an FMI Model RP-D-SSY pump. The eluent was monitored at 218 nm with a Schoeffel 770 detector which had been modified to bypass the heat exchanger.

The major peak, or in the case of the diastereomeric products the second major peak,[36,37] was cut and pooled for maximum purity. The solvent was stripped, and the bulk of the NH_4OAc in the residue was sublimed under high vacuum (~40 °C). The residue was lyophilized 3 times from H_2O to yield the pure analogue. Peptides were analyzed for purity (>95%) primarily by analytical HPLC as described above. TLC was performed on silica gel plates in the solvent systems 1-BuOH/HOAc/H_2O (4:1:5, upper phase, BAW) and 1-BuOH/EtOAc/HOAc/H_2O (1:1:1:1, BEAW). Samples of ~50 μg were applied, developed, and visualized as described.

Estrus Suppression Assay. Adult female Sprague-Dawley rats, each weighing approximately 160 g, were distributed equally be weight into groups of 10 and housed 5 per cage. They were maintained in 14:10 light/dark cycle in air-conditioned quarters and given unlimited access to food and water. The rats were injected subcutaneously, twice daily (0800 and 1630 h), with 0.1 or 0.2 mL/injection of 0.1% BSA-saline vehicle containing the test compound in solution. At least four doses at 2-fold dilutions were tested per compound. All assays included negative (vehicle) and positive (**25**) controls. Injections were administered for 14 consecutive days, during which time daily vaginal lavages were taken from each rat to determine the stage of the estrous cycle. The percent of rats showing complete estrus suppression (i.e., only diestrous cytology) from the 4th day on was plotted against the log dose, and the ED_{50} for complete estrus suppression was calculated in units of micrograms per injection.

Acknowledgment. We gratefully acknowledge the technical assistance of Ms. C. Tsai, Ms. P. Kirchgatter, Ms. K. Bergstrom, Ms. A. Worden, and Mr. R. Seidenberg in conducting the bioassays. We are indebted to Ms. J. Fazzari and Ms. S. Williams for HPLC and amino acid analyses. Dr. S. Unger provided helpful discussion of QSAR considerations. The encouragement and advice of Dr. J. G. Moffatt is gratefully acknowledged.

Species- or Isozyme-Specific Enzyme Inhibitors. 6.[1] Synthesis and Evaluation of Two-Substrate Condensation Products as Inhibitors of Hexokinases and Thymidine Kinases

Alexander Hampton,* Ton T. Hai, Francis Kappler, and Ram R. Chawla

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received January 4, 1982*

Syntheses are described of P^1-(adenosine-5')-P^3-(glucose-6) triphosphate (Ap_3 glucose), Ap_4 glucose, and P^1-(adenosine-5')-P^3-(thymidine-5') triphosphate (Ap_3T). The compounds were not substrates of any of the enzymes used in the present studies. Ap_3 glucose and Ap_4 glucose were inhibitors of yeast hexokinase (HK) and the rat isozymes HK I–III; in general, they had less affinity for the enzymes than the substrates ATP and glucose. Inhibition constants (K_i values) of Ap_3T with rat mitochondrial thymidine kinase (M-TK) and rat cytoplasmic TK (C-TK) were determined for variable thymidine (TdR) with a constant saturating level of ATP and for variable ATP with constant saturating TdR. Ap_3T was a potent and selective inhibitor of M-TK [K_M (TdR)/K_i = 1.6, K_M (ATP)/K_i = 38 with variable ATP; K_M (TdR)/K_i = 0.06, K_M (ATP)/K_i = 1.4 with variable TdR] relative to C-TK [K_M (TdR)/K_i = 0.006, K_M (ATP)/K_i = 0.7 with variable ATP; K_M (TdR)/K_i = 0.001, K_M (ATP)/K_i = 0.12 with variable TdR]. Inhibition of M-TK and C-TK by Ap_3T differed qualitatively and quantitatively from inhibition under the same conditions by the metabolic feedback inhibitor TdR 5'-triphosphate.

