Thymidine Phosphotransferase and Nucleotide Phosphohydrolase of the Fern *Asplenium nidus*

GENERAL PROPERTIES AND INHIBITION BY ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

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1. Extracts of several plant species contained nucleoside-AMP phosphotransferase activity. The ratio of activity with thymidine to that with uridine as nucleoside substrate was essentially constant, both between species and throughout plant development. Evidence is presented that the total thymidine-AMP phosphotransferase activity of the leaves of *Asplenium nidus* (bird's-nest fern) and of *Helianthus tuberosus* (Jerusalem artichoke) increases during maturation. 2. Thymidine-AMP phosphotransferase was purified 22-fold from a very rich source of this activity, extracts of *A. nidus*. 3. A broad specificity towards both nucleoside and nucleoside 5'-monophosphate substrates is displayed by this preparation, and the evidence suggests that all could be due to a single enzyme. 4. Nucleosides that act as substrates will also inhibit phosphotransfer to other nucleosides, with K_i values close to the corresponding K_m values found when utilized as substrates. 5. Ca^{2+} -activated ATP phosphohydrolase was separated from the phosphotransferase by differential complexing to Blue Dextran in the presence of urea, whereas an AMP phosphohydrolase activity was closely associated with thymidine-AMP phosphotransferase through all separation techniques used. 6. Metal ions did not activate either of the latter two activities, and 1,10-phenanthroline was found to inhibit the phosphotransferase. 7. K_m values for AMP for the respective activities were 0.11 mM (thymidine phosphotransferase) and 0.20 mM (AMP phosphohydrolase) and for thymidine (phosphotransferase only) 0.88 mM. 8. 3':5'-Cyclic AMP was found to inhibit both phosphotransferase and AMP phosphohydrolase activities, with K_i values of 0.056 mM and 0.15 mM respectively. It is suggested that this inhibitor would be of value in revealing the existence of thymidine kinase in plant extracts with high thymidine phosphotransferase activity.

Thymidine kinase (ATP-thymidine phosphotransferase; EC 2.7.1.75) is generally considered to catalyse the first step in the salvage incorporation of thymidine into DNA of most organisms (Cleaver, 1967; Grivell & Jackson, 1968). This enzyme utilizes ATP as phosphoryl donor for dTMP synthesis. In some plant tissue extracts, however, AMP can serve as the phosphoryl donor for dTMP synthesis, in a reaction catalysed by a nucleoside-AMP phosphotransferase (Brunngraber & Chargaff, 1967; Deng & Ives, 1972). Thymidine-AMP phosphotransferase activity is not confined entirely to plants (Maley & Maley, 1963; Mitsugi *et al.*, 1964; Georgatos, 1967). The activity from plant extracts, unlike thymidine kinase, does not appear to be specific for thymidine and close to structural analogues, and has not been shown to be subject to regulatory control by dTTP

(Deng & Ives, 1972). Owing to uncertainties arising from the high activity of ATP hydrolysis in plant extracts, it is possible that thymidine kinase is also present in plant tissue (Melichar & Cihák, 1974), where, as in most other organisms, it could be responsible for salvage phosphorylation of thymidine leading to DNA synthesis.

The aim of the present investigation was to study the properties of the phosphotransferase purified from a very active source (the bird's-nest fern, *Asplenium nidus*) to gain an insight into the possible functions of this enzyme and its relationship with thymidine kinase.

Experimental

Plants

Asplenium nidus (bird's-nest fern) plants were collected in a rain-forest area north of Sydney, N.S.W., Australia, and grown in a shade-house at the Waite

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Agricultural Research Institute; *Helianthus tuberosus* (Jerusalem artichoke) and the *Epidendrum* species (crucifix orchid) were grown under field conditions at the Waite Agricultural Research Institute; barley (*Hordeum vulgare* cv. 'Clipper') was grown in acid-washed sand in the nutrient medium of Hoagland & Arnon (1950); lucerne (*Medicago sativa*) pollen was collected from a beehive fitted with a pollen trap, and the pollen placed in liquid N₂ within 30 min of deposition in the trap.

Materials

Soluble polyvinylpyrrolidone was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Insoluble polyvinylpyrrolidone (Polyclar AT) was obtained from Croda Federal Chemicals Ltd. (Richmond North, Vic., Australia) and was hydrated, washed and stored at 4°C in 0.1 M-potassium phosphate (pH 7.0). The ultrafiltration cell, model 52, with XM50 membrane, was purchased from Amicon Corp., Scientific Systems Division (Lexington, MA, U.S.A.). Pharmacia Fine Chemicals (Uppsala, Sweden) supplied Sephadex G-200 and Blue Dextran 2000, and Whatman (Maidstone, Kent, U.K.), the DE-32 DEAE-cellulose microgranular anion-exchanger. Hypatite C was obtained from Clarkson Chemical Co. Inc. (Williamsport, PA, U.S.A.); bovine serum albumin, Tris buffer and nucleic acid bases, nucleosides and nucleotides were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and cysteine, sodium diethyl dithiocarbamate and inorganic reagents were A.R.-grade from BDH (Poole, Dorset, U.K.). The Radiochemical Centre (Amersham, Bucks., U.K.) supplied [2-¹⁴C]thymidine, [2-¹⁴C]uridine, [8-¹⁴C]adenosine, [8-¹⁴C]guanosine, [8-¹⁴C]-ATP, [8-¹⁴C]AMP and ³H₂O, and Schwarz-Mann supplied the [2-¹⁴C]dTMP.

