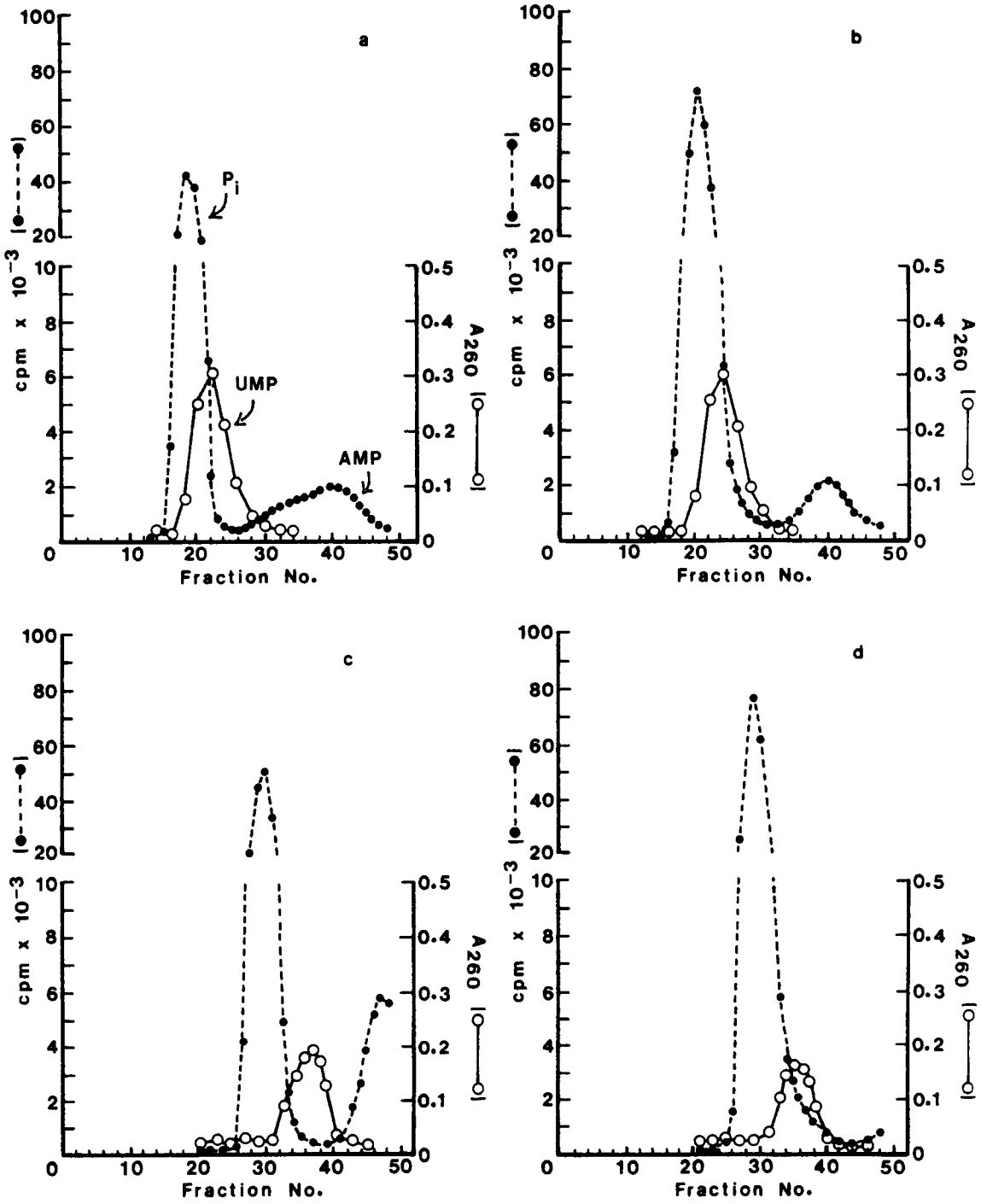

Figure 3-32. Elution profiles from Dowex columns of UMP recovered from [^{32}P]ATP uridine phosphorylation assays using 12-hour Arthur or Lemhi seedling crude extract as enzyme source. (a) 12-h Arthur, [α-^{32}P]ATP; (b) 12-h Arthur, [γ-^{32}P]ATP; (c) 12-h Lemhi, [α-^{32}P]ATP; (d) 12-h Lemhi, [γ-^{32}P]ATP.

