Use of Adenine Nucleotide Derivatives to Assess the Potential of Exo-Active-Site-Directed Reagents as Species- or Isozyme-Specific Enzyme Inactivators. 2.[1] Isozyme-Specific Inactivation of a Mammalian Enzyme and Its Significance in the Possible Design of Fetal Isozyme Targeted Antineoplastic Agents

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Potential exo-substrate-site alkylating agents of pyruvate kinase were prepared by attaching iodoacetamidoalkyl substituents [$-(CH_2)_nNHCOCH_2I$] to N^6 of adenosine 5′-diphosphate (ADP), a substrate of the enzyme. The known muscle (M), liver (L), and kidney (K) isozymes of rat pyruvate kinase were separated from each other by electrofocusing and treated with 5 mM ADP derivatives for 8 h at 22 °C and pH 7.0. When n was 2–7, none of the isozymes was progressively inhibited (inactivated); when n was 8, the M and K isozymes were not inactivated, whereas the L isozyme was 82% inactivated. Experiments with mixtures of the L form with either the K or M forms showed that the isozyme-selective effect was not due to activation or deactivation of the inhibitor. Inactivation was abolished by replacement of the iodine by hydrogen, indicating that alkylation of the L isozyme is involved. That the effect is ADP site directed is suggested by the ability of the inhibitor to function as a substrate of the L isozyme, by protection against the inactivation by 5 mM ADP, and by failure of the corresponding ATP and AMP derivatives (5 and 10 mM, respectively) to inactivate, presumably because they bind weakly to the ADP site. Kinetic analyses showed that the half-time of inactivation of the L isozyme approaches a limit value of 23 min with increasing inhibitor level and that inactivation involves reaction of not more than one molecule of inhibitor per catalytically active unit of isozyme. Isozyme-specific inhibitors of fetal-type isozymes predominant in tumor tissue are of potential chemotherapeutic importance as starting points in the design of antineoplastic agents. The above findings comprise direct evidence for the design of an isozyme-specific inhibitor of a mammalian enzyme, and they substantiate previous indications that one method by which such inhibitors can be obtained is through a study of potential substrate-site-directed exo-site enzyme reagents.

The isozyme patterns in normal and neoplastic rat tissues of more than 12 enzymes have now been studied in some detail, and it has been found that in highly neoplastic tissue the predominant isozymic form of each enzyme is almost invariably the type characteristic of early fetal tissue, whereas in those normal tissues examined the predominant isozymic form is sometimes the fetal type but frequently is one or another of the adult types found in differentiated tissues.[2-7] Further work in this relatively new field will presumably reveal additional instances of such isozyme patterns. These differences between the isozymic compositions of normal and neoplastic tissues might conceivably permit the design of useful antineoplastic agents which owed their effectiveness to an ability to inhibit the fetal isozyme but not the remaining isozymes of their respective target enzymes.

