Inhibition of Adenylate Kinase by P^1-(*lin*-Benzo-5′-adenosyl)-P^4-(5′-adenosyl) Tetraphosphate and P^1-(*lin*-Benzo-5′-adenosyl)-P^5-(5′-adenosyl) Pentaphosphate†

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ABSTRACT: P^1-(*lin*-Benzo-5′-adenosyl)-P^5-(5′-adenosyl) pentaphosphate and P^1-(*lin*-benzo-5′-adenosyl)-P^4-(5′-adenosyl) tetraphosphate have been synthesized from *lin*-benzoadenosine 5′-monophosphoromorpholidate and adenosine 5′-tetraphosphate and adenosine 5′-triphosphate. These mixed dinucleoside polyphosphates are potent inhibitors of porcine muscle adenylate kinase, with association constants of 2×10^5 M^{-1} for the pentaphosphate and 2×10^6 M^{-1} for the tetraphosphate, respectively, as determined by kinetics and fluorescence experiments. The increase in fluorescence intensities and fluorescence lifetimes of both inhibitors upon binding to adenylate kinase results from a breaking of the intramolecular stacking interaction observed when these ligands are free in solution and implicates their binding to the enzyme in an "open" or "extended" form. These results and the dimensional requirements of these inhibitors are discussed in relation to our current knowledge of the active site of adenylate kinase and to the known inhibitors of adenylate kinase, P^1,P^5-bis(5′-adenosyl) pentaphosphate and P^1,P^4-bis-(5′-adenosyl) tetraphosphate.

Adenylate kinase, the widely occurring enzyme that catalyzes the reaction $ATP{\cdot}Mg^{2+} + AMP \rightleftarrows ADP{\cdot}Mg^{2+} + ADP$, is important in maintaining equilibrium among the several species in the adenine nucleotide pool (Noda, 1973). The spatial relationship between the two substrates when bound to adenylate kinase has been examined by using A(5′)p_5(5′)A[1] and A(5′)p_4(5′)A [Anderson et al. (1979), Feldhaus et al. (1975), Lienhard & Secemski (1973), and Purich & Fromm (1972); for synthesis, Reiss & Moffatt (1965)]. The 1000-fold greater inhibition of A(5′)p_5(5′)A compared with that of A(5′)p_4(5′)A suggests that the additional phosphate of the former which lengthens the phosphate chain by about 2.7 Å [by using sodium triphosphate, $Na_5P_3O_{10}$, as a model (Corbridge, 1960; Davies & Corbridge, 1958)] is crucial for strong inhibition.

We have previously examined the spectroscopic properties of *lin*-benzoadenine nucleotides and their interaction with a wide variety of enzymes [Barrio et al. (1979), and references 1–9 cited therein]. In the present investigation, we have synthesized *lin*-benzo-A(5′)p_5(5′)A and *lin*-benzo-A(5′)p_4(5′)A and have examined their interaction with adenylate kinase to determine the effect of stretching one of the terminal adenines by 2.4 Å.

Materials and Methods

Ultraviolet absorption spectra were obtained on a Beckman Acta MVI spectrophotometer. Fluorescence spectra were obtained on a Spex Fluorolog spectrofluorometer. Fluorescence lifetimes were determined on a cross-correlation fluorometer (Spencer & Weber, 1969) interfaced to a Monroe programmable calculator. ^{31}P NMR were recorded on a Varian Associates XL-100-15-NMR system equipped with a Digital NMR-3-data system, operating at 40.5 MHz for ^{31}P and 100 MHz for ^1H. Broad-band proton decoupling centered at about δ 4.0 was used in some cases. Deuterium from the D_2O solvent was used for field-frequency stabilization. Phosphoric acid (85%) was used as the primary ^{31}P reference. Calf intestine alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) and snake venom phosphodiesterase (orthophosphoric-diester phosphohydrolase, EC 3.1.4.1) were obtained from Boehringer Mannheim. Rabbit muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) and rabbit muscle lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) were products of Sigma Chemical Co.