Evidence, summarized previously,[2] indicates that fetal isozyme-selective inhibitors of key enzymes in cell metabolism are of interest as potential starting points in the design of antineoplastic drugs. In systems so far studied, it was found that isozyme-selective inhibitors could be generated fairly frequently by attaching a single small substituent at various atoms in turn of a substrate of the target enzyme.[1,3,4] This procedure usually led to inhibitors of weak or moderate potency. In the case of one target enzyme (adenylate kinase), it has proved possible to produce a potent isozyme-selective inhibitor by incorporating the substituent into a two-substrate condensation product which itself is a potent but nonselective inhibitor of isozymes of that enzyme.[5] This report describes the synthesis of several two-substrate condensation products and their evaluation as inhibitors of thymidine kinase (TK) or hexokinase (HK), certain forms of which are of interest as targets in cancer chemotherapy. The potential value of specific inhibitors of either the cytoplasmic form (C-TK) or the mitochondrial form (M-TK) of TK has been discussed previously.[1] That HK may be an appropriate target in cancer chemotherapy is suggested by evidence that the level of this enzyme in experimental hepatomas tends to increase in parallel with increasing growth rate and degree of malignancy.[6] Of the four main isozymic variants of HK, HK II and III are minor components of most rat normal tissues[7,8] and major components of most rat tumor tissues[8,9] so far examined. Potent and selective inhibitors of HK II and III, hence, might exhibit antineoplastic activity or potentiate the activity of other agents.

The synthesis is described of P^1-(adenosine-5')-P^3-(glucose-6) triphosphate (Ap_3 glucose), which is a condensation product of the HK substrates ATP and glucose. The tetraphosphate homologue, Ap_4 glucose, has also been synthesized, as well as P^1-(adenosine-5')-P^3-(thymidine-5')

(1) For paper 5 in this series, see Hampton, A.; Chawla, R. R.; Kappler, F. *J. Med. Chem.* 1982, *25*, 644.
(2) Hampton, A.; Kappler, F.; Maeda, M.; Patel, A. D. *J. Med. Chem.* 1978, *21*, 1137.
(3) Hampton, A.; Kappler, F.; Chawla, R. R. *J. Med. Chem.* 1979, *22*, 1524.
(4) Hampton, A.; Picker, D. *J. Med. Chem.* 1979, *22*, 1529.
(5) Hampton, A.; Kappler, F.; Picker, D. *J. Med. Chem.* 1982, *25*, 638.
(6) Weber, G. *Cancer Res.* 1976, *36*, 3189.
(7) Katzen, H. M. *Adv. Enzyme Regul.* 1967, *5*, 335.
(8) Farron, M. *Enzyme* 1972, *13*, 233.
(9) Shatton, J. B.; Morris, H. P.; Weinhouse, S. *Cancer Res.* 1969, *29*, 1161.

0022-2623/82/1825-0801$01.25/0 © 1982 American Chemical Society

Table I. Physical Properties of Nucleotides and Synthetic Precursors

compd	yield, %	UV λ_{max} (pH 6), nm ($\epsilon \times 10^{-3}$)	electrophoresis pH 3.5	electrophoresis pH 7.5	R_f system A[a]	R_f system B[a]	R_f system D[a]	HPLC t_R,[b] min	phosphate to base ratio	formula	anal.
Ap_3 glucose	34	260 (15.0)	0.83	0.97	0.17	0.19	0.50	21	2.95	$C_{16}H_{23}N_5O_{18}P_3Na_3 \cdot 0.5H_2O \cdot 1.5CH_3OH$	C, H, N, P
Ap_4 glucose	17	260 (15.1)	0.80	0.87	0.10	0.16	0.19	39	3.97	$C_{16}H_{23}N_5O_{21}P_4Na_4 \cdot 3H_2O \cdot 0.5CH_3OH$	C, H, N, P
Ap_3T	36	262 (21.0)			0.23	0.33		24	1.47	$C_{20}H_{25}N_7O_{17}P_3Li_3 \cdot 7H_2O \cdot 0.5C_2H_5OH$	C, H, N, P
TMP					0.27	0.41		4			
ADP			0.91	0.95	0.12	0.37	0.25	10			
ATP			1.00[c]	1.00[c]	0.10	0.24	0.10	26			
glucose 6-phosphate					0.16	0.15	0.62				

[a] Composition given under Experimental Section. [b] Conditions are given under Experimental Section. [c] An assigned value.

Table II. Inhibition of Hexokinase-Catalyzed Reactions[a]

compd	concn, mM	inhibition, % HK I	HK II	HK III
Ap_3 glucose	0.3	9.3	4.4	3.1
	0.6	5.6	10.1	6.2
Ap_4 glucose	0.3	4.7	28.0	6.5
	0.6	8.0	30.0	8.7

[a] The ATP level was 1.12 mM; the remaining conditions are given under Experimental Section.

triphosphate (Ap_3T), a condensation product of thymidine (TdR) and ATP, the substrates of TK.