The first stage in the development of a chemotherapeutic agent of this type would clearly involve the design of a compound which could selectively inhibit a fetal isozyme in a cell-free system. At the outset of the present work, however, no direct evidence had been presented for the design of such an isozyme-specific inhibitor. In 1962 Baker[8] had proposed that isozyme-specific inhibitors might be obtainable systematically through a study of substrate derivatives which possess affinity for a substrate site of the target isozyme and which bear a substituent with an electrophilic group to render them capable of forming a covalent bond with the target isozyme outside the substrate site. It was reasoned that neighboring group effects operating in the nucleophilic displacement would tend to amplify structural differences between the isozymes and thereby help to achieve the desired selectivity of inhibition. Initial evidence supporting the feasibility of this approach was provided by the development of several compounds which selectively inactivate either rabbit muscle or beef heart lactate dehydrogenase,[9] two enzymes in which an isozymic difference is combined, however, with a species difference. Subsequently, the approach produced several compounds which appeared to inactivate dihydrofolate reductase extracted from mouse L1210 leukemia tissue but not that from a normal mouse tissue,[10] and indirect evidence suggested that the selectivity might result from hypothetical structural differences between the dihydrofolate reductase obtained from the two tissues. In contrast to the lactate dehydrogenase studies, no kinetic or other evidence was presented to characterize the nature of the inactivations. In this laboratory, we recently initiated a series of studies aimed at assessing the ability of Baker's approach to produce isozyme-specific inhibitors effective in cell-free systems. The overall approach adopted in this assessment was suggested from earlier work[11] which had shown that moderately large substituents could be attached to the N^6 of the adenine nucleotides ADP and ATP without preventing their binding to the adenine nucleotide substrate sites of various phosphokinases such as adenylate kinase, pyruvate kinase, and hexokinase. We therefore decided to synthesize a series of N^6-substituted adenine nucleotide derivatives bearing electrophilic groups and to study them as potential irreversible inhibitors of isozymic forms of a number of adenine nucleotide utilizing phosphokinases. This plan was adopted in order to be able to carry out a relatively wide survey of the effectiveness of Baker's suggested approach in cell-free systems using a minimal number of synthetic substrate derivatives. On the other hand, the above adenine nucleotide derivatives were not selected for study because they were thought to be of promise as potential precursors of isozyme-specific drugs effective in vivo. To the contrary, it is well recognized that nucleotides are poorly membrane permeable and, in addition, are readily susceptible to enzymatic dephosphorylation in vivo; moreover, a given adenine nucleotide derivative might well inhibit more than one of the many enzymes which utilize adenine nucleotides as substrates or effectors.

The present paper describes an evaluation of Baker's suggested approach to isozyme-specific inhibitors which has been carried out with the liver (L), muscle (M), and kidney (K) isozymes of rat pyruvate kinase. This enzyme catalyzes the reaction of adenosine 5′-diphosphate (ADP) with phosphoenolpyruvate to form adenosine 5′-tri-

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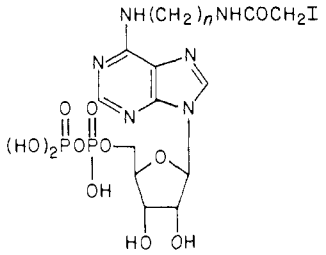
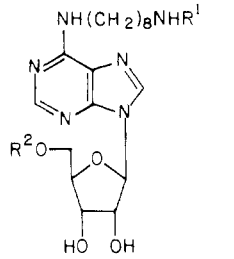
phosphate (ATP) and pyruvate. A series of N^6-iodoacetamidoalkyladenosine 5′-diphosphate derivatives (structure 1) was prepared as potential ADP site-directed



1a, n = 2 1e, n = 6
b, n = 3 f, n = 7
c, n = 4 g, n = 8
d, n = 5



2a, R^1 = H; R^2 = PO_3H_2
b, R^1 = $COCH_3$; R^2 = PO_3H_2
c, R^1 = $COCH_3$; R^2 = $P_2O_6H_3$

exo-site reagents. Alkyl substituents were selected for attachment to N^6 because their relatively high flexibility was expected to enhance the ability of the substituent to respond to structural differences between the three isozymes. The relatively reactive iodoacetyl moiety was chosen as the electrophile because it provided sufficiently stable ADP derivatives and, yet, had a high probability of enzyme alkylation (like iodoacetamide, the substituents of 1 should be capable of alkylating the nucleophilic groups of lysine, glutamate, aspartate, histidine, cysteine, tyrosine, and methionine residues). One of these derivatives extensively inactivated the L isozyme but under the same conditions did not inactivate the M or K isozymes. Evidence is presented that the selective inactivation is ADP site directed and involves alkylation of the L isozyme outside the ADP site. A brief account of most of the present findings has been presented previously.[12]