The [2-¹⁴C]thymidine and [2-¹⁴C]uridine were found to contain 0.5–1% of labelled contaminants that had mobilities similar to those of dTMP and UMP respectively during paper electrophoresis at pH 3.5. Because these contaminants greatly decreased the sensitivity of the phosphotransferase assay, 0.2 ml portions of the [2-¹⁴C]thymidine and [2-¹⁴C]uridine were purified by elution with water through small columns (4 mm × 5 mm diam.) of Dowex-1 (Cl⁻ form). The respective contaminants were decreased approximately tenfold, with 80% recovery of the labelled nucleosides.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), after precipitation with 5% (w/v) trichloroacetic acid, with bovine serum albumin as standard.

Nucleoside phosphotransferase assay

Thymidine phosphotransferase activity was assayed as a routine with incubation mixtures containing 0.07 M-Tris/HCl (pH 7.5), 0.07 mg of bovine serum albumin/ml, 7.1 mM-AMP, 1.4 mM-[2-¹⁴C]thymidine (0.4 mCi/mmol) and enzyme, in a total volume of 0.07 ml. The reaction mixtures were incubated at 38°C for 30 min in test tubes (13 mm × 100 mm) covered with Parafilm, and the reactions were terminated by heating in a boiling-water bath for 1.5 min. After cooling in ice, the tubes were centrifuged at 2000g for 1 min to return condensed moisture to the bulk of the reaction mixture. Samples (usually 0.025 ml) were analysed by paper electrophoresis on Whatman 3 MM paper (1 h at 26.5 V/cm in 0.1 M-sodium citrate, pH 3.5) together with authentic samples of non-radioactive marker substances. The separated marker areas (thymidine and dTMP product) were located with short-wavelength u.v. radiation, were cut out and the amount of ¹⁴C in each was determined in a Unilux Mark I liquid-scintillation spectrometer with a scintillation solution containing 2,5-diphenyloxazole (3 g/litre) and 1,4-bis-(5-phenyloxazolyl-2-yl)benzene (0.2 g/litre) in toluene. The counting efficiency was 64%.

The concentration of thymidine used in routine assays, 1.4 mM, was not enzyme-saturating, since this condition would have required a concentration exceeding 10 mM. The choice of the concentration and specific radioactivity of labelled compound finally used was a compromise between maximizing the absolute reaction velocity and maximizing the assay sensitivity. Consequently, reaction rates observed under these conditions were only about 60% of the maximum velocity expected for conditions of substrate saturation. To produce linear reaction progress curves, reaction mixtures were incubated with sufficiently small amounts of enzyme that the concentrations of thymidine was changed by less than 5% during the course of routine assay reactions. Where nucleoside phosphotransferase activity other than that utilizing thymidine as acceptor was determined, the [2-¹⁴C]thymidine in the reaction mixture was replaced by the appropriate [¹⁴C]-labelled nucleoside, and the corresponding u.v.-visible markers were used for paper electrophoresis.

5'-Nucleotide phosphohydrolase assay

(a) *Radiochemical method.* Reaction mixtures contained 0.07 M-Tris/HCl (pH 7.5), 0.25 mg of bovine serum albumin/ml, 1.59 mM-[8-¹⁴C]AMP (0.5 mCi/mmol) and enzyme, in a total volume of 0.07 ml. Enzyme was added to initiate the reaction, and the remainder of the assay carried out as described for nucleoside phosphotransferase, except that AMP and adenosine were used as u.v.-visible markers for paper electrophoresis.

(b) *Colorimetric method.* For some kinetic measurements, nucleotide phosphohydrolase activity was determined by measurement of P_i released from AMP. Reaction mixtures contained 0.045 mM-Tris/HCl (pH 7.5), 0.09 mg of bovine serum albumin/ml, variable amounts of AMP and enzyme, in a total volume of 1.11 ml. After incubation at 38°C for 45 min, P_i released was determined spectrophotometrically by the method of Chen *et al.* (1956).