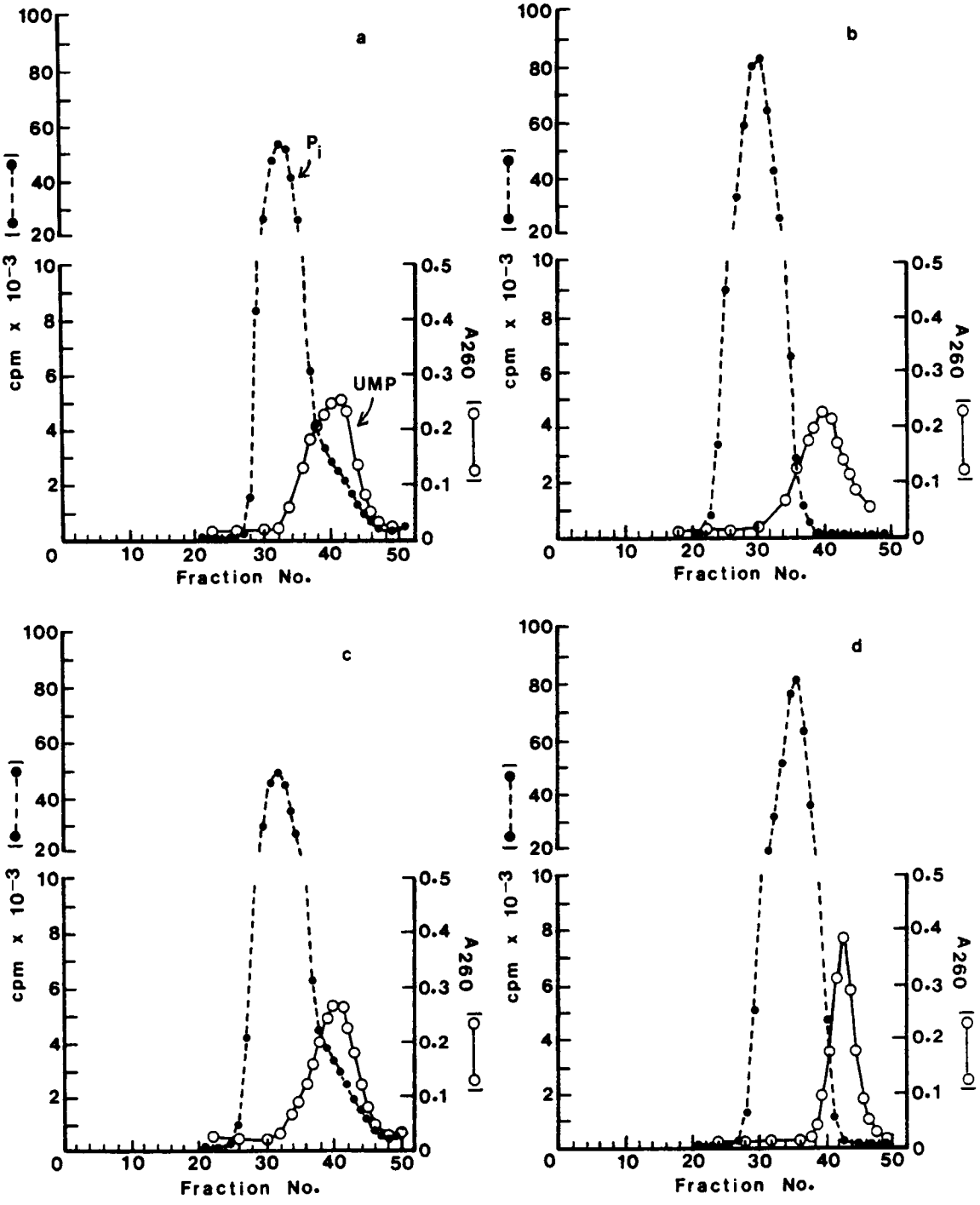

Figure 3-33. Elution profiles from Dowex columns of UMP recovered from [^{32}P]ATP uridine phosphorylation assays using 96-hour Arthur or Lemhi seedling crude extract as enzyme source. (a) 96-h Arthur, [α-^{32}P]ATP; (b) 96-h Arthur, [γ-^{32}P]ATP; (c) 96-h Lemhi, [α-^{32}]ATP, (d) 96-h Lemhi, [γ-^{32}P]ATP.

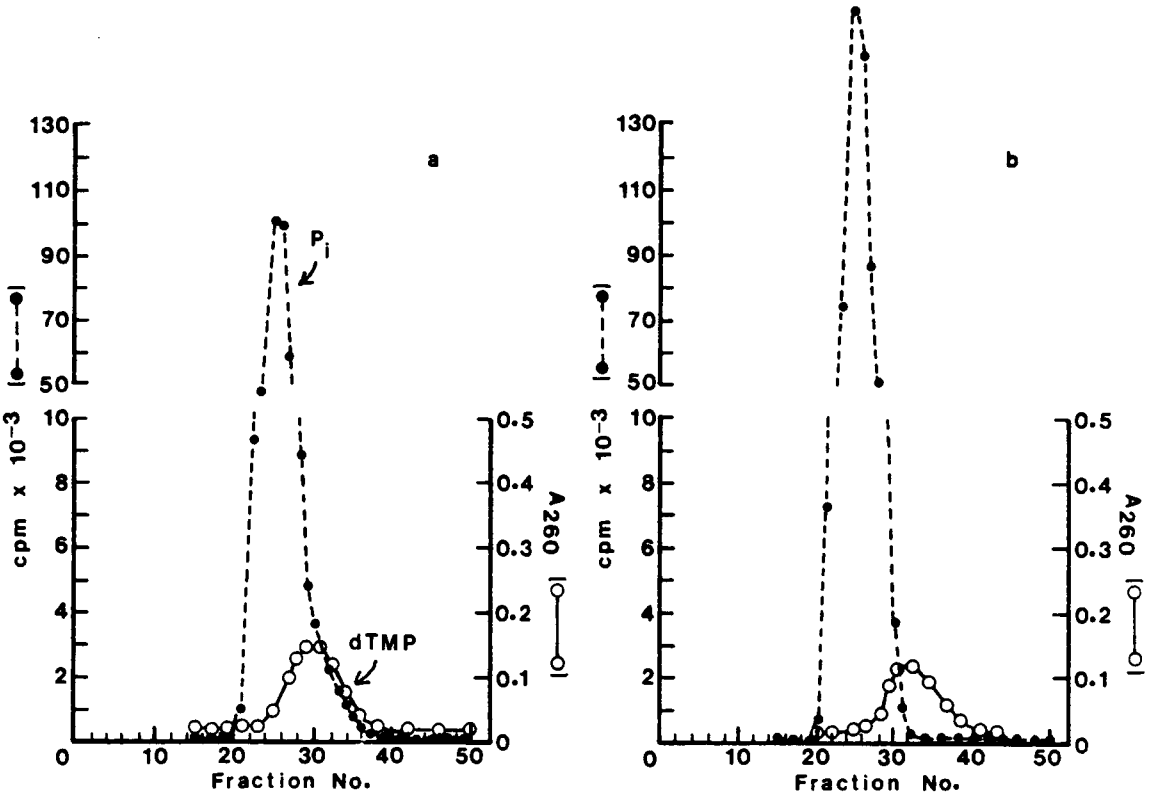

Figure 3-34. Elution profiles from Dowex columns of dTMP recovered from [^{32}P]ATP thymidine phosphorylation assays using 96-hour Lemhi seedling crude extracts as enzyme source. (a) 96-h Lemhi, [α-^{32}P]ATP; (b) 96-h Lemhi, [γ-^{32}P]ATP.