Synthesis of lin-Benzo-A(5′)p_5(5′)A and lin-Benzo-A-(5′)p_4(5′)A. Adenosine 5′-tetraphosphate (Na^+ salt, 0.2 mmol) was converted to its pyridinium salt by passage through a Dowex 50 W-X8 column (pyridinium form). Fractions were evaporated at 10–15 °C, and tri-*n*-octylamine (0.6 mmol) in methanol was added to the residue. Solvent was removed and the solid was dried by repeated evaporation with dry DMF. To the residue was added a solution of *lin*-benzoadenosine 5′-phosphoromorpholidate, prepared from *lin*-benzo-AMP (19.8 mg, 0.05 mmol) by using the general method of Moffatt & Khorana (1961), in dry DMF (3 mL), and the mixture was stirred at room temperature for approximately 2 weeks. The DMF was removed in vacuo, and the residue was treated with 50 units of alkaline phosphatase (0.25 M glycine, pH 9.5), which hydrolyzed all terminal phosphates. The solution was then applied to a DEAE-cellulose column (40 × 2.5 cm) which was eluted at 4 °C with a linear gradient of triethylammonium bicarbonate (pH 7.5; 0.05–0.5 M; 2 × 1250 mL) to provide *lin*-benzo-A(5′)p_5(5′)A (eluted at 1.8–1.9 L of gradient; 14%

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[1] Abbreviations used: A(5′)p_n(5′)A, P^1,P^n-bis(5′-adenosyl) *n*-phosphate (IUPAC–IUB Commission on Biochemical Nomenclature, 1977). The prefix lin refers to the linear disposition of the three rings in the "stretched-out" (by 2.4 Å) version of the adenine nucleus; "benzo" in the trivial name refers to the additional ring which, only when central, contains no nitrogen. This terminology is adaptable to derivatives similarly related to adenosine (*lin*-benzoadenosine), adenylic acid (*lin*-benzo-AMP), adenosine diphosphate (*lin*-benzo-ADP), adenosine triphosphate (*lin*-benzo-ATP), etc. The chemical name for *lin*-benzoadenosine is 8-amino-3-(β-D-ribofuranosyl)imidazo[4,5-*g*]quinazoline. *lin*-Benzo-A-(5′)p_n(5′)A, P^1-(*lin*-benzo-5′-adenosyl)-P^n-(5′-adenosyl) *n*-phosphate, is identical with A(5′)p_n(5′)A except that a *lin*-benzoadenosine has replaced one adenosine. Only 5′,5′-linked polyphosphates are examined in this paper. Other abbreviations used: DMF, dimethylformamide; TLC, thin-layer chromatography.

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yield) and *lin*-benzo-A(5′)p_4(5′)A (eluted at 1.5–1.6 L of gradient; 8% yield) among other products.

Characterization of Dinucleoside Polyphosphates. Samples for ^{31}P NMR, *lin*-benzo-A(5′)p_5(5′)A, *lin*-benzo-A(5′)p_4(5′)A, A(5′)p_5(5′)A (Boehringer Mannheim), and A(5′)p_4(5′)A (Sigma), were treated with 40 units of alkaline phosphatase and chromatographed on a DEAE-cellulose column using a linear gradient of 0.05–0.5 M triethylammonium bicarbonate. ^{31}P NMR spectra were obtained with samples containing the appropriate dinucleoside polyphosphate (13–20 mM) and EDTA (2 mM) in D_2O. The pH was adjusted to 10–10.5 by the addition of tetramethylammonium hydroxide. Both compounds were characterized by the ratio of UV absorbances corresponding to the two heteroaromatic components.

Aliquots of *lin*-benzo-A(5′)p_5(5′)A and *lin*-benzo-A(5′)p_4(5′)A (0.03–0.1 μmol) were also characterized by treatment with 0.3 unit of alkaline phosphatase alone and with 0.3 unit of snake venom phosphodiesterase and subsequent quantitation of the inorganic phosphate (Lowry & Lopez, 1946). The hypochromicities of *lin*-benzo-A(5′)p_5(5′)A and *lin*-benzo-A(5′)p_4(5′)A at 331 nm were determined by comparison of the absorbance spectra before and after hydrolysis by phosphodiesterase as 19 and 20%, respectively. By taking into account the known extinction coefficient of *lin*-benzo-AMP (Leonard et al., 1976), it was possible to assign extinction coefficients at 331 nm of 8800 for *lin*-benzo-Ap$_5$A and of 8600 for *lin*-benzo-Ap$_4$A.

Isolation of Porcine Adenylate Kinase (ATP:AMP Phosphotransferase, EC 2.7.4.3). Adenylate kinase from pork shoulder was purified by using the isolation procedure for carp adenylate kinase (Noda et al., 1975) except that the pH precipitation was done as described by Heil et al. (1974). Adenylate kinase was pure after passage through a Sephadex G-75 column. Purified adenylate kinase had a specific activity of 2000 units/mg at 30 °C and gave one band on gel electrophoresis in the presence (Weber & Osborn, 1969) and absence (Schirmer et al., 1970) of NaDodSO$_4$.