ATP phosphohydrolase assay

Incubation mixtures contained 0.07 M-Tris/HCl (pH 7.5), 0.25 mg of bovine serum albumin/ml, 1.4 mM- $CaCl_2$, 0.25 mM-[8- ^{14}C]ATP (3.33 mCi/mmol) and enzyme. The reaction mixtures were incubated at 38°C for 30 min, the reaction was terminated, and paper electrophoresis carried out as for the nucleoside phosphotransferase assay, except that ATP, ADP, AMP and adenosine were used as u.v.-visible markers for paper electrophoresis. The sum of ^{14}C corresponding to the adenosine, AMP and ADP areas was taken as the measure of ATP phosphohydrolase activity.

dTMP kinase assay

The incubation mixture, in a total volume of 0.07 ml, contained 0.07 M-Tris/HCl (pH 7.5), 0.07 mg of bovine serum albumin/ml, 7.1 mM-ATP, 14.2 mM- $MgCl_2$, 60 mM-creatine phosphate, 0.06 mg of creatine phosphokinase/ml and 1.4 mM-[2- ^{14}C]dTMP (0.8 mCi/mmol). Enzyme was added to start the reaction and the assay completed as described for nucleoside phosphotransferase, except that dTDP and dTTP were used as u.v.-visible markers for paper electrophoresis.

Results

Sources and distribution

During the course of a study on the phylogenetic distribution of thymidine kinase, an exceptionally high activity of thymidine phosphorylation was detected in crude extracts of *Asplenium nidus*. Although initially this was thought to be a thymidine kinase activity, it was subsequently demonstrated that AMP (and not ATP) was in fact the phosphoryl-donating substrate for this enzyme. ATP acted as an apparent substrate with crude preparations because of the presence of highly active hydrolytic enzymes degrading ATP to AMP. The fern extracts showed significantly higher thymidine phosphotransferase activity when compared with extracts from other plants tested (Table 1). In addition, the thymidine-phosphorylating capability of the crude extracts from fern, and indeed crude extracts from some other plant sources, is much higher than the thymidine kinase activity in crude extracts of such an active organism as *Escherichia coli*, which can phosphorylate 0.003 μ mol of thymidine/min per mg of protein (Okazaki & Kornberg, 1964). Phosphorylation of uridine, with AMP as substrate, was also observed in several of the plant extracts. The ratio of the thymidine- and uridine-phosphorylation rates was remarkably constant (approx. 2-3) for the species investigated (Tables 1 and 2).

As shown in Table 2, a study was made of enzyme distribution within a range of non-senescent leaves at various stages of development, obtained from a single plant of *A. nidus*. Over a 45-fold range in leaf fresh weight, the specific activity of thymidine phosphotransferase activity decreased by a factor of two, whereas the total activity per leaf increased ninefold from meristematic tissue to the mature leaf. This

Table 1. Activities of thymidine phosphotransferase and other enzymes in a range of plant extracts

Plant material was ground in a pre-chilled mortar and pestle for 3 min (1 g fresh wt. to 4 ml of 0.5% soluble polyvinylpyrrolidone in 0.1 M-potassium phosphate, pH 7.0) and then homogenized in an all-glass homogenizer. Each homogenate was centrifuged at 5000g for 10 min, and the supernatant assayed for the various activities as described in the Experimental section.

Plant species and and tissue	Enzyme	Enzyme activity (μ mol/min per mg of protein)			
		Thymidine phosphotransferase	Uridine phosphotransferase	ATP phosphohydrolase	dTMP kinase
<i>Asplenium nidus</i> : immature leaf		1.07	0.34		
		0.90			
		1.67		4.8	0.014
<i>Hordeum vulgare</i> : tops		0.059	0.020		
	roots	0.046	0.009		
<i>Helianthus tuberosus</i> : leaf		0.22	0.10		
<i>Epidendrum</i> hybrid: leaf		0.054			
<i>Medicago sativa</i> : pollen		0.0023			

Table 2. Distribution of thymidine and uridine phosphotransferase activities in the various parts of *Asplenium nidus* and *Helianthus tuberosus*

For the fern *Asplenium nidus*, the young bud was considered to be the tightly coiled bud and the immature leaf, the same when uncoiled. The young mature leaf taken was approx. 75% the size of the mature leaf. The *Helianthus tuberosus* plant was dissected in early summer, was 40cm long (including roots) and was forming small tubers. The mature leaf was sampled from an area approx. two-thirds of the way down the aerial portions of the plant and obviously necrotic leaves below this were not assayed. Representative samples of plant tissue were extracted and assayed as described in Table 1.

Plant species and tissue	Wet weight (g)	Extracted protein (mg)	Enzyme activity (μmol/min)			Ratio of activities
			Thymidine phosphotransferase		Uridine phosphotransferase (units per (mg of protein))	
			(total units from tissue)	(units per mg of protein)		
<i>Asplenium nidus</i>						
Young bud	0.22	4.95	8.13	1.64	0.73	2.24
Immature leaf	3.35	30.2	32.9	1.09	0.53	2.06
Young mature leaf	7.90	71.1	64.7	0.91	0.35	2.64
Mature leaf	9.90	89.1	74.8	0.84	0.30	2.70
Senescent leaf	4.75	3.85	1.5	0.07	0.0	—
<i>Helianthus tuberosus</i>						
Young leaf	1.5	87.8	41.0	0.47	0.22	2.13
Expanded leaf	2.8	139	65.8	0.47	0.17	2.84
Mature leaf	5.0	189	55.5	0.29	0.09	3.10
Stem	19.8	178	1.7	0.01	0.0	—

