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## Dimensional Probes of the Enzyme Binding Sites of Adenine Nucleotides. Biological Effects of Widening the Adenine Ring by 2.4 Å<sup>†</sup>

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ABSTRACT: *lin*-Benzoadenine nucleotides, defined by the formal insertion of a benzene ring (actually four carbons) into the center of the purine ring system, have been synthesized and their chemical integrity and purity analyzed by high performance liquid chromatography, paper electrophoresis, and <sup>31</sup>P nuclear magnetic resonance spectroscopy. With these dimensional probes, the size restrictions of enzyme-active sites specific for purine cofactors have been tested with respect to enzyme binding and activity. The "stretched-out" (by 2.4 Å) adenine nucleotide analogues bind strongly and have generally slower enzymatic rates with a representative group of kinases, comprising pyruvate kinase, adenylate kinase, phosphofruc-

A desirable antecedent to the goal of understanding the interaction between enzymes and their requisite substrates or cofactors is the definition, in detail, of the active binding sites on or within the enzymes. In this regard, the binding sites for adenine are especially important because of the number and variety of enzymes that operate on or require an adeninecontaining cofactor or coenzyme (Watts, 1965; Stadtman, 1970; Yount, 1975). Adenine binding sites have been characterized only for certain dehydrogenases (Adams et al., 1970; Rossmann et al., 1971, 1974, 1975; Buehner et al., 1973, 1974; Öhlsson et al., 1974; Abdallah et al., 1975; Eventoff & Rossmann, 1975; Brändén et al., 1975; Greenfield et al., 1975; Holbrook et al., 1975; Smith et al., 1975; Harris & Waters, 1976; Suhadolnik et al., 1977) and related kinases (Cohn et al., 1971; Noda, 1973; Blake & Evans, 1974; Schulz et al., 1974; Tanswell et al., 1974; McDonald et al., 1975; Noda et al., 1975). Otherwise, assembly of accurate information with respect to adenine binding sites is at a primitive stage. We have introduced the concept of testing the dimensional restrictions of enzyme-active sites by synthesizing cofactor analogues tokinase, phosphoglycerate kinase, hexokinase, and acetate kinase. *lin*-Benzo-ADP acted as a substrate for primer independent polynucleotide phosphorylase (*Micrococcus luteus*) in the presence of  $Mn^{2+}$ . The nucleotides have also shown useful fluorescence properties and sensitivity to environmental conditions, e.g., divalent metal ions, stacking, etc. The useful fluorescence properties of *lin*-benzoadenosine 5'-mono-, 5'-di-, 5'-tri-, and 3',5'-monophosphates and their increased  $\pi$  interactions can be directed to a variety of studies of static and dynamic interactions with different moieties, complexations, the nature of enzyme binding sites, and conformational changes induced by surrogate coenzyme/enzyme binding.

which are related to the natural cofactors by defined dimensional changes in the molecules (Leonard et al., 1976; Scopes et al., 1977).

While many analogues of adenine nucleotides have been made and tested for enzyme activation and inhibition, the  $lin^1$ -benzoadenine nucleotides (1-4 and 6) are unique in retaining the terminal pyrimidine and imidazole rings of adenine intact, as well as the attached ribosyl phosphate(s). We have found that stretched-out versions of adenine nucleotides, which are 2.4 Å wider than normal due to the formal insertion of a benzene ring between the terminal rings, exhibit strong enzyme binding, act as cofactors in enzyme reactions, and have spectroscopic properties, including useful fluorescence, that help define their environments. There was no sure way to predict such findings a priori. We have recognized that the heteroaromatic ring system in 1-4 and 6 differs electronically from adenine, which is reflected, for example, in basicity, nucleo-

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<sup>&</sup>lt;sup>‡</sup> Presented in the B. R. Baker Memorial Lecture, Department of Chemistry, University of California, Santa Barbara, November 1976. Exo bulk tolerance in enzyme-inhibitor complexes is a concept indirectly related that was introduced by Baker (1967).