Inhibition of Adenylate Kinase by Dinucleoside Polyphosphates. Inhibition experiments in which competition was determined vs. ATP were performed at 30 °C; the reaction mixture contained 100 mM Tris-HCl (pH 8.5), 75 mM KCl, 10 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM PEP, 1 mM AMP, 0.2 mM NADH, inhibitor (as applicable), ATP (0.2–1.0 mM), 20 units of pyruvate kinase, 50 units of lactate dehydrogenase, and 0.08 unit of adenylate kinase. None of the effects discussed in this paper resulted from inhibition of pyruvate kinase or lactate dehydrogenase because doubling the concentrations of these two enzymes had no effect on the reaction rate. The reaction was initiated by the addition of adenylate kinase and was monitored at 340 nm. Inhibition experiments in which competition was determined vs. AMP were identical except that ATP concentration was 1 mM and AMP concentration was varied from 0.2 to 1.0 mM. Inhibition constants were determined by using 1.9×10^{-6} M *lin*-benzo-A(5′)p_4(5′)A, 8.7×10^{-6} M *lin*-benzo-A(5′)p_5(5′)A, 4×10^{-5} M A(5′)p_4(5′)A, or 5×10^{-8} M A(5′)p_5(5′)A. Inhibition constants were calculated (Dixon & Webb, 1964) by employing K_M values of AMP and ATP of 0.17 mM and 0.30 mM, respectively, which were determined by using the same assay conditions as described above except that no inhibitor was present.

Binding of lin-Benzo-A(5′)p_5(5′)A and lin-Benzo-A(5′)p_4A to Adenylate Kinase As Monitored by Fluorescence Spectroscopy. Adenylate kinase, stored as an $(NH_4)_2SO_4$ precipitate, was collected by centrifugation, dissolved in 10 mM potassium succinate and 1 mM dithiothreitol (pH 6.7), and dialyzed for 3 h at 5 °C against this buffer. Conductivity measurements showed that after dialysis the solution was approximately 10 mM $(NH_4)_2SO_4$. The adenylate kinase was quantitated at 277 nm by using $A^{0.1\%} = 0.538$ (Schirmer et al., 1970). At concentrations of 0.5 mg/mL adenylate kinase, only small losses (10% or less) in specific activity were observed with this procedure. The enzyme was then diluted into the buffer used for the binding experiments: 100 mM Tris (pH 8.5), 75 mM KCl, 10 mM dithiothreitol, and 5 mM $MgCl_2$. All experiments were performed at 5 °C, and the wavelength of UV excitation was 330 nm. In order to maximize the fluorescence signal, some measurements were made with a 20-nm excitation band-pass and with the fluorescence emission at 390 nm monitored by using a 40-nm emission band-pass and a Corning Glass 0-52 emission filter.

The data were analyzed according to the Scatchard equation (Scatchard, 1949):

$$\frac{\bar{\nu}}{[\textit{lin}\text{-benzo-A(5′)p}_n\text{(5′)A}]} = K - K\bar{\nu}$$

in which K is the association constant, [*lin*-benzo-A(5′)p_n(5′)A] is the free ligand concentration, and $\bar{\nu}$ is the concentration of bound adenylate kinase divided by the total concentration of adenylate kinase. The value of $\bar{\nu}$ is calculated from the experimental data by using

$$\bar{\nu} = \frac{F - F_f}{F_b - F_f}$$

where F_f is the fluorescence intensity of a solution containing only *lin*-benzo-A(5′)p_n(5′)A, F_b is the fluorescence intensity of a solution in which *lin*-benzo-A(5′)p_n(5′)A is totally bound to adenylate kinase, and F is the fluorescence intensity of a solution containing both *lin*-benzo-A(5′)p_n(5′)A and adenylate kinase (Pesce et al., 1971). All calculations were aided by the use of a Monroe programmable calculator.