Consequently, ^{32}P label that is incorporated into UMP is seen only as a shoulder on the right side of the base of a very large $^{32}P_i$ peak, rather than as a completely separate peak, as would be most satisfying. Nonetheless, in most cases one can distinguish between incorporation and lack of incorporation of ^{32}P into UMP.

As reported in the previous section, there appears to be a true uridine kinase present in wheat embryos during the early hours of germination, NPTase not yet having appeared at this time. If this is so, then the phosphoryl group transferred to uridine from ATP should be the γ-phosphoryl group; therefore, only when [γ-^{32}P]ATP is used as phosphoryl donor should one see incorporation of ^{32}P label into UMP. Figure 3-32 does not show this clearly because of the poor separation of UMP from the large $^{32}P_i$ peak. In Arthur, in Figure 3-32a, there is no evidence of a shoulder at the base of the $^{32}P_i$ peak that might represent label incorporation into UMP, and this is as expected since uridine kinase would not transfer the α-phosphoryl group following hydrolysis of the γ- and β-phosphoryl groups of ATP. In Figure 3-32b there is the faintest suggestion of a shoulder on the $^{32}P_i$ peak that could represent label incorporation into UMP from [γ-^{32}P]ATP, but the asymmetry of the $^{32}P_i$ peak is not terribly convincing, since it does not differ greatly from control values (assays using inactive enzyme). The evidence for γ-phosphoryl transfer to uridine is a little better with Lemhi 12 hour embryo extracts in Figures 3-32c and 3-32d. In Figure 3-32c, once again it can be seen that there is little to no incorporation

of ^{32}P-label into UMP when [α-^{32}P]ATP is used as phosphoryl donor. On the other hand, in Figure 3-32d, one can detect an asymmetry at the base of the $^{32}P_i$ peak, with a shoulder corresponding to labelled UMP occurring on the right. This suggests that γ-phosphoryl transfer has taken place from ATP to uridine, exactly what one would expect from a uridine kinase.

Figure 3-33 shows the results of similar experiments using [α- or γ-^{32}P]ATP, but this time with extracts from 96 hour wheat seedlings. In Figure 3-33a, note that a 96 hour extract from Arthur produces a sizable UMP shoulder on the $^{32}P_i$ peak when [α-^{32}P]ATP is used as phosphoryl donor, but that this shoulder is missing in Figure 3-33b where [γ-^{32}P]ATP is used as phosphoryl donor. Similar results are obtained with Lemhi 96 hour extracts (Figures 3-33c and 3-33d), where [α-^{32}P] phosphoryl incorporation into UMP is seen as an obvious asymmetry at the base of the $^{32}P_i$ peak. Use of [γ-^{32}P]ATP as donor produces no such shoulder. These results indicate that by 96 hours after seed sowing, uridine kinase is no longer active in the wheat seedling extracts; only NPTase is active at this point, along with the ever present ATP phosphohydrolyzing activity that removes the γ- and β-phosphoryl groups from ATP to provide an AMP substrate for the NPTase.

Figure 3-34 shows similar results for ^{32}P incorporation into dTMP in 96-h Lemhi extracts. In this case, asymmetry at the base of the $^{32}P_i$ peak, indicating label incorporation into dTMP, is found only when [α-^{32}P]ATP is used as phosphoryl donor. This is as

expected, since Hofman and Schwarz (45) have shown that thymidine kinase activity is absent in 96-hour Lemhi seedling extracts. Nucleoside phosphotransferase is the only thymidine-phosphorylating activity present at this stage of development.

DISCUSSION

Rogers and Quatrano (79) have suggested the use of a five stage scheme based on morphological characteristics to describe the development of a wheat caryopsis. Although different varieties of wheat might vary in duration of each stage, the qualitative characteristics remain similar from variety to variety.

In stage I, in which most of the grain length is achieved, the ovary wall (pericarp) is still fleshy, the endosperm liquid, and the embryo undifferentiated so that it cannot be dissected from the tissues around it. According to Buttrose (21), this is a time of free nuclear division in the endosperm, followed by cell wall formation. Stage I lasts from day 0 to roughly day 7 post-anthesis for the wheat variety used as an example by Rogers and Quatrano.

Stage II is described by Rogers and Quatrano as a time of rapid embryo growth. By the end of stage II, the scutellum, ventral scale, coleptile, colerhiza, primary root primordium, and leaf primordia are present. During this time the endosperm goes through a milky stage and begins to become slightly sticky (early dough stage). The pericarp starts to shrink. Stage II lasts from day 7 to day 14.

In stage III, the pericarp is reduced further, but is still flexible. The endosperm attains a soft-dough consistency as it begins filling with starch and protein. Aleurone grains begin to develop during this time within the aleurone tissue (22). Starch granules also accumulate in young aleurone cells during this time, but begin to disappear by late stage III (22). In the embryo, lateral root primordia become differentiated and a cap can be found on the primary root (79). For Rogers and Quatrano, stage III lasts from day 14 to day 21.

By stage IV, the pericarp has become thin and papery and remains so. The endosperm reaches hard-dough consistency and begins to adhere to the scutellum of the embryo. In the embryo there is further differentiation of the primary and lateral roots, and caps can be found on lateral roots. Primary leaves appear. Rogers and Quatrano observed stage IV to last from day 21 to day 31.

In stage V, the endosperm is hard and the embryo fully differentiated. This stage lasts from day 31 through day 50.