indicates that enzyme synthesis was continuing during leaf expansion, at a time when DNA synthesis (which requires dTMP, the product of the enzyme) would be expected to have decreased. Uridine phosphotransferase activity was distributed similarly to thymidine phosphotransferase, the ratio of the two activities remaining remarkably constant throughout. An essentially similar pattern of activity distribution was found within a single plant of *Helianthus tuberosus*, but with lower specific activities. Although little or no activity was detected in the root material of either the *A. nidus* or *Helianthus tuberosus* (Table 2), results obtained with barley (*Hordeum vulgare*) (Table 1) show that root tissue in some plants can have appreciable activity. The activity reported here for barley extracts exceeds that recorded by Deng & Ives (1972), probably reflecting the lower nucleoside concentration used by these authors. As source material for enzyme preparation, very young leaf buds from *A. nidus* were chosen because of their high specific activity.

Extraction and partial purification

A major consideration in extracting thymidine phosphotransferase activity for further purification was the potential problem of protein cross-linking after oxidation of polyphenols that were observed to be present in high concentration in crude extracts of *A. nidus*, even though experimental data showed that these compounds did not lower the specific activity of the phosphotransferase. In an attempt

to eliminate this problem, insoluble polyvinylpyrrolidone ('Polyclar') in a buffer solution (pH 7.0) containing cysteine and sodium diethyldithiocarbamate was included in the extraction procedure both to enhance the removal of polyphenolic materials and to offset oxidation of any remaining polyphenols (Loomis & Battaile, 1966; Anderson, 1968).

Young buds from *A. nidus* were frozen in liquid N_2 and ground to powder with a mortar and pestle, the frozen powder being stirred immediately into a suspension of Polyclar in buffer solution. For each g fresh weight of tissue, 6.25 ml of the suspension was used. This contained 0.5 g dry wt. of Polyclar in freshly prepared buffer of the following composition: 0.1 M-potassium phosphate (pH 7.0), 0.01 M-cysteine/HCl and 0.01 M-sodium diethyldithiocarbamate. After centrifuging at 5000g for 10 min, this extraction procedure yielded a colourless crude supernatant fraction that remained clear for at least 24 h at 0°C. Centrifugation of this fraction at 144000g for 2 h gave a supernatant containing approx. 98% of the initial activity (Table 3). The phosphotransferase activity was retained in an ultrafiltration cell with an XM50 membrane, concentrating the activity for the next step. The enzyme was applied to a column (57 cm $\times$ 2.3 cm diam.) of Sephadex G-200 in 0.05 M-Tris/HCl (pH 7.5), and fractions (6.5 ml) were collected. Phosphotransferase activity was not excluded from the gel, and was eluted in separate runs well after Blue Dextran but just before bovine serum albumin. At this stage the uridine phosphotransferase activity followed closely the elution

Table 3. *Purification of thymidine phosphotransferase*

The various steps were carried out as described in the text. It is noteworthy that only 30% of the XM50 residue was loaded on the Sephadex G-200 column, and that only 66% of the Sephadex G-200 fraction was applied to the DE-32 column. The values given for both the Sephadex G-200 and DE-32 fractions below apply to the enzyme solution after concentration by ultrafiltration.

Fraction	Volume (ml)	Protein (mg/ml)	Thymidine phosphotransferase activity		Relative recovery of activity (%)	Ratio of (thymidine phosphotransferase)/ (uridine phosphotransferase activities)
			(units/ml)	(units/mg of protein)		
Crude supernatant	9.6	2.15	3.69	1.72	100	2.83
140000g supernatant	9.8	1.43	3.53	2.47	98	2.67
XM50 residue	7.9	1.33	3.32	2.50	74	2.52
Sephadex G-200	2.1	0.7	1.34	1.92	24	2.72
DE-32	2.0	0.010	0.37	37.1	10	2.10

pattern of thymidine phosphotransferase, and an active AMP phosphohydrolase activity as well as an ATP phosphohydrolase was also associated with the phosphotransferase, all at an elution volume corresponding to a mol.wt. of approx. 80000–100000.

Peak fractions from the Sephadex G-200 column, corresponding to 66% of the total activity eluted, were combined, concentrated by ultrafiltration as before, and applied to a DE-32 column (6cm×1cm diam.) in 0.02M-Tris/HCl (pH 7.5). After washing the column with a further 70ml of 0.02M-Tris/HCl (pH 7.5), a linear gradient was applied to the column (100ml of 0.35M-Tris/HCl, pH 7.5, in the reservoir and 100ml of 0.02M-Tris/HCl, pH 7.5, in the mixing vessel), and fractions (6.5ml) were collected. Thymidine phosphotransferase was eluted in a symmetrical peak through the gradient. Uridine phosphotransferase and AMP phosphohydrolase activities were eluted in the same fractions as the thymidine phosphotransferase and were closely correlated with it. An active ATP phosphohydrolase was also correlated closely with the phosphotransferases, the ratio of this activity to the thymidine phosphotransferase being 0.9:1.0. Concentration of the peak fractions by ultracentrifugation gave a good recovery of thymidine phosphotransferase activity, with a useful increase in specific activity resulting from DE-32 fractionation. An overall 22-fold purification was achieved. The ratio of thymidine phosphotransferase to uridine phosphotransferase activities remained essentially constant through the various purification steps (Table 3).