Fluorescence Quantum Yield Determinations. Fluorescence quantum yields of *lin*-benzo-A(5′)p_5(5′)A and *lin*-benzo-A(5′)p_4(5′)A both bound to adenylate kinase and free in solution were determined relative to that of *lin*-benzo-AMP under experimental conditions identical with those used to determine the binding constant. Quantum yields of *lin*-benzo-A(5′)p_n(5′)A completely bound to adenylate kinase were determined by adding sufficient ligand to bind fully a known amount of adenylate kinase and subtracting out the fluorescence of unbound fluorophore. Alternatively, this same quantum yield could be obtained by plotting the reciprocal of the fluorescence intensity of a solution containing adenylate kinase and *lin*-benzo-A(5′)p_n(5′)A vs. the reciprocal of the concentration of adenylate kinase and extrapolating to infinite protein concentration (Pesce et al., 1971). Both methods gave identical results.

Results and Discussion

Synthesis and Characterization of lin-Benzo-A(5′)p_n(5′)A. We have synthesized *lin*-benzo-A(5′)p_5(5′)A and *lin*-benzo-A(5′)p_4(5′)A by reaction of *lin*-benzoadenosine 5′-monophosphoromorpholidate with adenosine tetraphosphate (tri-*n*-octylammonium salt) (Reiss & Moffatt, 1965; Moffatt & Khorana, 1961). Degradation of adenosine tetraphosphate during the course of the reaction results in the formation of *lin*-benzo-A(5′)p_4(5′)A (8% yield) as well as *lin*-benzo-A(5′)p_5(5′)A (14% yield). Under identical conditions, the substitution of ATP for adenosine tetraphosphate results in the formation of *lin*-benzo-A(5′)p_4(5′)A in higher yield, ca. 20%. The formation of P^1,P^2-bis(5′-*lin*-benzoadenosyl) di-

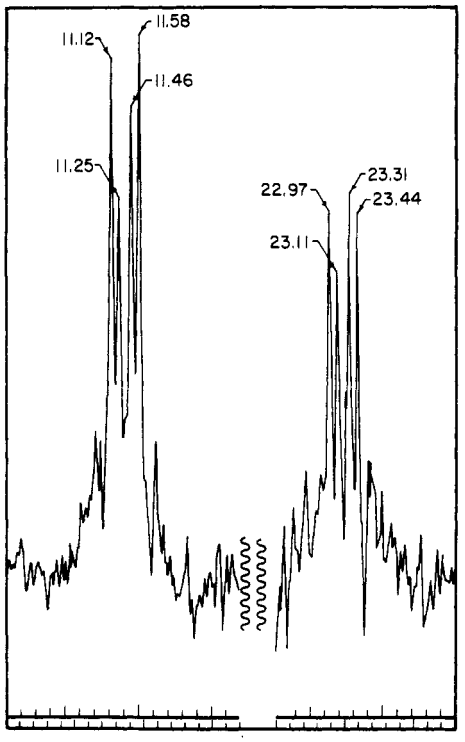


FIGURE 1: ^{31}P NMR spectrum of A(5′)p$_4$(5′)A in D_2O, proton decoupled as described under Materials and Methods.

phosphate (Scopes et al., 1977; Leonard et al., 1978) in this reaction could be kept to a minimum under the described conditions. Before fractionation of the reaction mixture on a DEAE-cellulose column, all terminal phosphates were hydrolyzed by alkaline phosphatase. The elimination of these terminal phosphates simplified the column chromatography.

lin-Benzo-A(5′)p$_5$(5′)A and *lin*-benzo-A(5′)p$_4$(5′)A were identified on the basis of the following evidence. Both compounds contain identical amounts of adenine and *lin*-benzoadenine since the absorbance ratio of 259 to 331 nm observed on hydrolysis is that of an equimolar mixture of AMP and *lin*-benzo-AMP (Sober, 1970; Leonard et al., 1976). Total phosphate digestion of *lin*-benzo-A(5′)p$_5$(5′)A and *lin*-benzo-A(5′)p$_4$(5′)A by snake venom phosphodiesterase and alkaline phosphatase yielded ratios of phosphate to *lin*-benzoadenosine residue of 4.8 and 3.9, respectively. No terminal phosphates were present as judged both by ^{31}P NMR and by treatment with alkaline phosphatase and determination of inorganic phosphate.