Chemistry. Ap_3 glucose and Ap_4 glucose were synthesized by application of the imidazolidate method utilized by Hoard and Ott for conversion of 2′-deoxynucleoside 5′-phosphates to the corresponding 5′-di- or -triphosphates.[10] Adenosine 5′-diphosphate (ADP) was converted by 1,1′-carbonyldiimidazole to the 2′,3′-*O*-carbonyl derivative of its β-phosphoroimidazolidate,[11] and this was treated in situ with 5 equiv of α-D-glucose 6-phosphate to give 2′,3′-*O*-carbonyl-Ap_3 glucose. The 2′,3′-*O*-carbonyl group was removed by a mild basic treatment,[11] and Ap_3 glucose was purified by anion-exchange chromatography on DEAE-cellulose and isolated in 34% yield as its trisodium salt. The compound appeared pure by elemental analysis and UV extinction coefficient and was homogeneous as judged by paper electrophoresis and chromatography, by HPLC, and by phosphate/base ratio (Table I) determined after sequential attack by snake venom phosphodiesterase and alkaline phosphatase. Tetrasodium Ap_4 glucose was obtained from ATP in 17% yield using the same method. HPLC analysis indicated the presence of a trace (0.03%) of ATP; Ap_4 glucose was homogeneous by the remaining criteria employed (Table I). Ap_3 glucose and Ap_4 glucose were earlier reported to have been prepared by the same route.[12] The products were not isolated or subjected to basic treatment to remove the 2′,3′-*O*-carbonyl groups which have been shown to be introduced during phosphoroimidazolidate formation with 1,1′-carbonyldiimidazole and incompletely removed during subsequent anion-exchange purification of the desired ribonucleoside 5′-polyphosphate.[11] We prepared homogeneous trilithium Ap_3T in 36% yield from condensation of preisolated ADP phosphoroimidazolidate with 2 equiv of thymidine 5′-phosphate (TMP). The structure was confirmed by elemental analysis and the finding that enzymatic digestion with a mixture of snake venom phosphodiesterase and alkaline phosphatase yielded 1 mol each of adenosine and thymidine and 3 mol of inorganic phosphate. Ap_3T could be prepared in similar yield by condensation of TMP phosphoroimidazolidate with 2 equiv of ADP. Ap_3T has been synthesized enzymatically by addition of TDP to a mixture of lysyl synthetase, ATP, and lysine.[13]

Studies with Hexokinases. Ap_3 glucose and Ap_4 glucose did not act as substrates of yeast hexokinase (HK) or the rat HK I, II, or III isozymes. In the presence of a saturating level of glucose and a level of ATP that was 1–2

(10) Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785.
(11) Maeda, M.; Patel, A. D.; Hampton, A. *Nucleic Acid Res.* **1977**, *4*, 2843.
(12) Danenberg, P. V.; Danenberg, K. D. *Biochim. Biophys. Acta* **1977**, *480*, 351.
(13) Zamecnik, P. C.; Stephenson, M. L. In "Regulatory Mechanisms for Protein Synthesis in Mammalian Cells"; San Pietro, A.; Lamborg, M. R.; Kenney, F. T., Eds.; Academic Press: New York, 1968; p 3.

Table III. Inhibition Constants of Ap_3 Glucose and Ap_4 Glucose with Yeast and Rat Hexokinases

source of hexokinase	K_i,[a] mM		K_M of ATP, mM
	Ap_3 glucose	Ap_4 glucose	
yeast	1.4	3.5	0.21
rat HK II isozyme	2.2		0.71

[a] Inhibitions were competitive with respect to ATP.