From gross observations made of pericarp and endosperm consistency and embryo characteristics in Arthur and Lemhi, it appeared that these wheat varieties followed very closely the developmental stages described by Rogers and Quatrano. The main difference was found in Lemhi, which seemed to go more rapidly through stages IV and V.

One thinks of thymidine phosphorylation in connection with DNA synthesis, since no other role for thymidine nucleotides comes

to mind. Likewise, uridine phosphorylation and RNA synthesis are associated, although uridine nucleotides do play a part in other biochemical pathways. However, thymidine and uridine phosphorylation are not necessarily linked temporally with nucleic acid synthesis. Nucleoside phosphorylation may occur well in advance of nucleotide utilization.

In both Arthur and Lemhi wheat varieties the major site of thymidine phosphorylating activity in the developing caryopsis is the ovary wall or pericarp. It is not clear if both thymidine kinase and AMP:nucleoside phosphotransferase (NPTase) are present, or whether the NPTase alone is phosphorylating the thymidine. The patterns of activity with both P-donors are suspiciously similar over the course of development, although the specific activity is higher when ATP is used as P-donor. At any rate, the thymidine phosphorylating activity in this parental tissue does not seem to correspond to any need for increased levels of thymidine nucleotides since the levels of DNA in the testa/pericarp remain constant after the first few days following anthesis (52). If most of the thymidine phosphorylating activity seen in the ovary wall is due to NPTase, then the phosphorylation of thymidine may not be the primary function of this non-specific nucleoside phosphorylating enzyme.

Thymidine phosphorylating activity in the ovule integuments (testa plus aleurone, in this case) is low in both Arthur and Lemhi during caryopsis development. Like the pericarp, the ovule integuments expand without cell division while the endosperm grows,

and the layer of cells that forms the aleurone also ceases divisions by about day 14.

In the endosperm, thymidine phosphorylating activity is very low in Lemhi, but a little higher in Arthur. The DNA content of wheat endosperm increases during the first three stages of development, even though cell division ceases by the end of stage II (9, 52), so there is a requirement for thymidine nucleotides during that time. The pyrimidine de novo pathway may provide the necessary quantities of dTTP, but the salvage pathway could supplement this by phosphorylating nucleosides translocated from other caryopsis tissues.

The DNA content of the embryo increases throughout development of the caryopsis (9). However, thymidine phosphorylating activity is most evident during stage III in both Arthur and Lemhi. It could be that a supply of thymidine nucleotides is made at this point and then utilized throughout the rest of embryo development. It is also likely that the de novo pathway is providing a portion of the thymidine nucleotides. In Arthur the thymidine phosphorylating activity appears to be thymidine kinase since there is very little NPTase activity. In Lemhi, where two peaks of thymidine phosphorylating activity occur, the first peak shows both NPTase activity and an apparent thymidine kinase activity. The second peak, however, appears to be thymidine kinase.

Whereas thymidine phosphorylating activity is relatively low throughout caryopsis development, uridine phosphorylating activity is clearly present in all tissues of the developing grain. It is

also evident that the predominant uridine phosphorylating activity is due to uridine kinase. Uridine phosphorylating activity with AMP as phosphoryl donor, i.e., NPTase, is practically non-existent in most of the tissues.

In the ovary wall and ovule integuments uridine phosphorylating activity is variable over the course of development in both wheat varieties. Jennings and Morton (52) have reported that the amount of RNA in the pericarp (ovary wall) and testa (seed integuments) remains the same throughout caryopsis development, but it is conceivable that there is a certain amount of RNA turnover during the time that these tissues remain hydrated and metabolically active. In the ovule integuments of Arthur there is a sudden dip in uridine kinase activity at day 26 following anthesis. It was on this day that the thin membranous layer thought to be the aleurone was not dissected with the ovule integuments as on all other days. Instead the aleurone layer was included with the starchy endosperm. The endosperm tissue on day 26 shows a concomitant increase in uridine kinase activity in the midst of a downward trend. It is possible that the aleurone component of the "ovule integuments" is the site of most of the uridine kinase activity found in this composite tissue. In Lemhi the same dissection change occurred on day 24 following anthesis, but the differences in uridine kinase activity in the two tissue groups is not so obvious. It should be noted that the total protein per individual in the ovule integument and endosperm tissues also showed a change in Arthur on day 26 and in Lemhi on day 24

(Figure 3-23, page 63). This change is consistent with the transfer of the protein-rich aleurone from the ovule integument sample to the endosperm sample on these specific days.

The RNA content of the endosperm in wheat increases until around day 19, and then decreases slightly (52). Uridine phosphorylating activity (uridine kinase) reaches a peak around day 15 in Arthur and falls off to a very low level by day 29. In Lemhi the peak is at day 18, falling to almost nothing by day 27. In this case there is a reasonable correspondence between the levels of uridine phosphorylating activity and the actual amount of RNA found in the tissue. It is also during this period of development that starch synthesis increases. Uridine nucleotides are required for the formation of UDP-glucose, a precursor for starch synthesis. Therefore the salvage pathway may be making a significant contribution to the ribonucleotide precursor pool. It is possible that the de novo pathway of uridine nucleotide synthesis is functioning as well, although nothing is known about its activity in developing endosperm proplastids, where most of the de novo enzymes are located (38, 39).

