properties are given in Table I.

P^1-[8-(Ethylthio)adenosine-5′]-P^5-[5′(*R*)-*C*-*n*-propyladenosine-5′] Pentaphosphate (4b) and P^1,P^5-Bis[8-(ethylthio)adenosine-5′] Pentaphosphate. 8-(Ethylthio)adenosine 5′-(tri-*n*-butylammonium trimetaphosphate)[6] (0.4 mmol) and 5′(*R*)-*C*-*n*-propyladenosine 5′-[tris(tri-*n*-butylammonium) diphosphate] (0.1 mmol) were condensed in DMF under conditions described for the synthesis of 8-SEt-Ap_5A.[6] Purification was effected by chromatography on a column of DEAE-cellulose (HCO_3^-) (4 × 40 cm), which was washed with water (500 mL) and eluted with a linear gradient of 0–0.5 M triethylammonium bicarbonate (4 L). Compound **4b** eluted at 0.36–0.39 M salt (690 OD_{265} units, 29% yield) and di(8-SEt)-Ap_5A eluted at 0.40–0.44 M salt (580 OD_{280} units). Both **4b** and di(8-SEt)-Ap_5A contained trace contaminants, which were removed by paper chromatography on Whatman 3MM paper in solvent system A. The compounds were eluted from the chromatograms with water, and this solution was passed through a column (2.5 × 5 cm) of DEAE-cellulose (HCO_3^-). Elution with 0.5 M triethylammonium bicarbonate gave a methanol-soluble triethylammonium salt, which was converted to a sodium salt as above, yielding 29 mg of **4b** and 21 mg of di(8-SEt)-Ap_5A as white powders. See Table I for physical properties.

P^1-[8-(Ethylthio)adenosine-5′]-P^5-[5′(*R*)-*C*-methyladenosine-5′] Pentaphosphate (4a). An anhydrous solution of 8-(ethylthio)adenosine 5′-[tetrakis(tri-*n*-butylammonium) triphosphate] (1 mmol) and dicyclohexylcarbodiimide (605 mg, 3.3 mmol) in Me_2SO (5 mL) was stirred at room temperature for 1 h. Piperidine (1 mL, 10 mmol) was added, and stirring was continued for 1 h, when TLC in solvent A showed a single spot of R_f 0.75. The precipitate of *N*,*N*′-dicyclohexylurea was removed by filtration and the filtrate was added to anhydrous diethyl ether (100 mL). The gummy solid was triturated several times with ether and then dissolved in Me_2SO (4 mL). To the Me_2SO solution of **2** was added 5′-(*R*)-*C*-methyladenosine 5′-[tris(tri-*n*-butylammonium) diphosphate] (0.25 mmol) in DMF (4 mL) containing tri-*n*-butylamine (1.2 mL). The solution was maintained at 35 °C for 4 days, at which time HPLC analysis showed **4a**, starting materials, and seven trace impurities, one of which was di(8-SEt)-Ap_5A. The mixture was diluted with 0.15 M aqueous $Et_3NH{\cdot}HCO_3$ (100 mL) and applied to a column with DEAE-cellulose bicarbonate (4 × 35 cm) that had been equilibrated with 0.15 M $Et_3NH{\cdot}HCO_3$. The column was washed with 0.15 M $Et_3NH{\cdot}HCO_3$ (0.75 L) and then eluted with a linear gradient of 0.15–0.5 M $Et_3NH{\cdot}HCO_3$ (4 L). The product eluted at 0.38–0.43 M salt. Although the product appeared as a symmetrical peak in the elution profile, only the center one-third of the peak was homogeneous on analysis by HPLC. The leading and trailing edges contained a trace impurity, which was removed by chromatography on Whatman 3 MM paper in solvent A. Conversion of the three combined fractions to the sodium salt via the triethylammonium salt as described above gave 120 mg (44%) of **4a** as a white powder [dried at 78 °C (0.01 mm) over P_2O_5]. Properties of **4a** are given in Table I.

Enzymatic Conversion of P^1,P^5-Di(nucleoside-5′) Pentaphosphates and 5 to Nucleosides and Inorganic Phosphate. Enzymic digestion of ca. 1 μmol of **4a,b** or di(8-SEt)-Ap_5A was carried out for 2 h at 22 °C in 1 mL of Tris buffer, pH 10.4, containing 10 mg of phosphodiesterase from *Crotalus atrox* (Type IV, Sigma Chemical Co.) and 0.02 mg of alkaline phosphatase from calf intestine (Type VII, Sigma Chemical Co.). Paper chromatography in *n*-BuOH–H_2O (86:14) showed 8-(ethylthio)adenosine (R_f 0.67), 5′(*R*)-*C*-methyladenosine (R_f 0.40), or 5′(*R*)-*C*-*n*-propyladenosine (R_f 0.60) as the only UV-absorbing spots. Elution of the spots from digestion of **4a,b** showed a 1:1 ratio of nucleosides. Compound **5** was digested as above, except that the phosphodiesterase was omitted. Paper chromatography in *n*-BuOH–HOAc–H_2O (5:2:3) gave a single spot (R_f 0.30) identical with the authentic phosphonic acid isostere of AMP.[12] Phosphate analyses were performed by the method of Lowry and Lopez.[22]