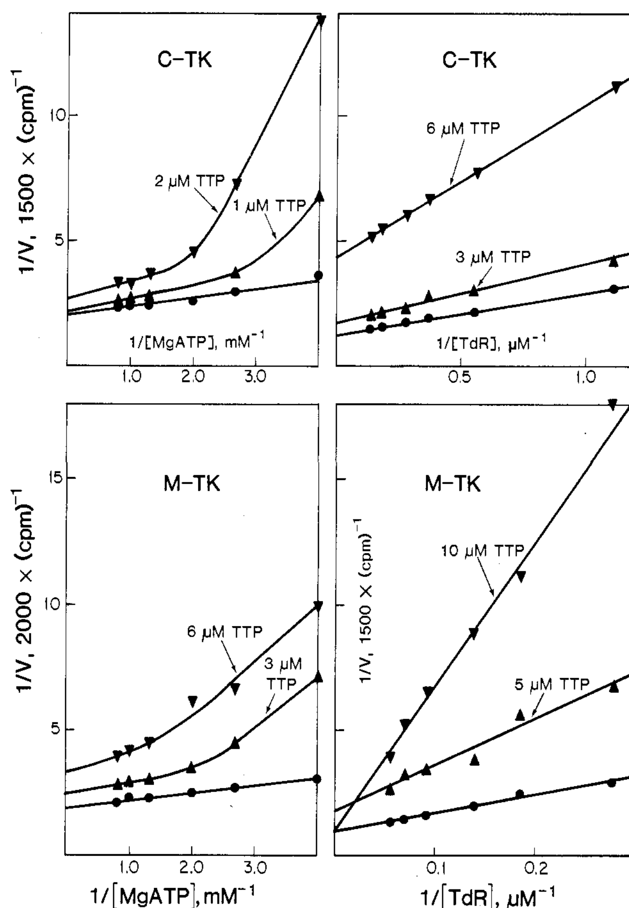

Figure 1. Inhibition of rat C-TK (*upper* two plots) and M-TK (*lower* two plots) by MgTTP. Velocities marked as circles were obtained in the absence of inhibitor. The conditions used were the same as detailed under Experimental Section for similar studies with $MgAp_3T$.

times its K_M value, both compounds were inhibitory to the rat isozymes (Table II) and appeared to possess weaker affinities than either ATP[14] (K_M = 0.44, 0.71, and 0.91 mM for HK I, II, and III, respectively, under the present conditions) or glucose[15] (K_M = 0.05, 0.2, and 0.007 mM, respectively). The inhibition of HK II by Ap_4 glucose (Table II) showed an apparent saturation effect with increasing inhibitor level. Kinetic analysis showed that Ap_3 glucose inhibited HK II competitively with respect to ATP and that the inhibition constant was 3-fold higher than the K_M of ATP (Table III). Ap_3 glucose and Ap_4 glucose had 7-17 times lower affinity than ATP for the ATP site of yeast HK as judged by K_i/K_M (ATP) ratios (Table III). Previous studies yielded K_i/K_M (ATP) values of ~2 for these inhibitors and yeast HK.[12] It was proposed[12] that Ap_3 glucose and Ap_4 glucose bind to the known[16] yeast HK–glucose complex via their ATP moieties and that their glucose moieties do not bind. Glucose binding appears to precede ATP binding during the catalytic cycles of both yeast and mammalian HK,[15] and it is possible that Ap_3 glucose and Ap_4 glucose may bind predominantly to the ATP site of HK I–III to give rise to the relatively weak inhibitions observed.

Studies with Thymidine Kinases. Ap_3T had no detectable substrate activity with rat C-TK or M-TK. Kinetic analyses of its inhibitory properties were carried out in the presence of saturating levels of the nonvaried substrate. The inhibition constants so obtained are given in Table IV. In the case of C-TK, Ap_3T exhibited moderate affinity for the ATP site [K_M (ATP)/K_i = 0.7 and 0.12 with variable ATP and TdR, respectively] and poor affinity for the TdR site [K_M (TdR)/K_i = 0.006 and 0.001, respectively]. In contrast, Ap_3T exhibited much stronger affinity for both the ATP and TdR sites of M-TK [K_M (ATP)/K_i = 38 and 1.4; K_M (TdR)/K_i = 1.6 and 0.06 with variable ATP and TdR, respectively]. The fully or partly competitive character of the observed inhibitions indicates that Ap_3T may be capable of binding simultaneously at both substrate sites of C-TK and M-TK. The evidence for this is stronger in the case of M-TK, where the affinity, particularly for the ATP site, appears to be substantially greater than that of the substrate. Several derivatives of TdR have been reported to possess differential affinity for human[17] or rat[1] M-TK in comparison with the corresponding C-TK. Ap_3T appears to represent a new type of selective inhibitor of M-TK with potent affinity for the ATP site as well as for the TdR site.

Mammalian thymidine kinases are subject to feedback inhibition by TTP through what is believed to be an allosteric mechanism.[18] The inhibitory properties of TTP with rat C-TK and M-TK were studied under the conditions used with Ap_3T in order to evaluate the possibility of interaction of Ap_3T at the TTP site. The inhibitions by TTP (Figure 1 and Table IV) in all cases differed from those by Ap_3T either qualitatively or quantitatively. Thus, with C-TK and varied ATP, TTP produced nonlinear double-reciprocal plots, whereas Ap_3T gave linear plots. With C-TK and varied TdR, TTP behaved as a potent noncompetitive inhibitor with a K_i value that varied with the TTP level, whereas Ap_3T was a weak competitive–noncompetitive inhibitor with a K_i value independent of Ap_3T level. With M-TK and varied ATP, TTP, in contrast to Ap_3T, gave nonlinear plots which upon extrapolation appeared to indicate noncompetitive inhibition. Finally, with M-TK and varied TdR, TTP was predominantly noncompetitive at 5 μM but competitive at 10 μM with a variable K_i value that was as much as 50-fold less than that of Ap_3T.