The ^{31}P chemical shifts and coupling constants of the products that we have determined to be *lin*-benzo-A(5′)p$_4$(5′)A and *lin*-benzo-A(5′)p$_5$(5′)A (see Materials and Methods) are consistent with these identifications, employing A(5′)p$_4$(5′)A and A(5′)p$_5$(5′)A as model compounds. Previously, we have shown that the ^{31}P chemical shifts and coupling constants of *lin*-benzoadenine nucleotides are similar to those of adenine nucleotides (Barrio et al., 1979). The ^{31}P chemical shifts of A(5′)p$_4$(5′)A (Figure 1) are centered at −11.35 (α-phosphorus) and −23.2 ppm (β-phosphorus). Both signals are doublets of doublets: $^2J_{POP}$ is 14 Hz while the smaller J, 5 Hz, results from β coupling. The ^{31}P chemical shifts of α-phosphorus and β-phosphorous of A(5′)p$_5$(5′)A (Rao & Cohn, 1977), *lin*-benzo-A(5′)p$_4$(5′)A, and *lin*-benzo-A(5′)p$_5$(5′)A are centered in positions similar to the corresponding phosphates of A(5′)p$_4$(5′)A; the central phosphorus of the two p$_5$ compounds has a chemical shift similar to that of the β-phosphorus of these compounds. The signals of all four compounds are resolved to an identical extent, but those of A(5′)p$_4$(5′)A possess a simpler pattern. The β coupling observed here with A(5′)p$_4$(5′)A suggests that splittings previously attributed solely to the effect of adenylate kinase on the environment of A(5′)p$_5$(5′)A (Rao & Cohn, 1977) may result partially from β splitting.

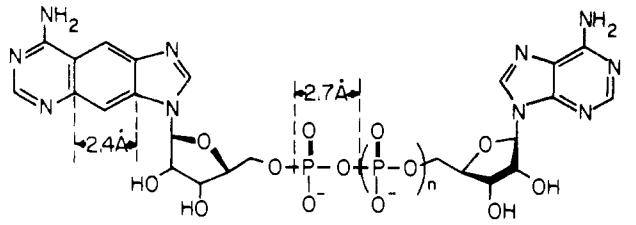
Table I: Association Constants of Dinucleoside Polyphosphates with Adenylate Kinase

compd	K (M^{-1})[a]
A(5′)p$_4$(5′)A[b]	8×10^4
A(5′)p$_5$(5′)A[b]	8×10^7
lin-benzo-A(5′)p$_4$(5′)A[b]	2.2×10^6
lin-benzo-A(5′)p$_4$(5′)A[c]	2.1×10^6
lin-benzo-A(5′)p$_4$(5′)A[d]	1.0×10^6
lin-benzo-A(5′)p$_5$(5′)A[b]	2.1×10^5
lin-benzo-A(5′)p$_5$(5′)A[c]	2.0×10^5
lin-benzo-A(5′)p$_5$(5′)A[d]	1.7×10^5

[a] Reciprocals of these association constants are the kinetic inhibition constants. [b] Determined by kinetic inhibition with varying ATP concentration. [c] Determined by kinetic inhibition with varying AMP concentration. [d] Determined by using fluorescence to monitor equilibrium binding to adenylate kinase.

Inhibition of Adenylate Kinase by Dinucleoside Polyphosphates. Compounds A(5′)p$_4$(5′)A and A(5′)p$_5$(5′)A are inhibitors of adenylate kinase (Table I), and we have confirmed that they are competitive with respect to ATP. The association constants which we have determined are within the range of the values previously reported for these compounds as competitive inhibitors of adenylate kinase with respect to both ATP and AMP (Feldhaus et al., 1975; Lienhard & Secemski, 1973; Purich & Fromm, 1972). It should be noted that adenylate kinase has a random bi bi mechanism [i.e., there is no order in which substrates must add or products must leave the enzyme (Purich & Fromm, 1972)], and the inhibition constant of any inhibitor competitive with either of the two substrates will be identical with a dissociation constant determined by means other than kinetics (Dixon & Webb, 1964). That binding of these inhibitors occurs at the active site and not at a noncatalytic substrate site is consistent with what is known about adenylate kinase, namely, that a noncatalytic site for substrate or substrate analogues has never been indicated (Noda, 1973).