Enzyme Kinetic Studies. Procedures for the determination of substrate and inhibitor constants with AK II and AK-M were the same as described previously.[6] All ATP derivatives and those Ap_5A derivatives with K_i values in excess of 0.10 mM were added in the form of 1:1 Mg complexes made by addition of $MgSO_4$ to the stock solutions.

Determination of substrate activity by HPLC analysis was accomplished by incubating the ATP analogue (0.5 mM), $MgSO_4$ (0.64 mM), and AMP (0.86 mM) in Tris buffer (0.1 M, pH 7.6) for periods up to 24 h at 22 °C with either AK II or AK-M (each at 10 × normal assay level). HPLC analysis was performed as described under "General" above but at pH 5 rather than at pH 3. This program separated ADP and ATP from the ADP and ATP derivatives, except for **5** and ATP which coeluted. However, the ADP analogue of **5** and ADP did separate as distinct peaks.

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Species- or Isozyme-Specific Enzyme Inhibitors. 9.[1] Selective Effects in Inhibitions of Rat Pyruvate Kinase Isozymes by Adenosine 5′-Diphosphate Derivatives

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Derivatives of adenosine 5′-diphosphate (ADP) with a substituent of 1–4 atoms at any of eight positions have been synthesized and evaluated as substrates and inhibitors of the liver (L), muscle (M), and kidney (K) isozymes of rat pyruvate kinase (PK). Inhibitory potencies of the compounds were expressed as K_M (ADP)/K_i or as K_M (ADP)/K_M when no K_i value was available. Nine of 14 ADP derivatives exhibited differential inhibitions. The M and K isozymes, which cross-react immunologically with each other but not with the L form, were inhibited differentially by 5 of the 14 derivatives. PK-K was preferentially inhibited by two derivatives, PK-L by three derivatives, and PK-M by two derivatives. Among the most selective and/or effective inhibitors were 3′-OMe-ADP [K_M (ADP)/K_i = 0.07 with PK-K; inhibitory potency, K/M/L, 7.6:6.0:1], N^6-Me,N^6-$(CH_2)_4$N(Me)COMe-ADP (prepared previously) [K_M (ADP)/K_M = 0.43 with PK-L; inhibitory potency, L/K/M, 3:2:1], and 8-NHEt-ADP [K_M (ADP)/K_i = 1.0 with PK-M; inhibitory potency, M/K/L, 7.1:1.2:1]. These and previous studies with two other enzymes indicate that monosubstituted substrate derivatives that bear short substituents (usually 1–4 atoms) at various positions are potentially useful probes in early stages of the attempted design of isozyme-selective inhibitors.

Evidence, summarized previously,[2] indicates that selective inhibitors for fetal-type isozymes are a class of compounds of potential utility in the design of new types of antineoplastic agents. It has been reported that in-

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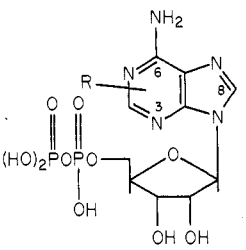
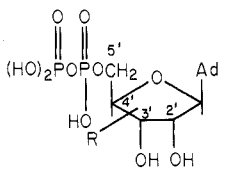
hibitors of rat thymidine kinases with varying degrees of isozyme selectivity were obtained when single substituents of 1–3 atoms were attached to seven of eight atoms of thymidine that were investigated.[3] In addition, isozyme-selective inhibitors of rat adenylate kinases were obtained by substituent attachment or isosteric group replacement at any of five atoms of the substrate adenosine 5′-triphosphate (ATP)[1,4,5] and at six of eight atoms of the substrate adenosine 5′-phosphate (AMP) for which data were obtained.[6] In the present work, the potential usefulness of such substrate derivatives as probes in the design of isozyme-selective inhibitors is explored with a third enzyme, pyruvate kinase (PK), which catalyzes reversible phosphoryl transfer between phosphoenolpyruvate and adenosine 5′-diphosphate (ADP) to give pyruvate and ATP. To this end, single substituents of 1–4 atoms were attached at any of seven positions of ADP (compounds **1–4** and **6–8**). A disubstituted ADP derivative (**5**) was also