Experimental Section

Chemical Synthesis. General. Adenosine 5′-triphosphate, D-glucose 6-phosphate, and thymidine 5′-monophosphate were purchased from Sigma Chemical Co. The monopotassium salt of adenosine 5′-diphosphate was from Boehringer Mannheim. *N*,*N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. Descending paper chromatography was carried out on Whatman No. 1 paper using the following

(14) Hampton, A.; Patel, A. D.; Chawla, R. R.; Kappler, F.; Hai, T. T. *J. Med. Chem.* **1982**, *25*, 386.
(15) Colowick, S. P. *Enzymes, 3rd Ed.*, **1973**, *9B*, 1.
(16) McDonald, R. C.; Steitz, T. A.; Engelman, D. M. *Biochemistry* **1979**, *18*, 338.
(17) Cheng, Y.-C.; Dutschman, G.; DeClercq, E.; Jones, A. S.; Rahim, S. G.; Verhelst, G.; Walker, R. T. *Mol. Pharmacol.* **1981**, *20*, 230.
(18) Roth, J. S. In "Protein Metabolism and Biological Function"; Bianchi, C. P.; Hilf, R., Eds.; Rutgers University Press: New Brunswick, NJ, 1970; p 141.

Table IV. Inhibition Constants of Ap_3T and TTP with Rat Cytoplasmic Thymidine Kinase (C-TK) and Rat Mitochondrial TK (M-TK)[a]

	C-TK			M-TK		
	K_i, μM (type of inhibn)[b]			K_i, μM (type of inhibn)		
compd	ATP varied	TdR varied	K_M, μM	ATP varied	TdR varied	K_M, μM
TdR			1.6			8.0
ATP			180			190
Ap_3T	260 (M)	1500 (M)		5.0 (C)	140 (M)	
TTP	c	6.5 (NC)[d]		c	3.7 (M)[e]	

[a] The values given were determined in the presence of a saturating level (6–10 × K_M) of the fixed substrate (TdR or MgATP). Other conditions are given under Experimental Section. [b] C = competitive; NC = noncompetitive; M = mixed competitive–noncompetitive. [c] Nonlinear double-reciprocal kinetic plots (Figure 1). [d] Calculated from the inhibition by 3 μM TTP (Figure 1). [e] Calculated from the inhibition by 5 μM TTP (Figure 1).

solvent systems: (A) 2-propanol–0.25 M NH_4HCO_3 (65:35), (B) isobutyric acid–NH_4OH–H_2O (66:1:33), and (C) 2-propanol–NH_4OH–H_2O (7:1:2). TLC on PEI-cellulose employed (D) 1 M LiCl. Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 (0.05 M citrate) and 7.5 (0.05 M $Et_3NH{\cdot}HCO_3$). UV spectra were obtained on a Cary Model 15 spectrophotometer. Elemental analyses were by Galbraith Laboratories Inc., Knoxville, TN. Where analyses are indicated by the symbols of the elements, analytical results were within ±0.4% of the theoretical value. High-pressure liquid chromatography was performed on a Waters Model 204 chromatograph equipped with a dual solvent-delivery system (Model M-6000 A) and a Waters Model 660 programmer. Compounds were analyzed on a μ-Bondapak NH_2 column (30 cm × 4 mm) utilizing a 1 mL/min flow rate with a linear gradient of ammonium dihydrogen phosphate (0.05 M, pH 3, to 0.25 M, pH 3) over 20 min. The eluate was monitored at 254 nm.