In the embryos of Arthur and Lemhi, uridine phosphorylating activity (uridine kinase) peaks at days 33 and 22, respectively. Activity then declines but, unlike in the endosperm, does not disappear. RNA increases throughout embryo development (9), so it is reasonable to find this type of activity present. It is interesting, though, that the bulk of the activity does not appear until late stage III and into stage IV of caryopsis development. This is

at a time when the RNA content of the endosperm is decreasing, possibly due to an increase in endosperm ribonucleases (9). It is conceivable that the ribonucleosides from degraded endosperm RNA are translocated to the developing embryo where they are rephosphorylated by the uridine kinase for use in embryo RNA synthesis. Some uridine kinase remains in the dry embryo, while the combined non-embryo tissues show very low uridine phosphorylating activity by the end of the sampling period. In a dehydrating seed, the rate of protein synthesis declines, even though the total amount of RNA does not change significantly. However, the number of polysomes decreases as the number of monosomes rises (69), suggesting that the mRNA becomes unavailable for polysome formation (84). During desiccation there is a decline in the production of proteins associated with seed development and the induction of synthesis of proteins to be involved in germination (31). Some of the mRNA molecules produced during this time of dehydration are apparently conserved, since mRNA's isolated from dry cereal and other seeds are capable of acting as templates for protein synthesis in cell-free wheat germ systems (23, 42), and since mRNA transcription inhibitors in the imbibition medium do not prevent protein synthesis (86). The uridine kinase which is conserved in the dry embryo may play a role in providing ribonucleotides for RNA synthesis that takes place in early stages of germination.

When a dried quiescent wheat embryo is exposed to water, a series of biochemical events leading to germination is set in motion.

The timing of these events varies, depending on whether or not the embryo is intact or excised, but the sequence is the same.

Imbibition of water by a dry seed or embryo proceeds rapidly at first as the outermost tissues are wetted, followed by a lag phase of variable time as successive layers are exposed to water, followed by another increase in fresh weight as cell growth resumes (29). The rate of respiration, which is very low in a dry embryo, also increases rapidly during the first several hours of imbibition, reaches a plateau for some period of time, then increases once again (84). The levels of ATP and other nucleotide triphosphates are very low in dry embryos, while the amount of AMP is fairly high. During the first several hours of imbibition in isolated wheat embryos, the level of ATP in the embryo increases dramatically while the AMP concentration falls. While some of the increase in the level of ATP is apparently due to phosphorylation of pre-existing AMP, the overall rise in adenine nucleotide levels suggests that de novo synthesis is involved as well (88).

Both RNA synthesis and protein synthesis resume very soon following the introduction of water, with RNA synthesis preceding protein synthesis somewhat (29, 37, 76). In the embryos of intact wheat grains allowed to imbibe at 2 C, mRNA synthesis can be detected within 15 minutes of transferring the seeds from the 2 C medium to a 22 C medium (37, 76). The mRNA synthesized soon after the transfer to 22 C combines with pre-existing proteins in the cell nuclei to form ribonucleoprotein particles (37).

Protein synthesis can be detected in isolated wheat embryos within 30 minutes of the beginning of imbibition (27). Early in imbibition polysomes begin to reform, using the conserved mRNA's as templates for protein synthesis (61). The synthesis of both rRNA and mRNA also begins soon after the onset of imbibition (87). RNA polymerases, especially RNA polymerase II, are found in the dry seed (63). The overall mRNA content of the embryo falls during the first hour or two of imbibition as conserved messengers are degraded, but recovers after a few hours (85). The new messengers that are synthesized earliest appear to code for the same proteins as do the conserved mRNA's, although the pattern of proteins produced in the embryo changes between 6 and 18 hours of germination (30, 84), during the time when cell growth resumes. At one point it had been reported that the rate of mRNA synthesis remained constant throughout the first 18 hours of germination in wheat, but that the rate of rRNA synthesis increased 10-fold between 6 and 16 hours (87). However, this work could not be repeated and subsequent work suggested that there was a two- to three-fold increase in RNA synthesis during the lag phase of germination, after which there was a steady increase in the rates of synthesis of both mRNA and rRNA throughout germination (49). The levels of RNA polymerase activity also begin to increase after about six hours imbibition (62). It has also been found that during the first stages of germination, a fraction of heterodisperse RNA rich in UMP is synthesized (36). This UMP-rich RNA is gradually modified until its base composition resembles that of total cellular

RNA. Figures 3-26 and 3-27 (page 66) show, not surprisingly, that a uridine kinase activity is present in germinating wheat seeds, most likely the same activity that is found in dry seeds.

It has been suggested that one of the factors controlling the rate of RNA synthesis during early germination is the level of purine and pyrimidine ribonucleoside triphosphates (28, 49). The dry wheat embryo has almost negligible levels of ribonucleoside triphosphates. But, during the first 40 minutes of imbibition in isolated embryos, the levels of all four ribonucleotide triphosphates increase manyfold (28). During the lag phase of germination, between one and five hours after the introduction of water, the purine ribonucleotide triphosphates continue to increase (60% and 30% for ATP and GTP, respectively) but the pyrimidine ribonucleotide triphosphates show an even greater increase (309% and 281% for UTP and CTP, respectively), at a time when the rate of RNA synthesis is also showing an almost 3-fold increase (28, 49). It is not known how active the de novo pyrimidine nucleotide pathway is during wheat germination. In a study with black gram seeds, uridine was an excellent precursor for formation of RNA and uridine nucleotides from the beginning of germination, while orotate was incorporated to a good degree only after 24 hours germination (6). Very likely the de novo pathway makes a significant contribution to the uridine nucleotide pool, since it is doubtful that uridine salvage alone could supply all of the precursors for RNA synthesis and other pathways requiring uridine nucleotides in germinating and growing embryos.