Separation of ATP phosphohydrolase activity

When the 144000g supernatant was subjected to column chromatography on Sephadex G-200 in the presence of Blue Dextran, complexing occurred between the thymidine phosphotransferase and Blue Dextran, resulting in the complete exclusion of

thymidine phosphotransferase, together with uridine phosphotransferase, AMP phosphohydrolase and ATP phosphohydrolase and Blue Dextran (Fig. 1a). However, chromatography on Sephadex G-200 equilibrated with 3M-urea/0.05M-Tris/HCl, pH 7.5, gave a very different elution profile. Although the ATP phosphohydrolase activity remained complexed with the Blue Dextran and was thus completely excluded, the thymidine phosphotransferase was no longer in a strong association with the Blue Dextran, and was eluted over a wider range (Fig. 1b), most of it not being excluded. The urea similarly eliminated the complexing between both uridine phosphotransferase and AMP phosphohydrolase activities and Blue Dextran. Both the uridine phosphotransferase and AMP phosphohydrolase activities were again eluted with thymidine phosphotransferase in the presence of urea. The peak fractions from the Sephadex G-200/urea chromatography were pooled and dialysed against 0.05M-Tris/HCl, pH 7.5, and rechromatographed on Sephadex G-200 in 0.05M-Tris/HCl, pH 7.5, with Blue Dextran. Complexing again took place (in this case in the absence of ATP phosphohydrolase) and Blue Dextran, along with thymidine phosphotransferase, uridine phosphotransferase and AMP phosphohydrolase, was excluded from the gel. In addition, when this dialysed material was subjected to DE-32 chromatography, the elution behaviour of thymidine phosphotransferase uridine phosphotransferase and AMP phosphohydrolase was as described for the partial-purification procedure.

The remarkably close correlation between thymidine phosphotransferase, uridine phosphotransferase and AMP phosphohydrolase activities through these elution profiles on Sephadex G-200 in the presence and absence of urea and Blue Dextran, together with the constancy of the ratios of thymidine phosphotransferase to uridine phosphotransferase activities through the purification, gave some indication that

Fig. 1. Gel filtration in the presence of Blue Dextran

The XM50 fraction (Table 3) enzyme preparation, together with 0.5% Blue Dextran, was subjected to gel filtration on a column (2.3 cm $\times$ 45 cm) of Sephadex G-200 in buffer of composition (a) 0.05 M-Tris/HCl, pH 7.5, and (b) 3M-urea/0.05M-Tris/HCl, pH 7.5. Enzyme activities were assayed in the fractions as described in the Experimental section. O, Thymidine-AMP phosphotransferase; Δ , uridine-AMP phosphotransferase; $\bullet$, ATP phosphohydrolase activity. Blue Dextran ($\blacktriangle$) was determined by E_{650} . The arrow indicates the peak of $^3\text{H}_2\text{O}$ elution. The relative activity of each enzyme is expressed as a fraction of thymidine-AMP phosphotransferase activity in the peak tube of Fig. 1(a). Fractions of volume 4.7 ml were collected.

these activities are closely associated and may even represent the same molecular species.

General properties

During storage at 0°C, the thymidine phosphotransferase activity was relatively stable. Thus both the crude supernatant and XM50-residue fractions (Table 3) retained 80% of initial activity after 6 months at 0°C. However, the enzyme was much less stable at 37°C; less than 5% of the activity of one preparation remained after 60 min at 37°C, and all had disappeared by 5 min at 63°C. The presence of either AMP or thymidine did not protect the enzyme against inactivation at 37°C, but bovine serum albumin did when added at concentrations of

Fig. 2. Reaction progress curves and the protective effect of bovine serum albumin on thymidine-AMP phosphotransferase

Reactions were carried out as described in the Experimental section, with the following modifications: (a) reactions were incubated for variable periods, in the presence (O) or in the absence (Δ) of 70 μg of bovine serum albumin/ml; (b) reactions were incubated for 20 min in the presence of various amounts of enzyme.

70 $\mu\text{g}/\text{ml}$ or greater. This is reflected in the reaction progress curve obtained under routine assay conditions (Fig. 2a), which was linear only in the presence of added bovine serum albumin. Thus albumin was added to all assays and reaction mixtures for kinetic studies. Under these conditions the reaction rate was strictly proportional to the amount of enzyme added (Fig. 2b). The optimum pH for thymidine phosphotransferase activity was found to be around 7.5.