1a, R = 2-Cl
b, R = 2-SMe
c, R = 2-NH_2
d, R = 2-NHMe
2a, R = 8-Br
b, R = 8-SMe
c, R = 8-SEt
d, R = 8-NHMe
e, R = 8-NHEt
f, R = 8-NH-*n*-Pr

(Ad = adenine-9-yl)

3, R = 2′-*O*-Me
4, R = 3′-*O*-Me
5, R = 2′,3′-*O*-CHMe
6, R = 4′-$CH_2CH{=}CH_2$
7, R = 5′(*R*)-Me
8, R = 5′(*S*)-Me

synthesized. The ADP derivatives were studied as substrates or inhibitors of the muscle (M), liver (L), and kidney (K) forms of rat PK. PK-K is the principal form in rat fetal tissue[7] and in poorly differentiated rat tumors originating from four different tissues.[7-9] Although it is present in many normal tissues, PK-K appears to be the sole isozyme in only 1 of 11 normal rat tissues examined.[10] That PK-K is a significant target in cancer chemotherapy is indicated by its high level in rapidly growing rat hepatomas[8,9] and by a parallelism observed between growth rate and level of PK activity in certain experimental tumors.[11] It may be noted that human brain tissue contains principally PK-M, whereas human fetal brain, meningiomas, and malignant gliomas contain mainly PK-K.[12] Human esophogeal cancer and mesothelioma tissue also appear to contain mainly PK-K.[13] In addition, PK in the serum of patients with carcinoma of the lung or stomach is reported to be immunologically more similar to human placental PK than to PK from other human tissues.[14]

Syntheses. Derivatives of adenosine 5′-phosphate (AMP) corresponding to **1a,b** and **3–8**, the preparation of which were described previously,[6] were converted by the action of *N,N*′-carbonyldiimidazole to their 5′-phosphoroimidazolidates; in the cases of **1a,b** and **6–8**, these intermediates were obtained as 2′,3′-*O*-carbonyl derivatives.[15,16] The 5′-phosphoroimidazolidates, as tri-*n*-butylammonium salts, were converted by reaction with tri-*n*-butylammonium phosphate in *N,N*-dimethylformamide to the corresponding derivatives of adenosine 5′-diphosphate (ADP). Following removal of 2′,3′-*O*-carbonyl groups, when present, by a mild basic treatment,[15] the ADP derivatives **1a,b** and **5–8** were obtained in 60–80% yield as trisodium salts that were homogeneous in the paper chromatographic, electrophoretic, and HPLC systems of Table I. The trisodium salts of **3** and **4** were obtained in lesser yield due to the formation of unidentified byproducts that were less polar than the AMP derivatives used in the syntheses. Application of the method to 2-amino-AMP gave no 2-amino-ADP (**1c**) and furnished instead two major unidentified products with altered UV absorption characteristics, suggesting possible derivatization of the 2-amino group. When tri-*n*-butylammonium 2-amino-AMP was treated at 22 °C for 24 h with 5 equiv of tri-*n*-butylammonium phosphoroimidazolidate in hexamethylphosphoric triamide (HMPT) solution, **1c** was obtained in 22% yield and 50% of the 2-amino-AMP was recovered. The poor yield of **1c** appeared to be associated with the limited solubility of tri-*n*-butylammonium 2-amino-AMP in DMF or HMPT.

The 8-alkylthio derivatives of ADP (**2b,c**) were obtained in ca. 70% yield by treatment of trilithium 8-bromo-ADP with the appropriate sodium alkylmercaptide in DMF–water at 70 °C. In the absence of DMF the displacement was slowed, and significant amounts of 8-bromo-AMP and 8-(alkylthio)-AMP were produced. The 8-(alkylamino)-ADP derivatives **2d–f** and 2-(methylamino)-ADP (**1d**) were prepared by treatment of trilithium 8-bromo-ADP and trisodium 2-chloro-ADP, respectively, with the appropriate alkylamine in DMF–water at 80 °C. The relatively poor yields of **2e–f** and **1d** (Table I) appear to result from the longer reaction times necessary, during which some hydrolysis to the corresponding derivatives of AMP took place.