<sup>&</sup>lt;sup>1</sup> Abbreviations used: 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 other parts of the names follow accepted IUPAC-IUB nomenclature. The chemical name for *lin*-benzoadenosine is 8-amino-3-( $\beta$ -D-ribofuranosyl)imidazo[4,5-g]quinazoline. Other abbreviations include: DMF, dimethylformamide; TLC, thin-layer chromatography.

philicity, and  $\pi$ -bonding characteristics, but the similarities in peripheral rings, in ring locus of protonation, and in relative position and conformation of ribosyl phosphate attachment embolden us to call the *lin*-benzoadenine nucleotides "dimensional probes". Their interactions with enzyme systems can tell us, inter alia, the effect of greater spacing between N7 and 6-NH<sub>2</sub>, N9 and N3. This work is part of a long-range goal, which is to provide enough purine ring analogues with known dimensional alterations that the limiting size of an enzyme binding "pocket", "slot", or surface for the substrate or cofactor can be accurately defined.

### Materials and Methods

Ultraviolet absorption spectra were obtained on a Beckman Acta M VI Spectrophotometer. Molecular fluorescence emission and excitation spectra were measured on a Spex Fluorolog spectrofluorometer. Fluorescence lifetimes were measured on a Model SLM-480 subnanosecond spectrofluorometer. Proton nuclear magnetic resonance spectra were recorded on a Varian Associates HA-220 spectrometer. <sup>31</sup>P nuclear magnetic resonance spectra were obtained on a Varian Associates XL-100-15-NMR system equipped with a Digital NMR-3 data system, operating at 40.5 MHz for <sup>31</sup>P and 100 MHz for <sup>1</sup>H. Broadband proton decoupling centered at about  $\alpha$  4.0 was used in all cases. Deuterium from the D<sub>2</sub>O solvent was used for field frequency stabilization. Phosphoric acid (85%) in a concentric capillary (2-mm o.d.) was used as primary <sup>31</sup>P reference. All spectra were obtained using 16K data points and a 2500-Hz bandwidth. Microanalyses were performed by Mr. J. Nemeth and his staff, who also weighed samples for quantitative ultraviolet absorption studies. The identity and purity of the new lin-benzoadenine nucleotides were established by <sup>31</sup>P NMR spectroscopy, high performance liquid chromatography, and electrophoresis. Hypochromism values were calculated as previously described (Browne et al., 1968; Leonard & Ito, 1974). Oscillator strengths were calculated using a program based on Simpson's rule.

Chemistry. lin-Benzoadenosine (1) was prepared by the method of Leonard et al. (1976) from 8methylthioimidazo[4,5-g]quinazoline ( $pK_a$  4.1 in 66% DMF), except that the deblocking of the sugar group after ribosidation was better carried out with ethanolic ammonia at room temperature for 24 h. Displacement of the methylthio group of the intermediate 8-methylthio-3-( $\beta$ -D-ribofuranosyl)-imidazo[4,5-g]quinazoline by an amino group to afford *lin*benzoadenosine was best accomplished at 150 °C for 24 h. The total yield after these two steps was nearly quantitative.

lin-Benzoadenosine 5'-Monophosphate (2). lin-Benzoadenosine (1) was converted to its 5'-monophosphate derivative essentially by the procedure described by Imai et al. (1969). Pyrophosphoryl chloride (0.45 mL, 3.2 mmol) was added dropwise to a cooled, stirred suspension (0-5 °C) of lin-benzoadenosine (200 mg, 0.64 mmol) in *m*-cresol (7.5 mL). After 4 h at 0-5 °C a clear yellow solution was obtained which was then poured onto 200 mL of ice water. After extraction with ether (4 × 50 mL) the aqueous acidic solution was concentrated to approximately half volume and allowed to stand at 5 °C overnight. The precipitated *lin*-benzoadenosine 5'monophosphate was collected by filtration: 192 mg (75%). An analytical sample was obtained by recrystallization from water;  $\lambda_{max}^{pH:8.5}$  260 nm ( $\epsilon$  18 500), 317 (8500), 331 (10 400), 346 (7800).

Anal. Calcd for C<sub>14</sub>H<sub>16</sub>N<sub>5</sub>O<sub>7</sub>P: C, 42.33; H, 4.06; N, 17.63. Found: C, 42.37; H, 4.03; N, 17.46.