The activity was not affected by the addition of 1.4 mM- or 14 mM-MgCl₂, nor by 14 mM-CaCl₂, nor by 1.4 mM-MnCl₂. However, 70–80% inhibition was observed with 14 mM-MnCl₂, -NiCl₂ or -CoCl₂. Although no activation was obtained by the addition of metal ions to the most purified enzyme fraction, the instantaneous (i.e. without preincubation) inhibition of thymidine phosphotransferase activity by 1,10-phenanthroline suggests that a metal ion is intimately involved in the catalytic process; 50% inhibition was

Table 4. Nucleoside substrate specificity

Nucleosides were tested as phosphoryl acceptors in place of thymidine, as indicated in the Experimental section, activities being expressed relative to that with thymidine as substrate. The values listed for glucose and ribose represent the limit of sensitivity for these determinations. A Sephadex G-200 enzyme fraction, with ATP phosphohydrolase activity removed, was used for these studies.

Nucleoside	Relative activity
Thymidine	1.00
5-Bromodeoxyuridine	0.90
Deoxyuridine	0.38
Uridine	0.68
Cytidine	0.20
Adenosine	0.36
Guanosine	0.48
Glucose	<0.05
Ribose	<0.10

observed at 1 mM-phenanthroline, which is rather higher than that given for several zinc-containing enzymes by Auld *et al.* (1975). That the metal ion is not Mg^{2+} is indicated by the instantaneous inhibition by 0.7 mM-1,10-phenanthroline of 26%, whereas the inhibition by 0.7 mM-1,10-phenanthroline in the presence of 14 mM- $MgCl_2$ was actually higher (68%). Zn^{2+} ions were not tested in this regard, as a precipitate formed when $ZnCl_2$ was added to the reaction mixture.

Substrate specificity

When $[2-^{14}C]$ thymidine was used as the nucleoside substrate for the reaction with AMP, only one labelled phosphorylated product, $[^{14}C]$ dTMP, was detected by paper electrophoresis in 0.1 M-sodium citrate, pH 3.5, and by paper chromatography (ascending mode, in isobutyric acid/conc. NH_3 /water, 66:1:33, by vol., as solvent at 28°C). In addition, this product was further phosphorylated by a partially purified dTMP kinase preparation from *Neurospora crassa*, which requires a pyrimidine nucleoside 5'-monophosphate as substrate, with a marked preference for dTMP.

The partially purified thymidine phosphotransferase activity also catalysed the phosphorylation of a wide range of purine and pyrimidine nucleosides in the presence of AMP, but not of the sugars ribose or glucose (Table 4). With thymidine as the nucleoside substrate, a wide range of purine and pyrimidine nucleoside 5'-monophosphates were found to substitute for AMP as phosphate donor for dTMP production (Table 5). However, the nucleotides dTTP, ATP and 3':5'-cyclic AMP would not serve as phosphoryl donors (Table 5). These data are consistent with the model of a single enzyme species

Table 5. Nucleotide substrate specificity

Nucleotides were tested both as phosphoryl donors substituted for AMP and for interaction in the presence of AMP. Activities are expressed relative to that with AMP alone as substrate, $[2-^{14}C]$ thymidine being the nucleoside acceptor in all cases. A Sephadex G-200 enzyme fraction with ATP phosphohydrolase activity removed was used for these studies.

Nucleotide	Relative activity	
	Added alone	Added with AMP
None	0.00	1.00
ATP	0.10	0.99
GMP	1.24	1.17
dAMP	1.00	0.94
CMP	0.47	1.00
UMP	0.72	0.96
dUMP	0.99	1.10
dTMP	0.88	0.97
dTTP	0.06	0.95
3':5'-Cyclic AMP	0.01	0.25

possessing a wide substrate-specificity range for both nucleoside and nucleoside monophosphate substrates. As also shown in Table 5, 3':5'-cyclic AMP is an effective inhibitor of thymidine phosphotransferase activity, a property that is further considered below.

Nucleotide phosphohydrolase activity

The nucleotide phosphohydrolase activity closely associated with thymidine-AMP phosphotransferase utilizes AMP as substrate and is also inhibited by cAMP (see below). The nucleosides that will serve as substrates for the thymidine-AMP phosphotransferase were found to inhibit nucleotide phosphohydrolase activity. Thus at 1.4 mM-AMP, the nucleosides adenosine, thymidine, cytidine and uridine (also at 1.4 mM) inhibited by 75%, 40%, 35% and 30% respectively. Addition of 5 mM- $MgCl_2$ or - $CaCl_2$ did not enhance phosphohydrolase activity.

Kinetic studies

A series of Lineweaver-Burk plots showing the reciprocal of reaction velocity versus the reciprocal of thymidine concentration for each of several AMP concentrations is presented in Fig. 3a. From these data, the K_m of the phosphotransferase for thymidine was calculated to be 0.84 mM. Similarly, in Fig. 3b, the data are replotted to show the reciprocal of initial velocity versus the reciprocal of AMP concentration at each of several thymidine concentrations. The data yielded a K_m for AMP of 0.11 mM.