While RNA and protein synthesis begin very early in wheat seed germination, the initiation of DNA synthesis associated with cell division is delayed somewhat. Although cell division in excised wheat embryos does not begin until 18 hours after the beginning of imbibition (20), incorporation of labelled thymidine into DNA in isolated wheat embryos has been reported as early as 4 to 5 hours after the beginning of imbibition (15, 76) and as late as 15 hours into imbibition (66). More recently it has been shown that some DNA synthesis, as measured by incorporation of labelled deoxyadenosine, does occur within the first hour of imbibition (89). It has been reported that proteins synthesized before 9 hours of germination are necessary for DNA replication in isolated embryos (66). Some DNA polymerase has been reported in the dry wheat embryo, but only from a cytoplasmic source, with none being found in isolated nuclei (25). A recent study has reported activity, during early wheat embryo germination, of a DNA polymerase that is different from the one involved in DNA synthesis associated with cell division (60). The function of this early DNA polymerase is not clear, although it may function in DNA repair and replication of extrachromosomal DNA. The later appearing DNA polymerase appears to be synthesized during the first 6 hours of germination, doubles over the next 6 hours and continues to increase even more steeply between 12 and 18 hours of germination (67). Enzyme activity that is capable of phosphorylating thymidine is not present during the early hours of germination in wheat, but appears at a later time (45; Figures 3-28 and 3-29, page 68, present study). The appearance of this activity tends to

coincide with beginning of DNA synthesis associated with cell division. The relatively small amounts of early DNA synthesis may be supported by existing thymidine nucleotide pools, or de novo pyrimidine enzymes (e.g., thymidylate synthetase) may be involved.

As just stated, neither the dry wheat seed (Figure 3-13, page 56; Figure 3-17, page 58) nor the early imbibing embryo (Figures 3-28 and 3-29, page 68) have any kind of thymidine phosphorylating activity. The thymidine phosphorylating activity that does appear after 36 hours in intact seeds is due to an AMP:nucleoside phosphotransferase rather than a thymidine kinase, even though use of ATP as phosphoryl donor does result in some thymidine phosphorylation (45). Uridine phosphorylating activity is found in both dry (Figure 3-5, page 49; Figure 3-9, page 51) and germinating (Figures 3-26 and 3-27, page 66) wheat seeds. In the dry embryos and in the early stages of germination, this activity is apparently the result of a uridine kinase since only ATP as phosphoryl donor is successful in uridine phosphorylation. Not until about 36 hours after the beginning of imbibition in these intact seeds does uridine phosphorylation occur using AMP as phosphoryl donor. After 36 hours of imbibition both ATP and AMP are successful phosphoryl donors, but it is not clear which enzyme activities are involved in the phosphoryl transfer at this point. It has been reported that in corn a uridine kinase is present during early germination, but that this activity falls off rapidly after 36 hours (33). It could also be the case in the present study that the initially occurring

uridine kinase activity diminishes and that the less specific AMP: nucleoside phosphotransferase then takes over in the phosphorylation not only of uridine, but also of thymidine and other nucleosides.

Experiments using [α-^{32}P]ATP and [γ-^{32}P]ATP were designed to show which phosphoryl group was actually transferred from ATP to uridine. If a true uridine kinase were involved, then radioactivity would show up in the nucleotide product only when [γ-^{32}P]ATP was used as phosphoryl donor. If an AMP:nucleoside phosphotransferase were the only activity involved in uridine phosphorylation, then radioactivity would appear in UMP product only when [α-^{32}P]ATP were used as phosphoryl donor. At 12 hours after the beginning of imbibition, extracts of embryos from Lemhi wheat show label in the UMP product primarily when [γ-^{32}P]ATP is used as phosphoryl donor, and hardly at all with the [α-^{32}P]ATP. This merely corroborates the data using unlabelled ATP and AMP that show a uridine kinase to be present and AMP:nucleoside phosphotransferase to be absent at 12 hours after the beginning of imbibition. The [^{32}P]ATP data using Arthur wheat embryos as enzyme source are unclear because of the incomplete separation of nucleotide from the large phosphate peak. Presumably, had separation been better, label would have shown up clearly in the UMP with [γ-^{32}P]ATP as donor but not with [α-^{32}P]ATP.

After 96 hours of germination, uridine phosphorylation can occur with either AMP or ATP as phosphoryl donor. It is known that an AMP:nucleoside phosphotransferase is present at this time and

that, in conjunction with phosphatases which cleave the γ- and β-phosphoryl groups, it can transfer the α-phosphoryl group from ATP to thymidine to form dTMP (45). It is probable that this same enzyme activity phosphorylates uridine to UMP in a similar fashion, whether AMP or ATP is used as phosphoryl donor. The question remains, however, whether uridine kinase is still present at this stage of germination. In the [^{32}P]ATP experiments using crude extracts of 96 hour seedlings as enzyme source (Figure 3-33, page 72), little to no label was found in the UMP product when [γ-^{32}P]ATP was used as phosphoryl donor. When [α-^{32}P]ATP was used, however, considerable label appeared in the UMP product. Results were the same for both Arthur and Lemhi varieties. The same experiment using thymidine as phosphoryl acceptor and Lemhi wheat seedlings as enzyme source yielded similar results, although there was more overlap of the dTMP and phosphate peaks. Labelled dTMP can still be detected as a shoulder on the right side of the phosphate peak.

This, then, is what appears to happen with nucleoside phosphorylation in wheat seeds upon introduction of water. The uridine kinase that appeared in the embryo tissue during later stages of seed development, and which remained in the dry embryo, is still present during the early hours of imbibition. However, the uridine kinase activity soon begins to decline and is virtually absent by 96 hours of germination, and probably a good bit before this. Meanwhile, AMP:nucleoside phosphotransferase, which is not present in

the dry seed or during early hours of imbibition, begins to appear at about 36 hours after introduction of water to the whole wheat seeds. This activity, which can phosphorylate both uridine and thymidine, continues to increase through 120 hours of germination. It is during the appearance of AMP:nucleoside phosphotransferase activity that thymidine phosphorylation is first detected. No thymidine kinase is present during wheat seed germination.

Figure 3-35 presents a summary of the stages of wheat grain development from anthesis through the first week of germination and growth. Included are comparisons of changes in RNA and DNA contents of different tissues at various stages of development and changes in uridine- and thymidine-phosphorylating activities in the tissues examined in this study.