We have measured the inhibition constants of *lin*-benzo-A(5′)p$_4$(5′)A and *lin*-benzo-A(5′)p$_5$(5′)A to adenylate kinase and have determined that they are competitive inhibitors with respect to ATP and AMP. It is apparent from our data (Table I) that although A(5′)p$_5$(5′)A is a better inhibitor (ca. 1000 times) than A(5′)p$_4$(5′)A, the reverse order holds for *lin*-benzo-A(5′)p$_4$(5′)A and *lin*-benzo-A(5′)p$_5$(5′)A, the former being a better inhibitor (ca. 10 times) than the latter. The finding that the *lin*-benzo-A(5′)p$_4$(5′)A inhibition constant is intermediate between the values for A(5′)p$_4$(5′)A and A(5′)p$_5$(5′)A suggests that the changes in inhibition constants, as a partial function of the distance between the two pyrimidine rings, are qualitatively similar whether the phosphate chain is lengthened (ca. 2.7 Å from p$_4$ to p$_5$) or one terminal base is widened (2.4 Å from A to *lin*-benzo-A) (Figure 2). Furthermore, the pyrimidine to pyrimidine distance in *lin*-benzo-A(5′)p$_4$(5′)A is longer than that of A(5′)p$_4$(5′)A only if the *lin*-benzoadenosine moiety is in the anti conformation as it is for the *lin*-benzoadenine nucleotides (Barrio et al., 1979). A tentative conclusion is thereby reached that this is the preferred conformation of the *lin*-benzoadenosine portion of *lin*-benzo-A(5′)p$_4$(5′)A bound to adenylate kinase. It has been demonstrated previously that AMP is bound to adenylate kinase in the anti conformation (Hampton et al., 1972). Since *lin*-benzo-ATP but not *lin*-benzo-AMP is a substrate (Leonard



n = 3 or 4

FIGURE 2: *lin*-Benzo-A(5′)p$_4$(5′)A and *lin*-benzo-A(5′)p$_5$(5′)A, showing both nucleosides in anti conformation.

Table II: Fluorescence Quantum Yields and Lifetimes of *lin*-Benzo-AMP, *lin*-Benzo-A(5′)p$_4$(5′)A, and *lin*-Benzo-A(5′)p$_5$(5′)A[a]

fluorophore	quantum yield, Φ	lifetime (ns)
lin-benzo-AMP	0.34[b]	3.5[b]
lin-benzo-A(5′)p$_4$(5′)A	0.04	1.5
lin-benzo-A(5′)p$_4$(5′)A bound to adenylate kinase	0.18	3.5
lin-benzo-A(5′)p$_5$(5′)A	0.05	1.5
lin-benzo-A(5′)p$_5$(5′)A bound to adenylate kinase	0.33	3.5

[a] See Materials and Methods for details. [b] Previously reported values have been in different buffers.

et al., 1978), it is reasonable to conclude that the ATP site of adenylate kinase accepts its substrates in the anti conformation. Although this is a satisfying self-consistent hypothesis, our data do not rule out the possibility of *lin*-benzo-AMP binding in a noncatalytic manner at the AMP site and, hence, of the *lin*-benzoadenosine portion of *lin*-benzo-A(5′)p$_4$(5′)A binding at the AMP site. The hypothesis will explain why *lin*-benzo-ATP is a poorer substrate for adenylate kinase than ATP. The pyrimidine rings of the adenine moieties of ATP and AMP when bound to adenylate kinase are separated by a distance which is ideal for catalysis, but when the two substrates are joined together as in A(5′)p$_4$(5′)A the distance is too short to form a potent inhibitor. *lin*-Benzo-A(5′)p$_4$(5′)A is a strong inhibitor of adenylate kinase, so that in the combination of *lin*-benzo-ATP with AMP bound to the enzyme the separation between the pyrimidine rings of the constituent bases is greater than optimal for phosphate transfer.

Fluorescence Properties of lin-Benzo-A(5′)p$_4$(5′)A and lin-Benzo-A(5′)p$_5$(5′)A in Solution and Bound to Adenylate Kinase. Under our experimental conditions, the fluorescence quantum yield and lifetime of *lin*-benzo-AMP are 0.34 and 3.5 ns, respectively. The substantially lower quantum yields and shorter lifetimes of *lin*-benzo-A(5′)p$_4$(5′)A and *lin*-benzo-A(5′)p$_5$(5′)A (Table II) result from the intramolecular stacking interaction between the *lin*-benzoadenine and adenine portions of these molecules in dilute solution. Similar results have been observed in related systems (Tolman et al., 1974; Barrio et al., 1972, 1973). The binding of *lin*-benzo-A(5′)p$_4$(5′)A and *lin*-benzo-A(5′)p$_5$(5′)A to adenylate kinase breaks this intramolecular stacking interaction and increases the quantum yield of the ligand. The differences in fluorescence intensity at 390 nm when excited at 330 nm have been used to monitor the binding of *lin*-benzo-A(5′)p$_4$(5′)A and of *lin*-benzo-A(5′)p$_5$(5′)A to adenylate kinase and to determine their association constants (Figure 3 and Table I). Treatment of either mixed dinucleoside polyphosphate in solution with phosphodiesterase brought the quantum yield up to that of