For analysis of phosphate content, a solution of the nucleotide (1.5–2 mM) in Tris buffer, pH 10.4 (1 mL), was treated with phosphodiesterase (1 mg, from *Crotalus Atrox*, type IV, Sigma Chemical Co.) and alkaline phosphatase (0.02 mg, calf intestine, type VII, Sigma Chemical Co.) at 22 °C overnight. The solution was analyzed for phosphate by the method of Lowry and Lopez.[19]

***P*¹-(Adenosine-5′)-*P*³-(glucose-6) Triphosphate (Ap_3 Glucose).** Glucose 6-phosphate or ADP (0.5 mmol) was converted to its tri-*n*-butylammonium salt by percolating its solution in water (50 mL) through a column of AG 50W-X8 resin (40 mL, pyridinium form). The combined eluates and washings were concentrated in vacuo to ~5 mL, and tri-*n*-butylamine (0.24 mL, 1 mmol) was added, followed by ethanol, to give a clear solution. Volatiles were evaporated in vacuo, and the residue was dried by repeated evaporations of ethanol and anhydrous DMF. The salt of ADP (0.5 mmol) was dissolved in anhydrous DMF (10 mL), and 1,1′-carbonyldiimidazole (405 mg, 2.5 mmol) was added. The mixture was stirred at 22 °C for 3 h and treated with MeOH (0.120 mL, 3 mmol). After 30 min, a solution of glucose 6-(tri-*n*-butylammonium phosphate) (2.5 mmol) in dry DMF (20 mL) was added. The reaction was monitored by TLC (solvent D) and electrophoresis at pH 3.6. The product and glucose 6-phosphate gave a positive test with a periodate–permanganate spray reagent for carbohydrate.[20] After 36 h, the mixture was treated with an equal volume of methanol and evaporated to dryness. The residue was dissolved in MeOH–water (1:1, 50 mL), and the solution was brought to pH 11 with triethylamine to remove cyclic 2′,3′-carbonyl residues.[11] After 2 h at 22 °C, the solvent was evaporated, and the residue was chromatographed on a column (3 × 30 cm) of DEAE-cellulose. Elution was carried out with a linear gradient of 0–0.25 M $Et_3NH{\cdot}HCO_3$ (4 L), and 20-mL fractions were collected. Fractions 50–80 and 86–135 contained peaks (ratio 2:3) of UV-absorbing material. Peak 1 reacted negatively to the carbohydrate spray. Peak 2 was freed of traces of peak 1 by rechromatography on a column of DEAE-cellulose (3 × 30 cm) eluted with a linear gradient of 0–0.2 M $Et_3NH{\cdot}HCO_3$ (4 L). Evaporation in vacuo of appropriate fractions gave ammonium Ap_3 glucose. Treatment of this with 1 M NaI in MeOH[10] gave sodium Ap_3 glucose (0.17 mmol, 34%) as a white powder that was homogeneous in the systems of Table I and contained no glucose 6-phosphate as indicated by the carbohydrate spray test. For physical properties and analyses, see Table I.

***P*¹-(Adenosine-5′)-*P*⁴-(glucose-6) Tetraphosphate (Ap_4 Glucose).** To a suspension of tri-*n*-pentylammonium ATP (1 mmol) (prepared as described for tri-*n*-butylammonium ADP) in anhydrous DMF (20 mL) was added 1,1′-carbonyldiimidazole (810 mg, 50 mmol). The mixture was stirred under anhydrous conditions. Chromatography in solvents A and D showed that conversion of ATP to its imidazolidate was complete after 24 h. The mixture was treated with methanol (0.240 mL, 6 mmol) for 30 min. Glucose 6-(tri-*n*-pentylammonium phosphate) (5 mmol) in DMF (20 mL) was added. The mixture was stirred for 36 h. The precipitate was removed and washed with DMF. Cyclic 2′,3′-carbonyl groups were removed from the DMF-soluble material as described for the synthesis of Ap_3 glucose, and the product was chromatographed on a DEAE-cellulose column (3 × 30 cm) with a linear gradient of 0–0.35 M $Et_3NH{\cdot}HCO_3$ (4 L). This gave three, partially overlapping, major UV-absorbing peaks of ca. 5:4:1 area ratio. Peak 3 was principally ATP. Peak 2 was rechromatographed on DEAE-cellulose to remove ATP. The sodium salt of Ap_4 glucose (0.17 mmol, 17%) was obtained as a white powder as described for the sodium salt of Ap_3 glucose. The product was homogeneous in the systems of Table I, except that HPLC revealed the presence of 0.03% of ATP. The carbohydrate spray test applied to paper chromatograms and electrophoretograms indicated the absence of glucose 6-phosphate. For properties and analyses of the product, see Table I.