Syntheses. A series of N^6-iodoacetamidoalkyladenosine 5′-triphosphates, the synthesis of which was previously described,[1] was prepared by an alternative route which involved converting a series of ω-aminoalkyladenosine 5′-monophosphates[1] to their ω-*N*-carbobenzyloxy derivatives, followed by conversion of these to the corresponding 5′-triphosphates, removal of the carbobenzyloxy groups, and iodoacetylation of the ω-amino groups.[13] Treatment of these ATP derivatives with yeast hexokinase in the presence of glucose gave the desired derivatives (**1a–g**) of ADP in good yields. The products were isolated as their trisodium salts which were homogeneous as indicated by the ultraviolet extinction coefficient, phosphate analysis, paper electrophoresis and chromatography, and anion-exchange column chromatography. The stability of the iodo group during the preparation of **1a–g** was confirmed by complete elemental analysis of **1g** and by treatment of **1a–g** for 1 h at 22 °C with concentrated NH_4OH, when, in each case, paper electrophoresis at pH 3.5 revealed a single component with the same UV spectrum as the starting material but with 60% as much mobility, this being indicative of conversion of the dianionic species **1a–g** to monoanionic species by transformation of iodoacetyl residues to glycyl residues. In further support of this interpretation, the products of the concentrated NH_4OH treatment were, in each case, ninhydrin positive, whereas the starting materials, **1a–g**, were ninhydrin negative. The acetamido analogue **2c** of the iodoacetamido derivative **1g** was prepared by N-acetylation of N^6-(8-amino-*n*-octyl)-adenosine 5′-phosphate (**2a**),[1] by a previously described general procedure,[1] and subsequent application to the resulting **2b** of the Hoard–Ott procedure[14] for phosphoanhydride synthesis, followed by mild basic treatment to remove 2′,3′-*O*-carbonyl residues.[15]

Table I. Substrate Properties of ADP Derivatives 1 and 2 with Rat Pyruvate Kinase Isozymes

compd	initial velocity rel to ADP[a]		
	M isozyme	L isozyme	K isozyme
ADP	100	100	100
1d	7	8	n.d.[b]
1e	19	9	n.d.
1f	25	12	n.d.
1g	54	13	21
2c	n.d.[b]	9	n.d.

[a] The initial concentration of ADP and its derivatives was 0.21 mM. [b] n.d. = not determined.

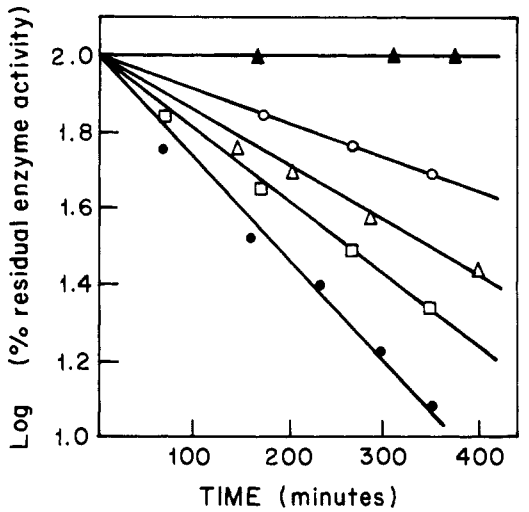


Figure 1. Rate of inactivation of the liver isozyme of rat pyruvate kinase at 22 °C by the following levels of **1g**: 3.0 (○), 5.0 (△), 7.0 (□), 10.0 (●), and 5.0 mM together with 5.0 mM ADP (▲).

Enzyme Studies. The liver (L), kidney (K), and muscle (M) isozymes of pyruvate kinase were obtained in partially purified and isozymically homogeneous form by electrofocusing according to described procedures.[16] Compounds **1d–g** and **2c** substituted for ADP as substrates for isozymes L and M (Table I), and **1g** was a substrate for isozyme K. In all cases, the initial reaction velocities were significantly less than with an equal initial concentration of ADP, and, in all cases, the reactions, when allowed to proceed to completion, produced 95% or more of the theoretical amounts of pyruvic acid as assayed with lactate dehydrogenase.