Enzyme Studies. Catalysis by pyruvate kinase (PK) requires the presence of K^+ and a divalent metal ion such as Mg^{2+}. The liver (L) and kidney (K) forms, and to a slight extent the muscle (M) form, are activated by fructose 1,6-diphosphate. Substrate and inhibitor properties of the present series of ADP derivatives were determined at a pH value and with levels of buffer, Mg^{2+}, K^+, and fructose 1,6-diphosphate reported to produce near-optimal activity of all three isozymes.[17] Phosphoenolpyruvate was employed in the studies at a level shown to produce near-

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Table I. Physical Properties of Adenosine 5'-Diphosphate Derivatives

compd	yield,[a] %	UV λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 1	UV λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 6.8	electrophoresis, pH 3.6, rel mobility[b]	R_f[b] syst A	R_f[b] syst B	HPLC retn time, min[b]	formula	anal.
ADP				1.0	0.06	0.2	12		
1a[d]	70			1.28	0.20	0.21	24		
1b[d]	73			1.05	0.19	0.33	31.5		
1c	22	215 (17.8) 253 (10.6) 292 (8.9)	215 (23.8) 255 (9.0) 280 (9.6)	0.85	0.05	0.27	8	$C_{10}H_{13}N_6O_{10}P_2Na_3 \cdot 2.5H_2O$	C, H, N, P
1d	45	255 (13.1) 299 (8.7)	258 (11.0) 289 (8.7)	0.88	0.10	0.35	14	$C_{11}H_{15}N_6O_{10}P_2Na_3 \cdot 0.5H_2O \cdot 0.6CH_3COCH_3$	C, H, N, P
2a[d]				0.98	0.08	0.28	15.5		
2b	74	283 (20.5)	281 (20.1)	0.92	0.09	0.32	23.5	$C_{11}H_{14}N_5O_{10}P_2SNa_3 \cdot 1.5MeOH$	C, H, N, P, S
2c	70	283 (20.9)	281 (19.9)	0.92	0.18	0.49	31.5	$C_{12}H_{16}N_5O_{10}P_2SNa_3 \cdot 2H_2O \cdot 0.5MeOH$	C, H, N, P, S
2d	70	277 (14.6)	279 (17.6)	0.95	0.06	0.27	17	$C_{11}H_{15}N_6O_{10}P_2Na_3 \cdot 1.75MeOH$	C, H, N, P
2e	48	277 (14.2)	279 (17.2)	0.89	0.13	0.41	21	$C_{12}H_{17}N_6O_{10}P_2Na_3 \cdot 0.75MeOH$	C, H, N, P
2f	45	277 (14.7)	279 (17.8)	0.85	0.19	0.51	29	$C_{13}H_{19}N_6O_{10}P_2Na_3 \cdot 2H_2O \cdot 0.5MeOH$	C, H, N, P
3	45		260 (15.1)	1.0	0.15	0.42	17	$C_{11}H_{14}N_5O_{10}P_2Na_3 \cdot 2H_2O$	C, H, N, P
4	40		260 (14.9)	1.0	0.16	0.40	15	$C_{11}H_{14}N_5O_{10}P_2Na_3 \cdot 3H_2O$	C, H, N, P
5	65		260 (15.2)	0.94	0.30	0.70	30	$C_{12}H_{14}N_5O_{10}P_2Na_3 \cdot H_2O \cdot 0.75MeOH$	C, H, N, P
6	60		260 (14.9)	0.93	0.28	0.54	30	$C_{13}H_{16}N_5O_{10}P_2Na_3 \cdot 2H_2O \cdot 0.5MeOH$	C, H, N, P
7[c]	80			1.0	0.10	0.38	15		
8	75		260 (15.0)	1.0	0.15	0.42	20	$C_{11}H_{14}N_5O_{10}P_2Na_3 \cdot H_2O \cdot MeOH$	C, H, N, P

[a] Yields are of purified trisodium salts. [b] For conditions, see Experimental Section. [c] Preparation and additional properties are described in ref 1. [d] A previously described compound; see Experimental Section for method of preparation.

maximal reaction velocity with the isozymes.[17] ADP and its derivatives were added as 1:1 Mg complexes in order to minimize variations in the level of free Mg^{2+} in the reaction mixtures. Table II lists the substrate constants obtained. All ADP derivatives showed substrate activity with the exception of 8-(methylthio)-ADP (**2b**), 8-(ethylthio)-ADP (**2c**), 8-(*n*-propylamino)-ADP (**2f**), and the endo diastereomer of 2',3'-*O*-ethylidene-ADP (**5**). The pronounced substrate activity of 2'-*O*-methyl-ADP with PK-M, together with the relatively low substrate activity [K_M (ADP)/K_M = 0.17; rel V_{max} = 22%] of 2'-deoxy-ADP with rabbit muscle PK,[18] suggests a possible role in catalysis for 0(2') of ADP. Table II also lists inhibition constants of the ADP derivatives. All the compounds tested behaved as competitive inhibitors with respect to ADP. Because ADP has differing K_M values with the three isozymes, the inhibitory potency of each compound for a given isozyme is expressed as K_M (ADP)/K_i or, to a first approximation, as K_M (ADP)/K_M when a K_i value is not available. In all but ten of the inhibitor–isozyme combinations tested, attachment of substituents to ADP produced inhibitors of weak or moderate potency [K_M (ADP)/K_i or $K_M < 1$]. A similar tendency for substrate substituents to produce inhibitors of only moderate potency was observed in previous studies in which AMP, ATP, or thymidine were substrates.[1,3-6,19]