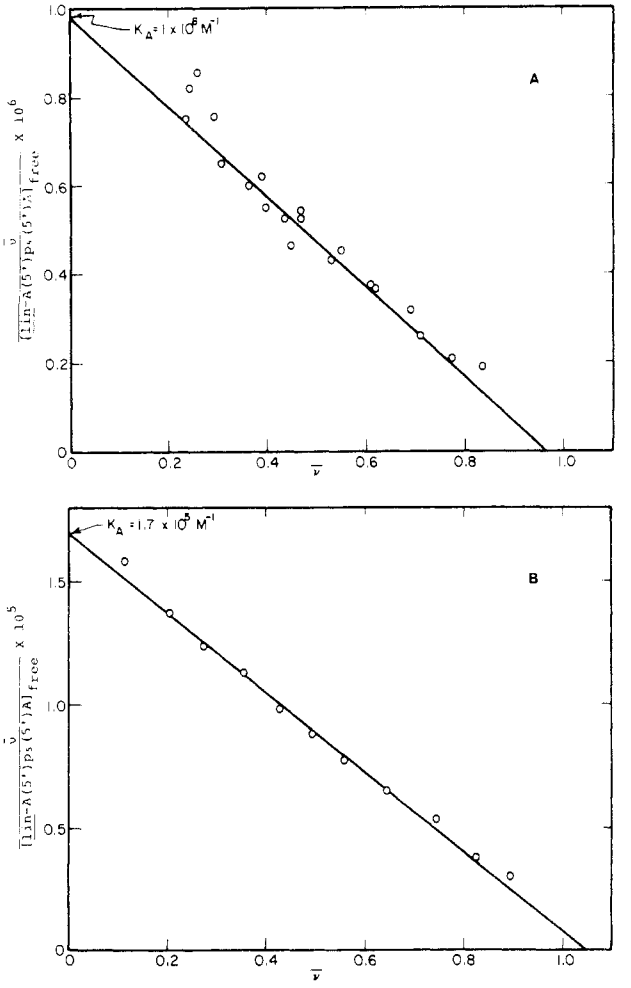


FIGURE 3: Scatchard plots of the binding of (A) *lin*-benzo-A(5′)p$_4$(5′)A and (B) *lin*-benzo-A(5′)p$_5$(5′)A to adenylate kinase. See text for details.

lin-benzoadenosine 5′-monophosphate. That *lin*-benzoadenosine 5′-monophosphate (Scopes et al., 1977) and adenosine 5′-monophosphate are the products of this reaction is indicated by a knowledge of the mechanism of action of snake venom phosphodiesterase (Razzell, 1963).

The quantum yield of *lin*-benzo-A(5′)p$_4$(5′)A bound to adenylate kinase is approximately half that of bound *lin*-benzo-A(5′)p$_5$(5′)A. There are at least two explanations for the difference in fluorescence intensities between these two compounds when bound to adenylate kinase. One suggests fluorescence dependence upon degree of hydrogen bonding from the amino group of the *lin*-benzoadenosine to a nucleophilic center at the ATP binding site as implicated in the case of aspartate transcarbamylase (VanDerLijn et al., 1978). No evidence supporting or refuting this explanation is found in other investigations of the ATP binding site of adenylate kinase. Previous investigations do support an alternative explanation. It is known that Tyr-95 is at the ATP binding site (Sachsenheimer & Schulz, 1977; Pai et al., 1977) and that the NMR chemical shift of the H-8 of ATP, upon binding to adenylate kinase, is shifted upfield, a change attributed to stacking interaction (McDonald et al., 1975). *lin*-Benzo-A(5′)p$_4$(5′)A may derive its stronger association to adenylate kinase, compared with that of *lin*-benzo-A(5′)p$_5$(5′)A, from mimicking more closely the interactions of ATP with the enzyme, hence leading to stronger stacking interaction with Tyr-95, resulting in partial quenching of the *lin*-benzoadenine fluorescence.