A 15 mM solution of lin-benzoadenosine 5'-monophosphate

in 150 mM Tris-acetate buffer (pH 8.6) containing 40 mM  $MgCl_2$  was treated with 5'-nucleotidase (Sigma). The reaction was followed by TLC and by electrophoresis. Complete conversion of the nucleotide to *lin*-benzoadenosine was observed.

lin-Benzoadenosine 5'-Diphosphate (3). A mixture of the lin-benzoadenosine 5'-phosphoromorpholidate, prepared from lin-benzoadenosine 5'-monophosphate (2; 93 mg, 0.23 mmol) using the general method of Moffatt & Khorana (1961), and tri-n-butylammonium phosphate (325 mg, 1.15 mmol; dried by repeated evaporations with anhydrous pyridine) in dry dimethylformamide (9 mL) was stirred at room temperature for 2 days (lin-benzoadenosine 5'-phosphoromorpholidate is insoluble in pyridine). The dimethylformamide was removed in vacuo, and the residue was taken up in triethylammonium bicarbonate buffer and applied to a column of DEAE-cellulose  $(4.5 \times 45 \text{ cm})$  (bicarbonate form). The column was eluted with a linear gradient of 3 L of 0.05 M  $\rightarrow$  3 L of 0.3 M triethvlammonium bicarbonate buffer, pH 8.0. Appropriate fractions were pooled and evaporated in vacuo (repeated evaporation with methanol/water to remove last traces of buffer) to provide lin-benzoadenosine 5'-diphosphate which was converted to its lithium salt by passage through a Dowex 50W-X8 column (Li form): 76 mg (66% from lin-benzoadenosine 5'-monophosphate).

Chemical Synthesis of lin-Benzoadenosine 5'-Triphosphate (4). A solution of 4-morpholine-N,N'-dicyclohexylcarboxamidinium lin-benzoadenosine 5'-phosphoromorpholidate (35 mg, 0.05 mmol) and bis(tri-n-butylammonium) pyrophosphate (110 mg, 0.20 mmol) in dry dimethylformamide (3 mL) was stirred at room temperature for 16 h. The dimethylformamide was removed in vacuo and the residue taken up in triethylammonium bicarbonate buffer and applied to a column of DEAE-cellulose (bicarbonate form). Elution with a linear gradient, 0.1 M  $\rightarrow$  0.4 M, of triethylammonium bicarbonate buffer (pH 8.0) provided lin-benzoadenosine 5'-triphosphate, which was converted to its lithium salt as described above: 10 mg (35%).

Enzymatic Synthesis of lin-Benzoadenosine 5'-Triphosphate (4) from lin-Benzoadenosine 5'-Diphosphate (3). This conversion was performed in a medium containing 125 mM Tris-HCl buffer (pH 7.5), 125 mM KCl, 20 mM MgCl<sub>2</sub>, 2.5 mM phosphoenolpyruvate, 1 mM lin-benzoadenosine 5'-diphosphate (3), and pyruvate kinase. A typical conversion utilized 25 mg of the diphosphate. After the conversion was complete, in less than 40 min, the total reaction mixture was applied to a column of DEAE-cellulose ( $4.5 \times 45$  cm, bicarbonate form). Elution by linear gradient of triethylammonium bicarbonate buffer (pH 8.0) provided lin-benzoadenosine 5'triphosphate which was converted to its lithium salt as described above: yield, ~100%.

*lin-Benzoadenosine* 3',5'-Monophosphate (6). The procedure employed was that described by Marumoto et al. (1975). Trichloromethylphosphonic acid dichloride (700 mg, 2.96 mmol) in triethyl phosphate (1.5 mL) was added dropwise to an ice-cooled stirred suspension of *lin*-benzoadenosine (1; 100 mg, 0.32 mmol) in triethyl phosphate (8 mL). The reaction mixture was stirred at 5 °C for 24 h and then poured onto ice-water (20 mL). The solution was adjusted to pH 3 with 2 N sodium hydroxide, concentrated to one-third volume, and applied to a column of activated charcoal. The column was washed with water and then eluted with ethanol:water:concentrated ammonia (9:10:1). The eluate was concentrated and applied to a column of DEAE-cellulose (bicarbonate form). Elution with a linear gradient of triethylammonium bicarbonate buffer (pH 8.0; 0.01-0.2 M) provided *lin*-benzoade-

nosine 5'-trichloromethylphosphonate (5; 74 mg,  $Et_3N^+H$  salt). This material was used directly in the cyclization step.