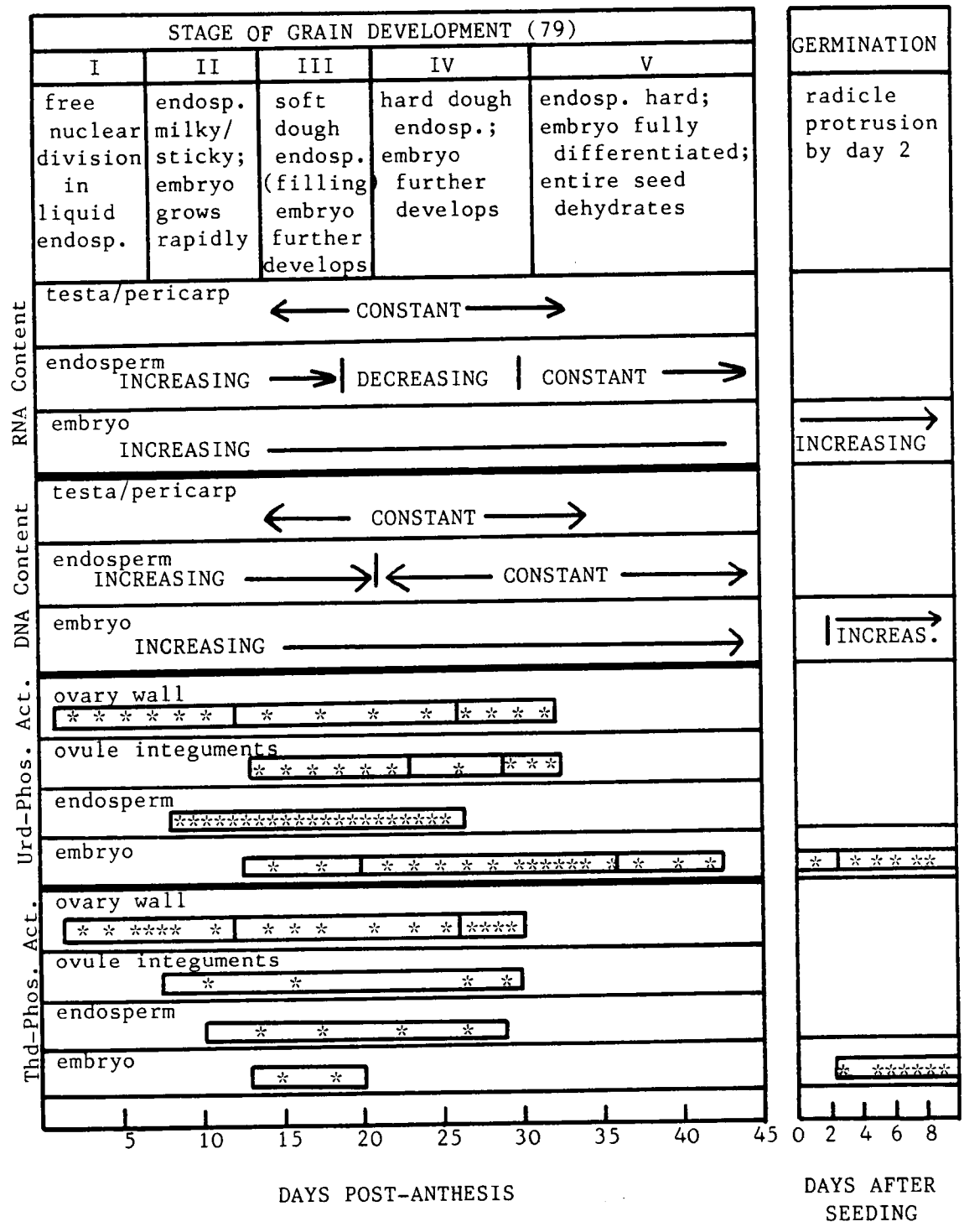

Figure 3-35. Stages of development of a wheat grain, with RNA and DNA contents, and uridine- and thymidine-phosphorylating activities of various tissues.

CHAPTER IV

CONCLUSION

The nucleoside kinases are specific enzymes with regard to phosphoryl acceptors. Uridine kinase will catalyze phosphoryl transfer from ATP to uridine and cytidine only, and thymidine kinase (from bacterial or animal sources) will phosphorylate deoxythymidine and deoxyuridine (2). Nucleoside phosphotransferase (NPTase), on the other hand, will catalyze phosphoryl transfer to any of the normal nucleosides, both ribose and deoxyribose forms. It should be noted, however, that uridine is one of the poorest nucleoside substrates for this enzyme. Prasher et al. (72) estimate that purified barley NPTase is almost thirty times more efficient at phosphorylating deoxythymidine than uridine. In fact, NPTase is generally more efficient with deoxyribonucleosides than with ribonucleosides.

What role do these enzymes play in seed and seedling development? Certainly NPTase activity is very low in most of the developing seed tissues. Uridine kinase is the enzyme that handles uridine reclamation in all of the true embryonic tissues of the developing wheat caryopsis. The interesting thing about this enzyme is the way it is developmentally regulated. During the stage of development when RNA is increasing in the endosperm (through day 19 post-anthesis) (52), uridine kinase reaches a peak. Thereafter the activity

declines, even as the RNA content of the endosperm decreases. In the embryo, uridine kinase reaches a peak fairly late in development. Activity declines somewhat as the seed dries, but does not disappear. In fact, this enzyme appears to be one of a number of activities that is carried in the embryo through desiccation, to be reactivated during the early stages of imbibition and seed germination. But then it disappears again. It has been reported that the RNA content of the wheat embryo increases throughout development (9). If that is the case, then why is the bulk of uridine kinase activity in that tissue seen only in later developmental stages? In order for the amount of RNA to increase in a tissue, uridine nucleotides must be continually supplied. It may be the case that the de novo pathway of pyrimidine nucleotide synthesis provides most of the uridine nucleotides necessary for RNA synthesis in the embryo. To my knowledge, no attempt has been made to measure enzymes of the de novo pathway in developing seeds. What, then, is the role of uridine kinase in developing seed tissues? In order for this enzyme to be effective, it must have a supply of uridine as substrate. The present data from endosperm and embryo tissues plus information about RNA content of those tissues suggests a plausible scenario for uridine kinase involvement. In the endosperm, RNA increases through stage II until about day 19 (52), then decreases. Bewley & Black (9) suggest that, after the RNA is used for a flurry of storage protein syntheses, it is degraded by increased endosperm ribonucleases. The degraded nucleic acid nucleotides, transported to the embryo as nucleosides