The three pyruvate kinase isozymes were exposed, at pH 7.0 for 8 h, 22 °C, to the action of 5 mM levels of the Mg salts of compounds **1a–g** in aqueous triethanolamine buffer containing $MgCl_2$ and KCl; the pH of this solution and the concentrations of its other components have been reported to permit near-optimal activity of all three isozymes.[17] In addition, a level of fructose 1,6-diphosphate sufficient to fully activate the L and K isozymes was included in the test mixtures (the M form is only slightly activated by this modulator).[17] No inactivation of any isozyme was effected by **1a–f** under these conditions. Compound **1g** progressively reduced the activity of the L isozyme over the 8-h period by a total of 80%, but **1g** had no effect on the activity of the K isozyme after 8 h or of the M isozyme after 24 h. Replacement of the iodine of **1g** by hydrogen (giving **2c**) abolished inactivation of the L isozyme, indicating that the effect involves alkylation of the isozyme by the iodoacetyl group of **1g**.

In the presence of 5 mM ADP, 5 mM **1g** caused no detectable inactivation of the L isozyme during 8 h (Figure 1) Additional evidence that the inactivation is ADP site directed is the substrate activity of **1g** referred to above

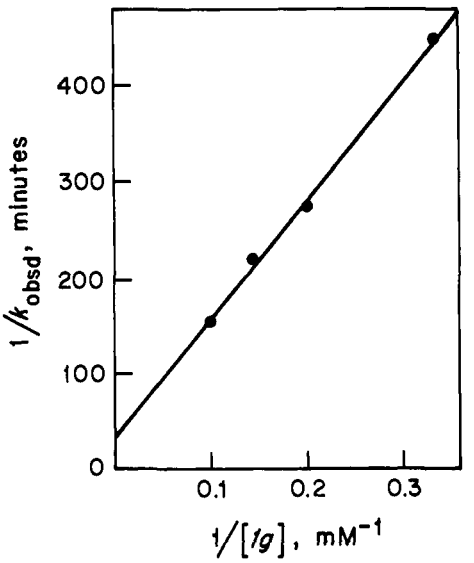


Figure 2. Inactivation of the liver isozyme of rat pyruvate kinase by **1g**; k_{obsd} is the apparent first-order rate constant for inactivation by a given level of **1g**.

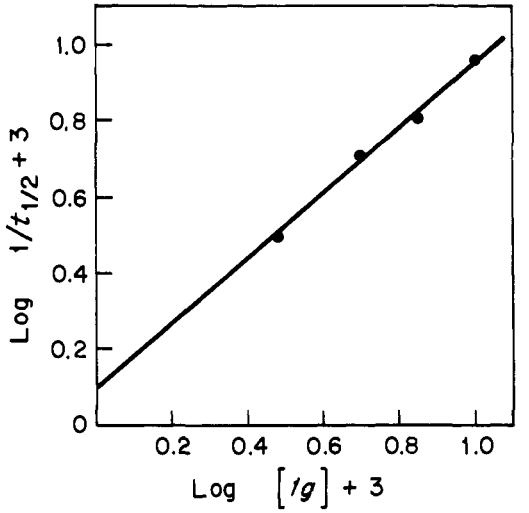


Figure 3. Determination of the number of molecules of **1g** required per catalytically active unit of the liver isozyme of rat pyruvate kinase to cause inactivation; [**1g**] is expressed as molarity, and the half-time of inactivation ($t_{1/2}$) is in minutes.

and the finding that the previously described[1] AMP and ATP analogues of **1g**, tested as their Mg salts at 10 and 5 mM levels, respectively, did not bring about measurable inactivation during the 8-h period, presumably because they bind more weakly than **1g** to the ADP site. In addition, kinetic analysis (Figure 1) indicated that inactivation by **1g** was pseudo first order and exhibited the rate saturation expected for a process in which reversible binding of **1g** precedes alkylation of the isozyme (eq 1, E

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E\text{–}I \text{ (inactive)} \qquad (1)$$

= enzyme, I = inhibitor). The half-time ($t_{1/2}$) of the L isozyme inactivation approached an extrapolated value of 23 min as the **1g** level approached infinity, and the apparent enzyme–inhibitor dissociation constant, K ($=k_{-1} + k_2/k_{-1}$), was 36 mM by the kinetic analysis described previously[1] and illustrated in Figure 2. A plot of log (**1g** concentration) vs. log (reciprocal $t_{1/2}$ of inactivation) (Figure 3) had a slope of 0.86 to indicate that inactivation requires no more than one molecule of inhibitor per catalytically active unit of isozyme according to a kinetic approach utilized in several analogous instances of enzyme inactivation.[18]