*lin*-Benzoadenosine 5'-trichloromethylphosphonate (5) (Et<sub>3</sub>N<sup>+</sup>H salt, 74 mg, 0.12 mmol) with potassium *tert*butoxide (125 mg, 1.12 mmol) in dry DMF (6 mL) was stirred at room temperature for 24 h. The reaction mixture was poured onto ice-water (10 mL) and the solution adjusted to pH 3 with 1 M hydrochloric acid. The solution was then applied directly to a short column of activated charcoal. The column was washed with water (170 mL) and then eluted with ethanol: water:concentrated ammonia (9:10:1; 200 mL). The eluate was evaporated in vacuo, taken up in water, and chromatographed on a column of DEAE-cellulose as indicated above to provide 40 mg (33%) of *lin*-benzoadenosine 3',5'-monophosphate (Et<sub>3</sub>N<sup>+</sup>H salt), which was converted to its lithium salt.

*lin*-Benzo-cAMP, on paper electrophoresis in 0.1 M phosphate buffer, pH 7.5, showed a relative mobility of 0.27 compared with that of *lin*-benzo-ADP (3) = 1.00 and that of *lin*-benzo-AMP (2) = 0.50, also distinguishable from that of 5 = 0.25. *lin*-Benzo-cAMP, a substrate for cyclic nucleotide 3',5'-phosphodiesterase, was converted to *lin*-benzo-AMP (2) under enzymatic conditions (Drummond & Yamamoto, 1971a) that left 2 unchanged, and the conversion  $6 \rightarrow 2$  did not proceed in the absence of enzyme.

 $P^{1}$ ,  $P^{2}$ -Di-lin-benzoadenosine 5'-Pyrophosphate (7) from 2. 4-Morpholine-N, N'-dicyclohexylcarboxamidinium linbenzoadenosine 5'-phosphoromorpholidate (61 mg, 0.08 mmol) in dry DMF (7 mL) was treated with trifluoroacetic acid (15  $\mu$ L). After 24 h most of the phosphoromorpholidate had been converted to the dimer. The DMF was removed by evaporation in vacuo, and the residue was dissolved in triethylammonium bicarbonate buffer and applied to a DEAE-cellulose column (bicarbonate form). Elution with a linear gradient of triethylammonium bicarbonate buffer (pH 8.0; 0.01 M to 0.3 M) provided  $P^{1}$ ,  $P^{2}$ -di-lin-benzoadenosine 5'-pyrophosphate (Et<sub>3</sub>N<sup>+</sup>H salt; 30 mg, 37%).

2-H Exchange in lin-Benzoadenine Nucleotides. Slow 2-H exchange occurs when lin-benzoadenine nucleotides remain in D<sub>2</sub>O solutions (pD  $\cong$  8.5) at room temperature. Deuterium exchange also occurs in solutions left at 0-5 °C, with complete conversion within a period of several months. When quick and complete exchange is needed, a given D<sub>2</sub>O solution of nucleotide (pD  $\cong$  8.5) can be warmed gently at 60-80 °C for 2-3 h. This procedure should be carefully executed with di- and triphosphates to minimize their phosphate hydrolyses.

Enzyme Studies. For the enzyme studies, pyruvate kinase (rabbit muscle), adenylate kinase (rabbit muscle), hexokinase (yeast), glucose-6-phosphate dehydrogenase (yeast), phosphofructokinase (rabbit muscle), phosphoglycerate kinase (yeast), lactate dehydrogenase (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), glyceraldekinase (*E. coli*) were all purchased from Sigma Chemical Co. Enzyme assays were performed with a Beckman Acta M VI spectrophotometer at 25 °C, monitored at 370 nm rather than the customary 340 nm to minimize the background absorbance by the *lin*-benzoadenosine moiety.

Pyruvate Kinase. The assay mixture, in a final volume of 1 mL, contained: 100 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM phosphoenolpyruvate, 0.2 mM NADH, lactate dehydrogenase, pyruvate kinase and ADP (0.04-0.4 mM) or *lin*-benzoadenosine 5'-diphosphate (0.2-2.0 mM). The reaction was initiated by the addition of the pyruvate kinase.

Adenylate Kinase. (a) ADP and lin-Benzoadenosine 5'-Diphosphate (3). The reaction mixture, in a final volume of 0.1 mL, contained 50 mM Tris (pH 8.0), 10 mM MgCl<sub>2</sub>, 7 mM ADP, and 1.5 mM lin-benzoadenosine 5'-diphosphate.