Calculation of relative inhibitory potencies for the isozymes (Table II) indicated that most of the ADP derivatives exhibited isozyme-selective inhibition to varying degrees. Little or no selectivity was shown by 2-SMe-ADP (**1b**), 2-NHMe-ADP (**1d**), 8-Br-ADP (**2a**), 2'-*O*-Me-ADP (**3**), or 5'(*S*)-*C*-Me-ADP (**8**). In cases wherein comparison of all three isozymes was possible, preferential inhibition of PK-L was shown by **1c**, **2d**, and N^6-Me, N^6-$(CH_2)_4$N(Me)$COCH_3$, preferential inhibition of PK-M was shown by **2e** and **7**, and preferential inhibition of PK-K was shown by **4** and **6**. In addition, **2f** and **5** inhibited PK-M more than PK-L. In all cases except **2e**, **4**, **6**, **7**, and N^6-Me, N^6-$(CH_2)_4$N(Me)$COCH_3$, the ADP derivatives inhibited PK-M and PK-K to a similar degree. This accords with the structural similarity of mammalian M and K isozymes shown by their immunological cross-reactions[20-22] and by their amino acid compositions, which are similar and distinctly different from that of PK-L.[23]

It has been reported that a high proportion of AMP or ATP derivatives with a single short (1 to 3 atoms) substituent show differential affinity for the AMP or ATP sites, respectively, of adenylate kinases and behave as species- or isozyme-selective inhibitors[1,4-6] and that a high proportion of monosubstituted thymidine derivatives likewise act as species- or isozyme-selective inhibitors of thymidine kinases.[3,19] The present results extend this type of finding to the ADP site of a third enzyme, pyruvate

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Table II. Substrate and Inhibitor Constants of ADP Derivatives with Muscle (M), Kidney (K), and Liver (L) Isozymes of Rat Pyruvate Kinase (PK)

compd	PK-M			PK-K			PK-L			inhibitory potency [K_M (ADP)/K_i or K_m]			rel inhibitory potency	
	K_i,[a] mM	K_M,[b] mM	rel V_{max}	K_i, mM	K_M, mM	rel V_{max}	K_i, mM	K_M, mM	rel V_{max}	PK-M	PK-K	PK-L	M/L	K/L
ADP		0.52	100		0.50	100		0.30	100					
1b		0.11	77		0.089	67		0.061	72	4.7	5.6	5.0	0.94	1.12
1c		0.26	77		0.25	90		0.090	95	2.0	2.0	3.3	0.61	0.61
1d		0.19	77		0.15	85		0.12	86	2.7	3.4	2.5	1.1	1.4
2a		2.9	6		2.9	6		2.3	14	0.18	0.17	0.13	1.4	1.3
2b	4.3		0	5.0		0	7.5		0	0.12	0.10	0.040	3.0	2.5
2c	3.7		0[c]	3.9		0[c]	8.4		0[c]	0.14	0.13	0.036	3.9	3.6
2d		0.56	55		0.58	49		0.16	40	0.93	0.86	1.9	0.49	0.45
2e	0.50[d]		<1	2.9	2.0	0.7	2.2[e]		<1	1.0	0.17	0.14	7.1, 13[f]	1.2, 2.2[f]
	0.87[g]						3.9[h]			0.60		0.077		
2f	0.9		0				1.6[i]		0	0.58		0.19	3.1	
							3.8[j]					0.079	7.3	
3		0.70	90		0.56	94		0.35	100	0.74	0.89	0.86	0.86	1.04
4	9.3		>0[c]	7.0		>0[c]	32		>0[c]	0.056	0.071	0.0094	6.0	7.6
5	1.6		0				2.2[k]		0	0.325		0.14	2.3	
							4.6[m]					0.065	5.0	
6		1.3	7		0.59	11		0.53	52	0.40	0.85	0.57	0.70	1.5
7	4.5[n]		>0[c]	8.0		>0[c]	5.4[p]		>0[c]	0.12	0.063	0.056	2.1, 4.2[f]	1.1, 2.2[f]
	7.0[q]						10.9[q]			0.074		0.028	2.6, 1.3[f]	
8		2.7	15		2.9	15		1.3	26	0.19	0.17	0.23	0.83	0.74
N^6-Me,N^6-R-ADP[r]		3.6[s]			1.7[s]			0.7[s]		0.14	0.29	0.43	0.33	0.67