In conclusion, we have synthesized *lin*-benzo-A(5′)p_4(5′)A and *lin*-benzo-A(5′)p_5(5′)A and determined their inhibition and association constants with adenylate kinase. A comparison of the inhibition constants of these two compounds with those of A(5′)p_4(5′)A and A(5′)p_5(5′)A indicates that qualitatively similar results are obtained whether one lengthens the phosphate chain [A(5′)p_5(5′)A] or widens one terminal base [*lin*-benzo-A(5′)p_4(5′)A] (Figure 2). The increase in the quantum yields of *lin*-benzo-A(5′)p_4(5′)A and *lin*-benzo-A(5′)p_5(5′)A when bound to adenylate kinase results from breaking the intramolecular stacking interaction of these two compounds when free in solution. The different quantum yields of *lin*-benzo-A(5′)p_4(5′)A and *lin*-benzo-A(5′)p_5(5′)A when bound to adenylate kinase are indicative of different modes of association at the enzyme active site.

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References

Anderson, C. M., Zucker, F. H., & Steitz, T. A. (1979) *Science 204*, 375–380.

Barrio, J. R., Secrist, J. A., III, & Leonard, N. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2039–2042.

Barrio, J. R., Tolman, G. L., Leonard, N. J., Spencer, R. D., & Weber, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 941–943.

Barrio, J. R., Liu, F.-T., Keyser, G. E., VanDerLijn, P., & Leonard, N. J. (1979) *J. Am. Chem. Soc. 101*, 1564–1569.

Corbridge, D. E. C. (1960) *Acta Crystallogr. 13*, 263–269.

Davies, D. R., & Corbridge, D. E. C. (1958) *Acta Crystallogr. 11*, 315–319.

Dixon, M., & Webb, E. C. (1964) *Enzymes*, pp 313–335, Academic Press, New York.

Feldhaus, P., Fröhlich, T., Goody, R. S., Isakov, M., & Schirmer, R. H. (1975) *Eur. J. Biochem. 57*, 197–204.

Hampton, A., Harper, P. J., & Sasaki, T. (1972) *Biochemistry 11*, 4965–4969.

Heil, A., Müller, G., Noda, L., Pinder, T., Schirmer, H., Schirmer, I., & von Zabern, I. (1974) *Eur. J. Biochem. 43*, 131–144.

IUPAC–IUB Commission on Biochemical Nomenclature (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2222–2230.

Leonard, N. J., Sprecker, M. A., & Morrice, A. G. (1976) *J. Am. Chem. Soc. 98*, 3987–3994.

Leonard, N. J., Scopes, D. I. C., VanDerLijn, P., & Barrio, J. R. (1978) *Biochemistry 17*, 3677–3685.

Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121–1123.

Lowry, O. H., & Lopez, J. A. (1946) *J. Biol. Chem. 162*, 421–428.

McDonald, G. C., Cohn, M., & Noda, L. (1975) *J. Biol. Chem. 250*, 6947–6954.

Moffatt, J. G., & Khorana, H. G. (1961) *J. Am. Chem. Soc. 83*, 649–658.

Noda, L. (1973) *Enzymes, 3rd Ed. 8*, 279–305.

Noda, L., Schulz, G. E., & von Zabern, I. (1975) *Eur. J. Biochem. 51*, 229–235.

Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37–45.

Pesce, A. J., Rosén, C.-G., & Pasby, T. L. (1971) *Fluorescence Spectroscopy*, pp 203–240, Marcel Dekker, New York.

Purich, D. L., & Fromm, H. J. (1972) *Biochim. Biophys. Acta 276*, 563–567.

Rao, B. D. N., & Cohn, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5355–5357.

Razzell, W. E. (1963) *Methods Enzymol. 6*, 236–258.

Reiss, J. R., & Moffatt, J. G. (1965) *J. Org. Chem. 30*, 3381–3387.

Sachsenheimer, W., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 23–36.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Schirmer, I., Schirmer, R. H., Schulz, G. E., & Thuma, E. (1970) *FEBS Lett. 10*, 333–338.

Scopes, D. I. C., Barrio, J. R., & Leonard, N. J. (1977) *Science 195*, 296–298.

Sober, H. A. (1970) *Handbook of Biochemistry*, p G-60, CRC Press, Cleveland, OH.

Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361–376.

Tolman, G. L., Barrio, J. R., & Leonard, N. J. (1974) *Biochemistry 13*, 4869–4878.

VanDerLijn, P., Barrio, J. R., & Leonard, N. J. (1978) *J. Biol. Chem. 253*, 8694–8696.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.