Table II. Properties of Compounds of Structure 1

compd	yield, %	UV λ_{max}[a] (H_2O), nm ($\epsilon \times 10^{-3}$)	electrophoresis, pH 3.5, rel mobility[b]	R_f[c]	phosphate/base ratio
1a	82	268 (17.2)	1.70	0.53	1.91
1b	80	268 (17.5)	1.67	0.55	1.95
1c	85	268 (17.2)	1.63	0.58	1.95
1d	76	268 (17.6)	1.62	0.60	2.06
1e	86	268 (17.3)	1.60	0.65	1.95
1f	80	268 (17.2)	1.59	0.68	1.98
1g	85	268 (17.3)	1.58	0.71	1.95

[a] Calculated for anhydrous trisodium salts. [b] AMP = 1. [c] Solvent system: 1-butanol–acetic acid–water (5:2:3).

Paper electrophoretic analysis at pH 3.5 gave no evidence that any of the three isozyme preparations converted **1g** to other compounds under the conditions of the inactivation studies. However, in order to eliminate the possibility that the isozyme-specific effect could have been caused either by activation of **1g** by the L isozyme preparation or by deactivation of **1g** by the M preparation, the L isozyme was treated with **1g** (7 mM) for 2 h at 22 °C under the usual conditions, whereafter an approximately equal amount of M isozyme activity was added. In addition, the converse experiment was conducted in which the M isozyme was first exposed to **1g** (7 mM), followed by addition of the L isozyme. Under the first set of conditions the M isozyme activity did not decrease during an additional 3.5 h, and under the second set prior treatment of **1g** with the M isozyme preparation had no effect on the rate of inactivation of the L isozyme during a 4-h period of observation. Similar results were given by mixtures of the L and K isozymes.

The above findings are all consistent with the view that the inactivation of the L isozyme involves binding of the ADP moiety of **1g** to the ADP site of the isozyme, followed by alkylation of an amino acid residue by the iodoacetyl group. Compound **1g** can also bind to the ADP site of the M and K isozymes as revealed by its substrate properties (Table I), and the specificity of action would therefore appear to be associated with the nature of the interaction of the N^6 substituent with the isozymes: the amino acid which becomes alkylated in the L isozyme is presumably either absent from the M and K isozymes or is less reactive or less accessible due to steric and/or electronic factors.

Experimental Section

Chemical Synthesis. General. The procedures for paper chromatography and paper electrophoresis, for determination of ultraviolet spectra, and for phosphate analysis of nucleotides have been described.[1] Eluates from ion-exchange chromatographic columns were monitored continuously at 254 nm in a 3-mm path-length cell with a Pharmacia Model 150 photometer. All the nucleotides prepared were eluted as single, smooth peaks.

General Preparation of N^6-Iodoacetamidoalkyladenosine 5′-Diphosphates 1. To a solution of each ATP derivative corresponding to 1 (0.015 mmol) in 1 mL of 0.05 M Tris–HCl (pH 7.6) containing 0.2 M glucose were added 0.067 mL of 0.1 M $MgCl_2$ and 5 μL of Boehringer yeast hexokinase (10 mg/mL of suspension). The solution was stored in the dark for 2 h at 22 °C and then diluted to 10 mL with H_2O. Compound 1 was purified by chromatography on a DEAE-cellulose (HCO_3^-) column (1.5 × 10 cm) using a linear gradient of aqueous triethylammonium bicarbonate (0–0.25 M, 1 L). The fractions containing the diphosphate were combined and converted to the corresponding trisodium salt by previously described methods.[1] All compounds were chromatographically and electrophoretically homogeneous. See Table II for properties. The UV maximum (268 nm) in H_2O rules out 1,N^6- and N^6,7-dialkyladenosine 5′-diphosphate

structures for **1** (arising from self-alkylation) because 1,N^6- and N^6,7-dialkyladenosines have maxima at 261 and 277 nm, respectively.[19] Moreover, these dialkyladenosines exist as free bases at pH 8,[20] thus indicating that the iodine of **1** must be attached covalently and not be present as iodide ion, because the present series of compounds were all isolated at pH 8 following anion-exchange chromatography. Anal. ($C_{20}H_{30}IN_6P_2O_{13}Na_3$, **1g**) C, H, N, P, I.