Data describing the interaction of three different nucleoside substrates are presented in Fig. 4.

Fig. 3. Initial-velocity studies with thymidine-AMP phosphotransferase

Reactions were undertaken as described in the Experimental section, and the data presented as double-reciprocal plots. (a) The initial concentration of thymidine was varied at each of the following AMP initial concentrations: ●, 0.05 mM; ○, 0.11 mM; ▲, 0.43 mM; △, 1.73 mM. (b) The data are replotted to show the relationships when the AMP concentration was varied at each of the following thymidine initial concentrations: ●, 0.31 mM; ○, 0.78 mM; ▲, 2.09 mM; △, 7.45 mM.

The double-reciprocal plots obtained by using thymidine, uridine or adenosine as the sole ¹⁴C-labelled nucleoside substrate are given in Fig. 4(a). From these, the K_m values were calculated to be 0.88 mM, 4.71 mM and 1.28 mM respectively. Fig. 4(b) shows a Lineweaver-Burk plot for [¹⁴C]thymidine as nucleoside substrate at various concentrations of unlabelled uridine. It reveals that uridine is a competitive inhibitor of thymidine phosphorylation ($K_i = 2.75$ mM). This value is close to the K_m for uridine. Similarly, adenosine was shown to be a competitive inhibitor of thymidine phosphorylation (Fig. 4c), the calculated K_i (1.07 mM) being very similar to the K_m (1.28 mM) for adenosine.

Fig. 4. Initial-velocity studies with various nucleosides as substrate and inhibitor of thymidine-AMP phosphotransferase

The reactions were carried out as described in the Experimental section, and the data are presented as double-reciprocal plots. (a) The enzyme catalysed the phosphorylation of isotopically labelled uridine (▲), thymidine (□), or adenosine (●) added as sole substrate in turn. (b) The concentration of [¹⁴C]thymidine as substrate was varied in the absence and in the presence of uridine of 0.79 mM (△) and 1.85 mM (▲). (c) The concentration of labelled thymidine as substrate was varied in the absence and in the presence of adenosine at 0.51 mM (○) and 2.06 mM (●).

The kinetics of inhibition of thymidine-AMP phosphotransferase activity by cyclic AMP are shown in Fig. 5. The cyclic AMP strongly inhibited the reaction in a mode that was non-competitive with respect to thymidine ($K_i = 0.087$ mM; Fig. 5a). The

Fig. 5. Inhibition of thymidine-AMP phosphotransferase by 3':5'-cyclic AMP

The reactions were carried out as described in the Experimental section, and the data are presented as double-reciprocal plots. (a) The initial concentration of thymidine was varied with a constant AMP concentration of 0.11mM at each of the following cyclic AMP concentrations: ●, no cyclic AMP; ○, 0.05mM; △, 0.204mM. (b) With a constant initial concentration of thymidine, the AMP concentration was varied at each of the cyclic AMP concentrations listed in (a). Initial velocity (*v*) was recorded as nmol/10min per assay.

inhibition by the cyclic nucleotide was non-competitive also with respect to AMP ($K_i = 0.056$ mM; Fig. 5b). However, a 'mixed' inhibition mechanism could also be operating, with a significant competitive component.

The nucleotide phosphohydrolase activity of the DE-32 fraction was also inhibited by cyclic AMP (Fig. 6) in a mode that was strictly non-competitive, although, as before, it is possible that there is a significant competitive component. The K_i for this inhibition (0.14mM-cyclic AMP) is of the same order as that observed for the inhibition of thymidine phosphotransferase activity by cyclic AMP.

Fig. 6. Inhibition of AMP phosphohydrolase activity by 3':5'-cyclic AMP

The reactions were undertaken with the radiochemical assay method described in the Experimental section, and the data are presented as double-reciprocal plots. The initial concentration of AMP was varied in the presence of the following cyclic AMP concentrations: ●, 0; ○, 1.05mM; △, 2.63mM. Initial velocity (*v*) was recorded as nmol/10min per assay.

Table 6. Kinetic parameters for phosphotransferase and phosphohydrolase activities

The G-200 fraction, in conjunction with radiochemical assays, was used for all determinations, except for the K_i and first-listed K_m estimations for the nucleotide phosphohydrolase, for which the DE-32 fraction was used. The second K_m listed for nucleotide phosphohydrolase was obtained with the G-200 fraction, by using the colorimetric assay method. All K_m and K_i values are given in mM units.

Substrate or inhibitor ...	Nucleotide phosphohydrolase activity		Thymidine phosphotransferase activity				
	AMP	cAMP	Thymidine	Uridine	Adenosine	AMP	cAMP
K_m	0.29		0.84	4.71	1.28	0.11	
	0.20		0.88				
K_i (thymidine varied)				2.75	1.07		0.087
K_i (AMP varied)		0.14					0.056

A summary of the various kinetic parameters obtained for the phosphotransferase and phosphohydrolase activities is given in Table 6.