(b) AMP and ATP or *lin*-Benzoadenosine 5'-Triphosphate (4). The reaction mixture, in a final volume of 0.1 mL, contained 50 mM Tris (pH 8.0), 4 mM MgCl<sub>2</sub>, 4 mM AMP, and either 4 mM ATP or 4 mM *lin*-Benzoadenosine 5'-Triphosphate.

(c) *lin*-Benzoadenosine 5'-Diphosphate (3). The reaction mixture, in a final volume of 0.1 mL, contained 50 mM Tris (pH 8.0), 1 mM MgCl<sub>2</sub>, and 2 mM *lin*-benzoadenosine 5'-diphosphate. All reactions (a-c) were initiated by the addition of adenylate kinase. After 1 h the reaction mixtures were analyzed either on a DEAE-Sephadex A25 column equilibrated in 300 mM Tris (pH 7.5) using a pressure of 160 lb/in.<sup>2</sup> (flow rate of 48 mL/h) or on cellulose thin-layer chromatography (75% isobutyric acid, 24% H<sub>2</sub>O, 1% NH<sub>4</sub>OH). The column chromatography was monitored at 260 nm and at 340 nm and checked against standards.

Hexokinase. The assays were carried out by coupling the production of glucose 6-phosphate with glucose-6-phosphate dehydrogenase. The reaction rate was determined by monitoring the reduction of NADP<sup>+</sup> at 370 nm. The assay mixture, in a final volume of 1 mL, contained: 100 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM glucose, 0.2 mM NADP<sup>+</sup>, glucose-6-phosphate dehydrogenase, hexokinase, and ATP or *lin*-benzoadenosine 5'-triphosphate (0.04–0.4 mM). For assays with the *lin*-benzoadenosine 5'-triphosphate, 20 times more hexokinase was used than with ATP.

Phosphofructokinase. The assay mixture, in a final volume of 1 mL, contained: 100 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.3 mM fructose 6-phosphate, 2 mM phosphoenolpyruvate, 0.2 mM NADH, pyruvate kinase, lactate dehydrogenase, phosphofructokinase, and ATP (0.01–0.10 mM) or *lin*-benzoadenosine 5'-triphosphate (0.04–0.4 mM). For the phosphofructokinase inhibition studies, 12 mM MgCl<sub>2</sub> was used, all other components being the same as above, with appropriate concentrations of ATP and *lin*-benzoadenosine 5'-triphosphate. Reactions were initiated by addition of the phosphofructokinase.

*Phosphoglycerate Kinase.* The assay mixture, in a final volume of 1 mL, contained: 50 mM Tris-HCl buffer (pH 7.5), 30 mM MgCl<sub>2</sub>, 2 mM 3-phosphoglyceric acid, 0.2 mM NADH, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and ATP (0.1-1.0 mM) or *lin*-benzo-adenosine 5'-triphosphate (0.1-0.5 mM).

Acetate Kinase. The assay mixture, in a final volume of 1 mL, contained: 50 mM Tris (pH 7.5), 25 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 40 mM potassium acetate, lactate dehydrogenase, pyruvate kinase, and ATP (0.05-0.4 mM) or *lin*-benzoadenosine 5'-triphosphate (0.05-0.2 mM). The reaction was initiated by the addition of acetate kinase.

<sup>32</sup>P Incorporation in the  $\gamma$ -Phosphate of lin-Benzoadenosine 5'-Triphosphate. The assay mixtures (0.1 mL) contained: 50 mM Tris-HCl buffer (pH 8.3), 1 mM dithiothreitol, 1.5 mM ATP (lin-benzoadenosine 5'-triphosphate), 1.5 mM 3-phosphoglyceric acid, 6.7 mM MgCl<sub>2</sub>, 0.1 mM K<sub>2</sub>H<sup>32</sup>PO<sub>4</sub>, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase. Radioactive phosphate was added last. The incorporation of <sup>32</sup>P into triphosphate was assayed by TLC of the reaction mixture on polyethylenimine sheets (Polygram, Brinkmann Instruments) which were developed with 0.3 M ammonium bicarbonate buffer (pH 8.0). The reactions were assayed at 5, 10, 20, 40, and 80 min. The appropriate sections of the chromatograms were cut out (i.e., for inorganic phosphate, ATP, and lin-benzoadenosine triphosphate) and the radioactivities were determined by scintillation counting to provide the percent of the total phosphate incorporated into the triphosphate (ca. 50%).