N^6-(8-Acetamido-*n*-octyl)adenosine 5′-Diphosphate (2c). N^6-(8-Amino-*n*-octyl)adenosine 5′-phosphate[1] (200 mg, 0.42 mmol) was added to a stirred solution of *N*-acetoxysuccinimide (250 mg, 1.6 mmol) and sodium bicarbonate (53 mg, 0.63 mmol) in 2-methoxyethanol–water (5:3, 80 mL). The solution was kept at 22 °C for 18 h and then evaporated. The residue was purified by chromatography on a DEAE-cellulose (HCO_3^-) column (2.5 × 16 cm) using a linear gradient of triethylammonium bicarbonate (0–0.2 M, 2 L). Compound **2b** was obtained as a white solid (90% yield) which was homogeneous on paper electrophoretograms run at pH 3.6 and 7.6. To compound **2b** (0.3 mmol) in dry DMF (10 mL) was added 1,1′-carbonyldiimidazole (250 mg, 1.5 mmol). The solution was stirred for 1 h at 22 °C when paper electrophoresis (pH 7.6) showed total conversion to a monoanionic species (mobility 0.3 vs. 0.7 for **2b**). Methanol (100 μL) was added, and after 0.5 h at 22 °C tributylammonium phosphate (1 mmol) was added and the mixture stirred for 18 h. The mixture was treated with an equal volume of methanol and evaporated. The residue was purified by chromatography on a DEAE-cellulose (HCO_3^-) column (2.5 × 19 cm) using a linear gradient of triethylammonium bicarbonate (0–0.4 M, 2 L); 15-mL fractions were collected at a flow rate of 180 mL/h. Fractions 44–70 were combined and evaporated to give triethylammonium **2c** (89% yield determined at 267 nm). After treatment with 0.5% aqueous Et_3N,[15] it was converted to its sodium salt by a standard procedure[1] to give 105 mg (50%) of **2c** as a white powder: UV λ_{max} 267 nm (ε 17 300). Periodate titration[21] revealed a *cis*-glycol content of 95% of the theoretical amount. Anal. ($C_{20}H_{31}N_6P_2O_{11}Na_3 \cdot C_2H_5OH$) C, H, N, P.

Enzyme Kinetic Studies. Lactate dehydrogenase (type 1, rabbit muscle) was from Sigma Chemical Co. Pyruvate kinase catalyzed 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 in every case linear over at least 10 min and were proportional to the concentration of pyruvate kinase and independent of the concentration of lactate dehydrogenase.

Following the procedures of Ibsen and Trippet,[16,17] the M, K, and L pyruvate kinase isozymes were extracted from muscle, kidney, and liver, respectively, of a male Sprague–Dawley rat and partially purified by electrofocusing with LKB Models 2117-501 (M and K forms) and 8100-1 (L form) using ampholine mixtures of pH ranges 6–8 (for the M isozyme) and 5–8 (K and L isozymes). The K isozyme was electrofocused in the presence of 20% glycerol to stabilize its activity. The observed isoelectric points (pH 7.2, 6.6, and 5.9) agreed within experimental error with the reported[17] values (7.5, 6.8, and 5.7, respectively). A Corning Model 12 pH meter and a Thomas No. 4094-L15 combination glass electrode were used. The isozyme preparations were dialyzed as described[17] and stored at 4 °C; their activity remained constant for the duration of the investigation. For studies of substrate properties, each pyruvate kinase was employed in 1 mL of 0.05 M triethanolamine-HCl (pH 7.0) containing $MgCl_2$ (5 mM), KCl (67 mM), phosphoenolpyruvate cyclohexylammonium salt (0.56 mM), reduced nicotinamide adenine dinucleotide (0.4 mM), fructose 1,6-diphosphate (10 μM), ADP or ADP derivatives (0.21 mM), and lactate dehydrogenase (10 μg). Components of the assay were stored at 22 °C for 5 min, after which time the reaction was initiated by addition of ADP or an ADP derivative. The level of ADP for half-maximal velocity was 0.38 mM for the L isozyme, 0.50 mM for the M isozyme, and 0.57 mM for the K isozyme.