[a] All inhibitions were competitive with respect to ADP. [b] K_M = concentration of substrate for half-maximal velocity (V_{max}). [c] HPLC analysis was performed for PK-catalyzed conversion of an ADP derivative to an ATP derivative. No substrate activity was detected with the coupled enzyme assay. [d] With 1.0 mM 2e. [e] With 3.9 mM 2e. [f] Calculated from the two inhibitory potency values of PK-L. [g] With 0.67 mM 2e. [h] With 2.6 mM 2e. [i] With 4.7 mM 2f. [j] With 2.35 mM 2f. [k] With 7.2 mM 5. [m] With 3.6 mM 5. [n] With 10 mM 7. [p] With 10.7 mM 7. [q] With 6.7 mM 7. [r] R = $(CH_2)_4N(Me)COCH_3$. [s] Data from ref 24.

kinase, and serve to further indicate that such monosubstituted substrate derivatives are potentially useful probes in early stages of the design of isozyme-selective inhibitors. Recent studies have shown that the low or moderate inhibitory potency exhibited by many such substrate derivatives can sometimes be enhanced by modifying the substituent[3] or by elaborating the derivative into an inhibitor with affinity for two adjacent enzymic substrate sites.[1,5]

Experimental Section

Chemical Synthesis. General. Ethanethiol and 70% ethylamine in water were purchased from Aldrich Chemical Co. Methyl mercaptan was from Matheson. Methylamine, 40%, in water and *n*-propylamine were obtained from Eastman Kodak Co. Monopotassium ADP was purchased from Boehringer Mannheim. *N,N*-Dimethylformamide and hexamethylphosphoric triamide were distilled from calcium hydride and stored over molecular sieves. Paper chromatography was carried out by the ascending technique on Whatman No. 1 paper in (A) 1-butanol–acetic acid–water (5:2:3) (B) isobutyric acid–NH_4OH–water (66:1:33), and (C) 1-propanol–NH_4OH–water (55:10:35). Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 (0.05 M citrate) and at pH 7.5 (0.05 M triethylammonium bicarbonate). Ultraviolet spectra were obtained on Cary Model 15 and Varian Model 635 spectrophotometers, and ^1H NMR spectra were obtained on a Perkin-Elmer 24B spectrometer. Chemical shifts are given as parts per million downfield from $SiMe_4$. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN. Analytical results were within ±0.4% of the theoretical values. All compounds were dried over P_2O_5 at 25 °C, except **2b,d,e**, which were dried at 80 °C for 2 h. 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 Model 660 programmer. Compounds were analyzed with a Waters RCM-100 unit containing a μ-Bondapak C_{18} column eluted at 2 mL/min with 0.1 M KH_2PO_4–0.025 M Bu_4NHSO_4 (pH 5) with a linear gradient of 10–30% MeOH over 20 min.

The AMP derivatives corresponding to **1a–c** and **3–8** were prepared as previously reported.[6] The lithium salt of 8-Br-ADP[25] was prepared by bromination of ADP (4 mmol). The reaction required a longer time (3 days) than reported. The product was purified by elution from an AG1-X8 column (Bio-Rad, 5 × 30 cm) with a linear gradient of 0.01–0.3 M LiCl in 0.001 N HCl (8 L) and was obtained in 55% yield and free from ADP as judged by HPLC. 2-Cl-ADP (**1a**) and 2-SMe-ADP (**1b**) were prepared from 2-Cl-AMP and 2-SMe-AMP, respectively, by the phosphoroimidazolidate method described below. Compounds **1a** and **1b** were prepared previously via the corresponding mononucleotide phosphoromorpholidates.[26,27] The UV spectral properties and R_f value of **1a** and **1b** agreed with those reported.[26,27]

All ADP derivatives, except 8-Br-ADP, were purified by one of two procedures.

Method I. The crude product (0.4 mmol) was chromatographed at 5 °C on a DEAE-cellulose column (4 × 20 cm). Elution with a linear gradient of 0–0.35 M $Et_3NH{\cdot}HCO_3$ (4 L), vacuum evaporation of appropriate fractions, and several evaporations of EtOH from the residue gave the triethylammonium salt of the product, which was dissolved in sufficient MeOH so that on addition of 1 M NaI in MeOH (7.5 equiv) no precipitate formed. The methanol solution was evaporated to a minimum volume and diluted with acetone (ca. 10 volumes) to precipitate the trisodium salt.

Method II. When byproducts were present that coeluted with the desired product from columns of DEAE-cellulose, the crude product was chromatographed in solvents A or C on Whatman No. 17 paper (width 46 cm, 0.2 mmol/sheet). The band containing the product was eluted with water at 5 °C, and the eluate was

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applied to a column of DEAE-cellulose according to method I above. All products were homogeneous in the systems of Table I.