Synthesis of Poly(lin-benzoadenylic acid). The reaction medium contained the following components: lin-benzoadenosine 5'-diphosphate (2.5 mM), MnCl<sub>2</sub> (5 mM), Tris-HCl buffer (100 mM, pH 8.1), and 50 units of polynucleotide phosphorylase (*Micrococcus luteus* enzyme, kindly donated by Professor Olke Uhlenbeck) in a total volume of 200  $\mu$ L. The reaction mixture was incubated at 37 °C for 17 h and then extracted several times with chloroform-isoamyl alcohol (3:1). The aqueous layer was applied to a column of Sephadex G-50 (1.3 × 100 cm) and eluted with water. The polymeric material was excluded from the column and the appropriate fractions were pooled and concentrated to give a 1 OD (approximately) solution ( $\lambda$  331 nm; made 0.1 M in Tris-acetate, pH 8.6, buffer to keep the polymer in solution).

Molecular Weight Determination by Gel Electrophoresis. 16% Polyacrylamide gel electrophoresis, in 8 M urea and 10% glycerol, was performed using Tris-acetate-EDTA buffer, pH 6.0 (Loening, 1969). Molecular weight distribution monitored by ultraviolet absorbance at 260 and 331 nm indicates a polymer comprising ca. 80 nucleotide units.

#### **Results and Discussion**

Chemistry. Unprotected lin-benzoadenosine (1) was converted to lin-benzoadenosine 5'-monophosphate (2) by treatment in the cold with pyrophosphoryl chloride in *m*-cresol in an application of the general method of Imai et al. (1969) for 5'-monophosphates. The product (2) was characterized by high performance liquid chromatography (HPLC), electrophoresis, <sup>31</sup>P NMR spectroscopy, and microanalysis. The 5'-position of the phosphate was established by the complete reversion of the ribonucleotide to *lin*-benzoadenosine (1) on incubation with 5'-nucleotidase (Drummond & Yamamoto, 1971b), which is highly specific, and by <sup>31</sup>P NMR (Barrio et al., 1978). The *lin*-benzoadenosine 5'-diphosphate (3) was prepared by



the general method of Moffatt & Khorana (1961) from *lin*benzoadenosine 5'-phosphoromorpholidate obtainable from the 5'-monophosphate **2**. The same intermediate served as a precursor for *lin*-benzoadenosine 5'-triphosphate (**4**). Both the diphosphate and the triphosphate were characterized by TLC, HPLC, electrophoresis, and <sup>31</sup>P NMR spectra. When we discovered that *lin*-benzoadenosine 5'-diphosphate (**3**) was active as a cofactor with rabbit muscle pyruvate kinase and phosphoenolypyruvate, we utilized this convenient enzymatic route from di- to triphosphate. We were thereby able to maintain our supply of *lin*-benzoadenosine 5'-triphosphate for testing in various ATP-requiring enzyme systems. When the *lin*-benzoadenosine 5'-triphosphate was converted to 5'-di-

phosphate in such systems, we simply accumulated the residues, isolated the pure diphosphate by high performance liquid chromatography, reconverted it to triphosphate essentially quantitatively with pyruvate kinase and phosphoenolypyruvate, and purified the product by chromatography. The extensive enzymatic studies reported herein were facilitated by this recycling procedure.

*lin*-Benzoadenosine 3',5'-monophosphate (6) was synthesized from *lin*-benzoadenosine (1) by the general procedure of Marumoto et al. (1975) via 3'-O anionic displacement of the trichloromethyl group from *lin*-benzoadenosine 5'-trichloromethylphosphonate (5). The structure of the cAMP analogue 6 was established by its reconversion to *lin*-benzo-