Studies of the rate of inactivation of L and M pyruvate kinase were carried out in solutions which lacked ADP and PEP and included the usual levels of the other assay components, except for the pyruvate kinases which were present either at ten- (for the M isozyme) or twofold (L and K isozymes) the final assay level. The solubility of Mg–**1g** limited the level of **1g** in inactivation studies to 10 mM. Stock solutions of **1a–g** used for inactivation studies contained $MgCl_2$ in equimolar amounts to **1a–g**. At various time intervals aliquots (10 μL for the M isozyme, 20 μL for the L and K isozymes) were assayed under the above conditions except that 0.373 mM ADP was present. Mixtures lacking the ADP analogues were utilized as controls. Inactivation mixtures and their controls were maintained and assayed at 22 °C. In most experiments, the activity of the controls increased 5–20% in the first hour and then remained constant; the L and K isozymes lost no activity during a total of 8 h, and the M isozyme lost none during 24 h.

Acknowledgment. The authors wish to thank Dr. Sidney Weinhouse for helpful discussions. 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 (U.S. Public Health Service Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

References and Notes

(1) For paper 1, see A. Hampton, L. A. Slotin, and R. R. Chawla, *J. Med. Chem.*, **19**, 1279 (1976).
(2) W. E. Criss, *Cancer Res.*, **31**, 1523 (1971).
(3) W. E. Knox, "Enzyme Patterns in Fetal, Adult, and Neoplastic Rat Tissues", Karger, Basel, Switzerland, 1972.
(4) F. Schapira, *Adv. Cancer Res.*, **18**, 77 (1973).
(5) W. E. Knox, *Cancer Res.*, **34**, 2102 (1974).
(6) S. Sato and T. Sugimura, *Methods Cancer Res.*, **12**, 259 (1976).
(7) S. Weinhouse, J. B. Shatton, and H. P. Morris in "Cancer Enzymology, Miami Winter Symposia", Vol. 12, J. Schultz and F. Ahmad, Ed., Academic Press, New York, N.Y., 1976, pp 41–61.
(8) B. R. Baker, *Biochem. Pharmacol.*, 11, 1155 (1962).
(9) B. R. Baker and R. P. Patel, *J. Pharm. Sci.*, **53**, 714 (1964).
(10) B. R. Baker and N. M. J. Vermeulen, *J. Med. Chem.*, **13**, 1154 (1970).
(11) A. Hampton and L. A. Slotin, *Biochemistry*, **14**, 5438 (1975), and references cited therein.
(12) A. Hampton, F. Kappler, M. Maeda, and A. Patel, 175th National Meeting of the American Chemical Society, Anaheim, Calif., March 1978, MEDI 67.
(13) A. Hampton, M. Maeda, and A. Patel, unpublished results.
(14) D. E. Hoard and D. G. Ott, *J. Am. Chem. Soc.*, **87**, 1785 (1965).
(15) M. Maeda, A. D. Patel, and A. Hampton, *Nucleic Acids Res.*, **4**, 2843 (1977).
(16) K. H. Ibsen and P. Trippet, *Biochemistry*, 11, 4442 (1972).
(17) K. H. Ibsen and P. Trippet, *Arch. Biochem. Biophys.*, **156**, 730 (1973).
(18) P. F. Hollenberg, M. Flashner, and M. J. Coon, *J. Biol. Chem.*, **246**, 946 (1971).
(19) B. Singer, L. Sun, and H. Fraenkel-Conrat, *Biochemistry*, **13**, 1913 (1974).
(20) J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **85**, 193 (1963).
(21) D. H. Rammler and J. C. Rabinowitz, *Anal. Biochem.*, **4**, 116 (1962).