***P*¹-(Adenosine-5′)-*P*³-(thymidine-5′) Triphosphate (Ap_3T).** To an anhydrous solution of tributylammonium ADP (2 mmol) in DMF (5 mL) was added 1,1′-carbonyldiimidazole (1.6 g, 10 mmol) in DMF (5 mL). After 3 h, cellulose TLC in system A showed that conversion of ADP to its imidazolidate was virtually complete. Methanol (0.66 mL, 10 mmol) was added, and after 30 min the mixture was evaporated. A solution of the residue in 5 N NH_4OH (100 mL) was stored at 22 °C for 2 h and evaporated, and the residue was chromatographed on a column of DEAE-cellulose (HCO_3^-) (3 × 30 cm). The column was washed free of UV-absorbing material with water and then eluted with a linear gradient of 0–0.33 M $Et_3NH{\cdot}HCO_3$. The imidazolidate (85% yield, determined spectrophotometrically) appeared as a single peak at 0.1–0.17 M salt. It was homogeneous on chromatography in systems A (R_f 0.54), C (R_f 0.38), and D (R_f 0.47). To 1.5 mmol of this material in DMF (5 mL) was added $(n\text{-}Bu)_3NH^+$-TMP (3 mmol) in DMF (5 mL). After 48 h at 22 °C, TLC (system D) showed a major spot (R_f 0.34) along with TMP (R_f 0.61), ADP imidazolidate (R_f 0.47), and ADP (R_f 0.18). After volatiles were evaporated in vacuo, the residue was applied to a column of Dowex-1 (Cl^-) (3 × 15 cm), which was washed in succession with 1 L each of 0.025, 0.05, 0.1, 0.15, and 0.2 M LiCl in 0.01 N HCl. The eluate from 0.01 N HCl–0.2 M LiCl was neutralized with 1 N LiOH and concentrated to small volume. Addition of acetone–ethanol (4:1, 400 mL) precipitated a white solid, which was collected by centrifugation and washed with acetone–ethanol (4:1, 4 × 20 mL). The product (480 mg, 36%) was dried in vacuo at 22 °C over P_2O_5. It was homogeneous in chromatographic systems A and B and with HPLC (Table I). Anal. ($C_{20}H_{25}N_7O_{17}P_3Li_3{\cdot}7H_2O{\cdot}0.5C_2H_5OH$) C, H, N, P.

Ap_3T was treated with phosphodiesterase and alkaline phosphatase as described under General above. Paper chromatography in *n*-BuOH–H_2O (86:14) showed adenosine (R_f 0.26) and thymidine

(19) Lowry, O. H.; Lopez, J. A. *J. Biol. Chem.* **1946**, *162*, 421.

(R_f 0.48) as the only UV-absorbing materials. Elution of these spots gave a 1:1 ratio of nucleosides by spectrophotometric determination. Phosphate analysis of the enzymatic digest gave a phosphate/base ratio of 2.94:2.

Studies with Hexokinases. Adenosine 5′-triphosphate, lactate dehydrogenase (LDH; type II, rabbit muscle), and phosphoenolpyruvate (PEP) were from Sigma Chemical Co. Pyruvate kinase (PK) and yeast hexokinase (HK) were from Boehringer Mannheim, and NADH was from PL Biochemicals. Rat HK I–III isozymes were obtained by isoelectric focusing, followed by dialysis as described previously,[14] except that 0.5 mM dithiothreitol was included in the dialysis medium.

The assay mixture comprised 1 mL of 0.05 M Tris-HCl (pH 7.6) containing 6.6 mM $MgCl_2$, 10 mM α-D-glucose, 0.45 mM NADH, 0.55 mM cyclohexylammonium PEP, 20 mM KCl, PK (7 units), LDH (18 units), and variable MgATP. Reactions were started by addition of 0.028 unit of yeast HK of 50 μL of HK I, II, or III. In K_i determinations, the MgATP level was 0.6–4.0 × K_M. Stock solutions of ATP, Ap_3 glucose, and Ap_4 glucose were made in the Tris buffer and contained an equimolar amount of $MgCl_2$. The solutions for each experiment were freshly prepared. Reactions were followed at 22 °C with a Cary Model 15 spectrophotometer from changes in optical density at 340 nm in cells of 1-cm light path. Initial velocities were linear and proportional to the level of HK activity and independent of the level of PK or LDH. K_i values were obtained from replots of inhibitor concentrations vs. slopes of double-reciprocal plots of velocity vs. substrate level, all of which were linear. The inhibition studies used two inhibitor levels and six levels of MgATP. Inhibitions remained unchanged when higher levels of PK or LDH were used.