Conversion of Nucleoside 5′-Phosphates to Nucleoside 5′-Diphosphates (1a,b and 3–8). The requisite nucleoside 5′-monophosphate (0.4 mmol) was converted to the corresponding 5′-phosphoroimidazolidate, and this was reacted with tri-*n*-butylammonium phosphate according to the Hoard–Ott procedure.[16] After evaporation of the DMF used as solvent, the residue was dissolved in EtOH–water (1:1, 100 mL), and the solution brought to pH 11 with Et_3N to remove 2′,3′-cyclic carbonate residues.[15] After 4 h at 23 °C, volatiles were removed, and ethanol was evaporated from the residue to remove residual triethylamine. Crude **1a,b** and **5–8** were purified by method I above. The reaction mixture obtained from 2′-*O*-Me-AMP contained **3** and an unidentified byproduct that migrated faster than 2′-*O*-Me-AMP in solvent A in a ratio of ca. 4:1; **3** was purified by method II (solvent A) above. Crude **4** also contained a byproduct of relatively high R_f in solvent A and was purified by method II (solvent A) above. **7**: NMR (D_2O, 60 MHz) δ 1.4 (d, 3, J = 6 Hz, H-6′), 4.1 (m, 2, H-5′), 4.3–5.0 (m, 3, masked by H_2O, H-2′, H-3′, H-4′), 5.95 (d, 1, J = 5 Hz, H-1′), 7.9 and 8.3 (s, 1, H-2 and H-8). **8**: NMR (D_2O) δ 1.4 (d, 3, J = 6 Hz, H-6′), 4.1 (m, 2, H-5′), 4.3–4.9 (m, 3, masked by H_2O, H-2′, H-3′, H-4′), 5.95 (d, 1, J = 4.5 Hz), 7.95 and 8.4 (s, 1, H-2 and H-8). **5**: NMR (D_2O) δ 1.45 (d, 3, J = 5 Hz, CH_3-acetal), 4.05 (m, 2, H-5′), 4.4–5.4 (m, 4, masked by H_2O, H-2′, H-3′, H-4′ and H-acetal), 6.0 (d, 1, J = 3 Hz, H-1′), 7.9 and 8.2 (s, 1, H-2 and H-8). For other properties, see Table I.

2-Aminoadenosine 5′-Diphosphate (1c). The tri-*n*-butylammonium salt of 2-amino-AMP (0.5 mmol) was reacted with the imidazolidate of tri-*n*-butylammonium phosphate (2.5 mmol) according to the Hoard–Ott procedure.[16] An equal volume of anhydrous HMPT was used in place of DMF as solvent for the reaction. After 24 h, several grams of ice was added. The mixture was brought to pH 4.5 with acetic acid, stored at 23 °C for 6 h, then neutralized with Et_3N, and purified by method I to give a mixture of 2-amino-AMP (50%) and **1c** (22%). The yield was not improved by a longer reaction period (3 days). For properties, see Table I.

2-(Methylamino)adenosine 5′-Diphosphate (1d). 2-Cl-ADP·Na_3 (0.5 mmol) was treated with an aqueous 40% solution of methylamine according to the procedure described below for the synthesis of **2d–f**. The reaction was followed by electrophoresis in pH 3.6 buffer and by HPLC. The reaction was almost complete after 5 h, at which time the reaction mixture contained a small amount of 2-Cl-ADP, together with **1d** and 2-NHMe-AMP in a ratio of ca. 2.5:1. Compound **1d** was purified by method II (solvent C). Properties of **1d** are given in Table I.

General Method for the Synthesis of 8-(Alkylthio)-adenosine 5′-Diphosphates (2b,c). To a solution of NaOMe (3.5 mmol) in absolute methanol (1.2 mL) was added the appropriate thiol (8 mmol) (methyl mercaptan was liquefied with a dry ice condenser). The solution was stirred at 23 °C for 30 min. A solution of 8-Br-ADP·Li_3 (0.56 mmol) (**2a**) in water (11 mL) was added, followed by DMF (11 mL). The mixture was warmed to 70 °C, and the reaction was followed by the change in UV absorption maxima from 271 to 281 nm and then by HPLC. After 40–50 min, HPLC showed that conversion of **2a** was complete. The reaction mixture contained **2b** or **2c** and traces of the corresponding AMP derivatives. The mixture was neutralized with acetic acid, excess thiol was removed with a stream of nitrogen gas, and the product was isolated by method I above. **2b**: NMR (D_2O) δ 2.6 (s, 3, SCH_3-8), 4.25 (m, 2, H-5′), 4.4–5.1 (m, 3, masked by H_2O, H-2′, H-3′, H-4′), 5.9 (d, 1, J = 6 Hz, H-1′), 8.0 (s, 1, H-2). **2c**: NMR (D_2O) δ 1.4 (t, 3, J = 7 Hz, SCH_2CH_3-8), 3.2 (q, 2, J = 7 Hz, SCH_2CH_3-8), 4.25 (m, 2, H-5′), 4.4–4.8 (m, 2, masked by H_2O, H-4′, H-3′), 5.1 (m, 1, H-2′), 6.0 (d, 1, J = 6 Hz, H-1′), 8.0 (s, 1, H-2). For other properties, see Table I.