Discussion

After partial purification of thymidine-AMP phosphotransferase from the fern *Asplenium nidus*, a very rich source of this activity, the enzyme was found to have closely associated with it uridine phosphotransferase, AMP phosphohydrolase and Ca^{2+} -activated ATP phosphohydrolase activities. With regard to the nucleoside phosphotransferase activities, the ratio of rates with thymidine to that with uridine in crude extracts from various plants and from different parts of both *Helianthus tuberosus* artichoke and *A. nidus* was observed to be very constant. In addition, the constancy of the same ratio during the purification steps and in the various fractions collected during urea/Sephadex G-200 gel filtration and DE-32 chromatography all suggest that the nucleoside phosphotransferase activity is a single enzyme with a wide specificity range. This concept is strongly reinforced by the observation that phosphoryl transfer to one nucleoside is inhibited by other nucleosides, which themselves serve as substrates when tested separately. In addition, kinetic studies, the first reported for nucleoside phosphotransferase, show that the K_i values for uridine and adenosine as inhibitors of thymidine-AMP phosphotransferase activity are very similar to the K_m values obtained for these nucleosides as substrate.

The associated ATP phosphohydrolase activity was separated from nucleoside phosphotransferase activity by urea/Sephadex G-200 gel filtration in the presence of Blue Dextran. However, an AMP phosphohydrolase activity was not removed and, in fact, chromatography on DE-32 and urea/Sephadex G-200 with Blue Dextran gave identical elution patterns for AMP phosphohydrolase and thymidine phosphotransferase activities. Like the phosphotransferase, the AMP phosphohydrolase activity did not require a metal ion, and was markedly inhibited by 3':5'-cyclic AMP. The K_i for this inhibition was of the same order for the two activities. Whether or not this phosphohydrolase activity is carried out by the thymidine phosphotransferase enzyme (it may even be a partial reaction of this enzyme) must await the completion of further purification and kinetic studies. It is likely that the nucleotide phosphohydrolase activity described in the present work is not closely related to the non-specific phosphatase(s) of plant tissues described by Bielecki (1963, 1964), since it is much less stable to heat (inactivated by 1.5 min in a boiling-water bath). In addition, it may be relevant that, during the purification of a similar nucleoside phosphotransferase from carrot tissue,

Brunngraber & Chargaff (1967) observed that most of the phosphatase activity of crude extracts was removed, leaving a residual nucleotide phosphohydrolase activity that hydrolysed efficient donors for the transferase reaction.

The inhibition of a nucleotide phosphohydrolase (from wheat) by 3':5'-cyclic AMP has been reported by Polya (1974) and Polya & Ashton (1973). As in the present studies, the K_i for cyclic AMP was of the same order of magnitude as the K_m for AMP substrate. Uncertainties exist as to the role, if any, that cyclic AMP has as a 'second messenger' in plant cells (Barendse & Holtslag, 1975; Ownby & Ross, 1975), and even as to the very existence of cyclic AMP in plant tissues (Niles & Mount, 1974; Amrhein, 1974). In fact, it is possible that the experimentally observed inhibition has no physiological significance within the plant cell, but may simply reflect the close structural similarity between the phosphomonoester substrate (5'-AMP) and the phosphodiester inhibitor (3':5'-cyclic AMP).

It is highly probable that some at least of the published data on thymidine kinase activity in plant extracts are predominantly due to thymidine-AMP phosphotransferase activity coupled to ATP phosphohydrolase (Deng & Ives, 1972; Melichar & Cihák, 1974). An inhibitor of thymidine kinase, dTTP, was used by Brunori *et al.* (1974) to aid in discriminating between the phosphotransferase and kinase activities; however, this inhibitor suffers from the disadvantage that the dTTP can be degraded to thymidine, diluting the labelled thymidine substrate, and so producing an apparent inhibition. The present work suggests that an alternative means of discriminating between the kinase and phosphotransferase activities is to use cyclic AMP as an inhibitor of the latter. It has the advantage of a low K_i , and its addition cannot interfere with the specific radioactivity of the labelled thymidine substrate. Preliminary work in this laboratory carried out in part with this inhibitor has indicated, for example, that most of the thymidine kinase activity reported by Harland *et al.* (1973) in cultured *Helianthus tuberosus* explants is likely to be due to the thymidine-AMP phosphotransferase.

A complete understanding of the relationship of thymidine-AMP phosphotransferase to thymidine kinase, and its function, must await further work. However, the present investigation has provided us with a tool (3':5'-cyclic AMP) that should prove useful in elucidating the first point. On the second question, it may be significant that nucleoside-AMP phosphotransferase activity increases during maturation of the plant or a portion of the plant (when the rate of DNA synthesis should be decreasing). This fact, together with the apparent broad specificity range towards the nucleoside substrate and the high activity compared with the next likely enzyme in the

metabolic pathway, dTMP kinase (Table 1), suggests that the phosphotransferase does not function solely as a salvage mechanism specifically for DNA synthesis.

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