adenosine 5'-monophosphate (2) on incubation with beef heart nucleotide 3',5'-phosphodiesterase (Drummond & Yamamoto, 1971a). The enzyme is specific for 3', 5'-monophosphates and plays an important role in regulating intracellular cAMP. With this enzyme, the initial rate of hydrolysis of lin-benzoadenosine 3',5'-monophosphate (at 0.5 mM concentration) was approximately 5% of that for cAMP, but with excess enzyme complete  $6 \rightarrow 2$  conversion was possible. Identity of the cyclic monophosphate 6 was also established by its distinctive  ${}^{31}P$ NMR spectrum (see below), and by its chromatographic and electrophoretic properties in comparison with those of the ribonucleotides 2-4. The stereochemical configuration at C-1' was confirmed as  $\beta$ , as in *lin*-benzoadenosine (Leonard et al., 1976), by <sup>1</sup>H NMR spectroscopy (Schmidt et al., 1978). The 220-MHz <sup>1</sup>H NMR spectrum of lin-benzoadenosine 3',5'monophosphate in D<sub>2</sub>O revealed the anomeric proton as a singlet centered at  $\delta$  6.10 ppm approaching infinite dilution, consistent with sharp singlet signals generally observed for  $\beta$ anomers of 3',5'-cNMP type and in contrast to the doublets observed for 1'-H in  $\alpha$  anomers (Robins & MacCoss, 1977).

The <sup>31</sup>P NMR spectra of the *lin*-benzoadenine ribonucleotides **2**, **3**, **4**, and **6** are strikingly similar (Table I) in chemical shifts (from 85% H<sub>3</sub>PO<sub>4</sub>) and P-O-P coupling constants to the corresponding adenine ribonucleotides, respectively, AMP, ADP, ATP (Labotka et al., 1976), and cAMP. Observed under similar conditions, for example, the <sup>31</sup>P chemical shift for both *lin*-benzo-cAMP and cAMP is -1.7ppm, quite distinct from the chemical shift of +3.9 ppm for both *lin*-benzo-AMP and AMP. The <sup>31</sup>P shifts and coupling constants of *lin*-benzo-ADP and *lin*-benzo-ATP, similar to those of ADP and ATP, respectively, are useful for their

#### TABLE I: lin-Benzoadenine Nucleotides; <sup>31</sup>P NMR Data.<sup>a</sup>

	compound				
	2	3	4	6	7
chemical shift (ppm)	+3.9	$-5.8 (d, \beta)$ -10.3 (d, $\alpha$ )	$\begin{array}{c} -6.1 \ (\mathrm{d}, \gamma)^{b} \\ -11.1 \ (\mathrm{d}, \alpha) \\ -22.4 \ (\mathrm{t}, \beta)^{b} \end{array}$	-1.7	-11.0
coupling constant (Hz)	s <sup>c</sup>	${}^2J_{\rm POP} = 22$	$J_{\alpha-\beta} = 20 J_{\beta-\gamma} = 20$	s <sup>d</sup>	S
chemical shift (ppm)	AMP +3.9	ADP -7.3 (d, $\beta$ ) -10.8 (d, $\alpha$ )	ATP -5.7 (d, $\gamma$ ) <sup>b</sup> -11.0 (d, $\alpha$ ) -21.6 (t, $\beta$ ) <sup>b</sup>	<b>cAMP</b> -1.7	A <sub>P2</sub> A -11.1
coupling constant (Hz)	s <sup>c</sup>	${}^2J_{\rm POP} = 22$	$J_{\alpha-\beta} = 20 J_{\alpha-\gamma} = 20$	s	s

<sup>a</sup> Spectra recorded in D<sub>2</sub>O, nucleotide concentration (0.013–0.020 M), EDTA (0.002 M), adjusted to pH 10–10.5 by addition of (CH<sub>3</sub>)<sub>4</sub>N<sup>+</sup>OH<sup>-</sup>, chemical shifts in ppm from 85% H<sub>3</sub>PO<sub>4</sub>. <sup>b</sup> Large NOE signal enhancements were observed for the  $\beta$ - and  $\gamma$ -P resonances (Hart, 1976). <sup>c</sup> Undecoupled spectra: **2**, t, <sup>3</sup>J<sub>PH</sub> = 5 Hz; AMP, t (unresolved). <sup>d</sup> Undecoupled spectra: **6**, d, <sup>3</sup>J<sub>PH</sub> = 20 Hz; cAMP, d, <sup>3</sup>J<sub>PH</sub> = 19 Hz.