General Method for the Synthesis of 8-(Alkylamino)-adenosine 5′-Diphosphates (2d–f). A solution of trilithium 8-Br-ADP (0.55 mmol) in 40% aqueous alkylamine solution (5.5 mL) was diluted with DMF–water (1:1, 24 mL) and heated in a stainless-steel bomb at 80 °C. The reaction was followed by the change in UV absorption maximum from 265 to 279 nm and also by HPLC. The conversion of **2a** was complete after 50 min with methylamine and almost complete after 3 h with ethylamine and *n*-propylamine. In the latter cases the reaction mixtures contained **2e** or **2f** and the corresponding AMP derivatives in ratios of ca. 2.5:1. Volatiles were removed in vacuo, and ethanol was evaporated from the residue to remove residual alkylamine. **2d** was purified by method I; **2e** and **2f** were purified by method II (solvent A). **2f**: NMR (D_2O) δ 0.9 (t, 3, J = 7 Hz, $NHCH_2CH_2CH_3$), 1.5 (m, 2, $NHCH_2CH_2CH_3$), 3.3 (m, 2, $NHCH_2CH_2CH_3$), 4.3 (m, 2, H-5′), 4.4–5.0 (m, 3, masked by D_2O, H-2′, H-3′, H-4′), 5.9 (d, 1, J = 6 Hz, H-1′), 7.9 (s, 1, H-2). Other properties are given in Table I.

Enzyme Kinetic Studies. The M, K, and L pyruvate kinase isozymes were extracted from rat tissues and partially purified by isoelectric focusing as described previously,[2] except that fructose 1,6-diphosphate (FDP) was added to the crude centrifuged liver extract to a level of 100 μM, and incubation of the extract with FDPase[28,29] was omitted. When the mixture was electrofocused in the presence of 35% glycerol, the recovery (ca. 30%) of PK-L activity (pI 5.7–5.9) was 5–10 times higher than with the previously used procedure.

PK-catalyzed reactions were followed at 22 °C by measuring the rate of change of optical density at 340 nm in a Cary Model 15 spectrophotometer with 1-cm cells containing a final volume of 1 mL. Initial velocities were linear and proportional to the concentration of PK and independent of the level of lactate dehydrogenase (LDH). The assay system contained 0.05 M triethanolamine hydrochloride (pH 7.0), $MgCl_2$ (5 mM), KCl (67 mM), phosphoenolpyruvate cyclohexylammonium salt (0.56 mM), reduced nicotinamide adenine dinucleotide (NADH) (0.4 mM), fructose 1,6-diphosphate (50 μM), and rabbit muscle LDH (type 1, Sigma Chemical Co., 10 μg). Reactions were started by the addition of ADP or an ADP derivative. Stock solutions of ADP and ADP derivatives were made the same day studied and contained equimolar amounts of $MgCl_2$.

Substrate constants were determined from double-reciprocal plots of velocity vs. substrate level; all plots were linear. Five or more levels of substrate were employed. ADP derivatives that are reported in Table II to have no detectable substrate activity were tested for activity at a level of 1 mM or higher for 0.5 h with 10 times the level of PK activity used in assays in which ADP was the substrate.

Compounds were tested by HPLC analysis for possible substrate activity at levels of 1–3 mM in the assay medium lacking NADH and LDH for 16–48 h at 22 °C in the presence of a 20–50 times higher level of PK activity than used in the above spectrophotometric assay. Mixtures lacking phosphoenolpyruvate were analyzed when evidence for conversion of an ADP derivative to an ATP derivative was obtained. HPLC analyses were carried out with an NH_2 Bondapak column (30 cm × 4 mm) eluted with a 0.01–0.3 M gradient of $NH_4H_2PO_4$, pH 3.0. The eluant was monitored at 254 nm in tests with **4** and **7** and at 280 nm with **2c**.

Inhibition studies used five or more levels of ADP in the range 0.5–4.0 × K_M for each of two inhibitor levels that were in the range 0.6–4 × K_i. Inhibition constants (K_i values) were obtained from replots of inhibitor concentrations vs. slopes of double-reciprocal plots of velocity vs. substrate level.

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