Ap_3 glucose (0.5 mM) in the above assay mixture produced no absorbancy change at 340 nm during 2 h in the presence of yeast HK or HK II or III. Ap_3 glucose or Ap_4 glucose (0.5 mM) was stored for 16 h at 22 °C in a solution containing the Tris buffer, $MgCl_2$, KCl, and glucose of the assay mixture, together with yeast HK or HK I–III at 4 times their normal assay level. Paper electrophoresis and chromatography (systems A, B, and D) showed Ap_3 glucose and Ap_4 glucose as the sole UV-absorbing components, and the sugar spray test[20] gave no evidence for production of glucose 6-phosphate.

Studies with Thymidine Kinases. Adenosine 5′-triphosphate, adenosine 5′-diphosphate, thymidine 5′-monophosphate, and dithiothreitol were obtained from Sigma Chemical Co. [2-^{14}C]Thymidine (57 Ci/mol) was obtained from New England Nuclear Inc. Preparations of C-TK and M-TK were obtained as described earlier,[1] except that in the case of C-TK the two dialyses (16 h, 4 h) after isoelectric focusing were carried out against a mixture of Tris-HCl buffer (0.05 M) at pH 8.0, dithiothreitol (5 mM), and glycerol (50%, v/v) and the preparation was not concentrated thereafter. The preparation was stable at −20 °C for at least 4 weeks. M-TK employed in kinetic studies with variable MgATP was first dialyzed in 1-mL portions at 4 °C against the above buffer (50 mL) for 64 h and then with fresh buffer for an additional 24 h in order to remove final traces of MgATP used to stabilize the M-TK during isoelectric focusing. The activity increased by 25%; the M-TK preparations so obtained were stable for at least 10 days at −20 °C. The K_M values of ATP and TdR are given in Table IV.

Enzyme-catalyzed reactions were carried out with 10 μL of either isozyme preparation in 0.1 mL of 0.05 Tris-HCl buffer, pH 8.0, and were linear for 60 min, 37 °C, in the presence of 18 μM TdR and 2.5 mM MgATP, during which time 850 cpm of [^{14}C]TMP (corresponding to 4.8% conversion of [^{14}C]TdR) for C-TK and 1120 cpm (6% of [^{14}C]TdR) for M-TK were obtained in the assay. With C-TK, reactions were linear for 60 min in the presence of 1 mM MgATP and 1.8 μM TdR. Reaction rates were proportional to enzyme level.

In inhibition studies carried out at fixed TdR levels (20 μM for C-TK and 80 μM for M-TK), six levels of MgATP in the range 0.25–1.25 mM were used for each of two levels of inhibitor and also for mixtures which lacked inhibitor. For fixed MgATP levels (1 mM for both isozymes), six levels of TdR (3.6–18 μM for M-TK and 0.9–7.2 μM for C-TK) were used for each of two levels of inhibitor. TTP and Ap_3T were added as their 1:1 magnesium complexes. All enzyme-catalyzed reaction mixtures were made up in duplicate. The mixtures were rocked in a water bath at 37 °C for 60 min, and the formation of [2-^{14}C]thymidine 5′-phosphate was measured as described earlier.[21]

Possible substrate activity of Ap_3T with TK was studied by HPLC with a μ-Bondapak NH_2 column (30 cm × 4 mm) utilizing a 2 mL/min flow rate with a linear gradient of ammonium dihydrogen phosphate (0.01–0.3 M, pH 5.0). The column eluant was monitored at 254 nm. The assay mixture contained, in a total volume of 0.1 mL, 10 μL of either of the two isozyme preparations and $MgAp_3T$ (0.4 mM) in Tris-HCl buffer (0.05 M at pH 8.0). The mixture and two controls (lacking Ap_3T or isozyme) were rocked in a water bath at 37 °C for 1 h and then immersed in boiling water for 2 min and cooled in ice. Denatured protein was removed by centrifugation and 15 μL of supernatant was analyzed by HPLC. Ap_3T was the only UV-absorbing component detected, and it appeared with an unaltered peak height. The retention times for TMP, ADP, Ap_3T, and ATP were 6, 17.5, 18, and 23 min, respectively. Conversion of a minimum of 6% of the Ap_3T could have been detected.

Acknowledgment. This work was supported by U.S. Public Health Service Research Grant CA-11196 from the National Cancer Institute and grants to the Institute for Cancer Research (USPHS Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

(20) Lemieux, R. V.; Bauer, H. F. *Anal. Chem.* **1954**, *26*, 920.

(21) Hampton, A.; Kappler, F.; Chawla, R. R. *J. Med. Chem.* **1979**, *22*, 621.