for easier membrane passage, could be used in RNA synthesis in that tissue. The influx of uridine (and cytidine, since uridine kinase phosphorylates both) would provide substrate for uridine kinase in the embryo. What sparks the early appearance of uridine kinase activity in endosperm tissues is not as clear.

In germinating seeds, stored and maybe newly synthesized uridine kinase could utilize uridine derived from nucleic acid degradation in the embryo or the endosperm. Early stages of imbibition and germination are times of rapid RNA synthesis. During germination, constituents of the endosperm are transferred to a growing embryo. It is likely that nucleic acids of the endosperm are degraded and their component parts transported to the embryo as nucleosides, since these are taken up with higher efficiency than free purine or pyrimidine bases (54). One would expect that uridine nucleotides would be required throughout seedling growth, not only for RNA synthesis but also for the formation of UDP-sugars. It is interesting, then, to see uridine kinase vanish by 72-96 hours into germination. Perhaps by this time transport of uridine from nutritive tissues has diminished and available uridine has become incorporated into RNA. Further RNA and UDP-sugar synthesis could be supplied by the <u>de novo</u> pyrimidine synthetic pathway. Uridine kinase may vanish during later germination stages, but uridine phosphorylating activity does remain in the form of NPTase. Uridine is not an especially good substrate for NPTase, but that may not matter if uridine levels are low anyway.

What about thymidine phosphorylation? Should not there be a thymidine kinase analogous to uridine kinase that supplies thymidine

nucleotides for DNA synthesis? In the endosperm, DNA increases between days 8 and 19 post-anthesis (9, 52), and then remains constant. There is little corresponding increase in thymidine phosphorylation during this time. In the embryo, the DNA content increases throughout seed development (9). At least in this tissue there appears to be a small increase in thymidine phosphorylating activity during stage III of development. Results suggest that the activity may be due in part to thymidine kinase since thymidine phosphorylation is measurable even when NPTase activity is very low. But whichever enzyme is responsible, the salvage of thymidine does not seem to be very important in providing thymidine nucleotides for DNA synthesis. Since the endosperm DNA content remains constant after its initial increase, it is unlikely that much thymidine in this tissue would be available for transport to the embryo. The possibility that thymidine kinase is there at all during seed development is noteworthy because the presence of this enzyme in higher plants has been doubted. Most apparent thymidine kinase activity in plants has been shown to be a combination of ATP-hydrolase and NPTase activities. This is the case in wheat during seed germination.

There is considerably more thymidine phosphorylating activity during seed germination--NPTase, not thymidine kinase--than during seed development. Thymidine may be made available to the embryo by nutritive tissues during germination, as with uridine, but the enzyme activity responsible for making it available for incorporation into DNA does not appear until somewhat later in germination, even

as the major portion of DNA synthesis is delayed. Is it possible that NPTase is primarily responsible for deoxyribonucleoside phosphorylation? In general, NPTase phosphorylates deoxyribonucleosides more efficiently than ribonucleosides. It would be very economical to use one enzyme that could phosphorylate all four deoxyribonucleosides.

Not only is NPTase more versatile than nucleoside kinases, but it also can effect a "low energy" phosphoryl transfer. An NMP is the typical donor in the NPTase reaction, as opposed to an NTP in a kinase reaction. It is difficult to say how important this is. During the earliest stages of germination a great deal of ATP is generated. There would probably be no lack for the reaction catalyzed by uridine kinase. The level of ATP is probably lower during later germination stages, but not likely to be limiting to nucleoside salvage reactions.

One of the more peculiar features of NPTase, as compared to nucleoside kinases, is the nucleotidase function that exists alongside the phosphotransferase activity, and which must decrease the efficiency of the enzyme. What function this hydrolytic activity plays is unknown, but there are other group-transferring enzymes that also have a hydrolytic capability in addition to the transfer function (5, 44, 53, 72). Conceivably, the nucleotidase function of NPTase may be more active when NMP levels are high (little conversion to NTP). This activity would generate both inorganic phosphate and nucleosides. The latter could then be degraded further or transported

to another location. When NTP synthesis and utilization is active, the transferase function of NPTase could generate a variety of NMP's for incorporation into nucleic acid.

The de novo pathway of pyrimidine nucleotide biosynthesis presumably is active during both seed development and germination. At this point it is difficult to compare the contributions of the de novo and salvage pathways. A great deal more energy is required to make pyrimidine nucleosides from scratch than to recycle them, but when a net increase in nucleotides is needed, and nucleoside quantities are limited, then the de novo pathway must produce them. The major importance of the salvage enzymes may be their role in transport of nucleotides from one location to another. In the case of NPTase, this one enzyme could both hydrolyze NMP's on one side of a membrane and reassemble them on the other.

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VITA

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After a year of travel and factory work, she entered the Botany graduate program at The University of Tennessee, Knoxville. While on a teaching assistantship, she completed a Master's degree in June 1980, then began doctoral work. In September 1984, after completing the research part of her doctoral project, she moved to Ann Arbor, Michigan, with her husband, David.

In March 1985, a son, Daniel, was born, and the dissertation work slowed considerably. In May 1987, the family moved to Arvada, Colorado, where she completed the dissertation. She received her Ph.D. degree in August 1988.