TABLE II: lin-Benzoadeni	nine Nucleotides; Chemical Shifts at Infinite Dilution. <sup>a</sup>					
		2-H	4-H	6-H	9-H	1 <b>′-H</b>
NH2 н	lin-benzo-cAMP	8.56	7.93	8.37	8.50	6.12
N N N N N N N N N N N N N N N N N N N	lin-benzo-AMP 2	8.83	7.94	8.36	8.46	6.05
	lin-benzo-ADP 3	8.82	7.91	8.37	8.48	5.99
	lin-benzo-ATP 4	8.91	8.03	8.44	8.61	6.03
lin-benzo-AMP "dimer"	bis-5',5'-[ <i>lin</i> -benzo-AMP-2-d] 7-d <sub>2</sub>		7.42	8.10	7.74	5.71

<sup>a</sup> Shifts in ppm. Values were extrapolated to infinite dilution using a curve-fitting iterative procedure to be described. Experimental conditions: D<sub>2</sub>O, pD 8.5 (2 mM K<sub>2</sub>DPO<sub>4</sub>), 28 °C.

characterization. The symmetrical anhydride of *lin*-benzo-AMP linked through the 5'-phosphates, namely,  $P^1$ ,  $P^2$ -di*lin*-benzoadenosine 5'-pyrophosphate (7), was synthesized by



treatment of a salt of *lin*-benzoadenosine 5'-phosphoromorpholidate in dry dimethylformamide with trifluoroacetic acid and was isolated and purified by linear gradient chromatography. The singlet <sup>31</sup>P NMR signal confirmed that the two phosphates were equivalent in this molecule, and the <sup>31</sup>P chemical shift was practically identical with that of  $P^{1},P^{2}$ -diadenosine 5'-pyrophosphate (Table I).

The <sup>1</sup>H NMR chemical shifts, extrapolated to infinite dilution, for all of the aromatic hydrogens show a remarkable consistency throughout the series 2, 3, 4, and 6 (Table II). Details concerning the <sup>1</sup>H NMR spectra and the methods of assignment of chemical shifts for the individual hydrogens will be described in a later paper. One major point to be made at this time is that the 2-H signal (see Table II for the accepted Chemical Abstracts numbering of the ring system) lies at lowest field in all cases. On the basis of other model compounds, this suggests that the  $N-C_{1'}$  preferred conformation is anti, as in the corresponding adenine series (Schweizer et al., 1964). Another point to be made now is that the chemical shifts for all of the aromatic protons in the lin-benzo-AMP anhydride or "dimer" (7, with the <sup>2</sup>H's replaced by deuterium), which are at higher field than those of lin-benzo-AMP, are indicative of intramolecular complexing or "stacking" (Broom et al., 1967) of the heteroaromatic rings in  $P^1$ ,  $P^2$ -di-lin-benzoadenosine 5'-pyrophosphate (7) in very dilute solution.

The magnitude of the interaction between the two tricyclic ring systems in 7 could be determined quantitatively relative to that of two adenine rings in the corresponding  $P^1, P^2$ -diadenosine 5'-pyrophosphate (Scott & Zamecnik, 1969). Ultraviolet absorption spectra at pH 8.5 and 25 °C in aqueous solution were measured before and after hydrolytic cleavage by snake venom phosphodiesterase. The percent hypochromism was found to be 23 for 7 and 9 for  $P^1, P^2$ -diadenosine 5'-pyrophosphate. The strong intramolecular interaction between

TABLE III: Binding and Activity of Cofactor Analogues

	$K_{\rm M}$ (mM)			
enzyme	lin-benzo-ADP	ADP	(rel)	
pyruvate kinase (rabbit muscle)	0.74 <i>lin</i> -benzo-ATP	0.30 ATP	0.2	
hexokinase (yeast)	0.18	0.09	0.75 <i>ª</i>	
phosphofructokinase (rabbit muscle)	0.16	0.04	1.0	
3-phosphoglycerate kinase (yeast)	0.4	0.5	0.01	
acetate kinase (E. coli)	0.15	0.06	0.23	

"I wenty times more enzyme used in the case of *lin*-benzo-ATP.

the tricyclic rings in 7 was also shown by the dramatic change in fluorescence emission. The enzymatic cleavage of  $P^1, P^2$ di-lin-benzoadenosine 5'-pyrophosphate to lin-benzo-AMP (2) resulted in an increase in fluorescence intensity of approximately two orders of magnitude (Scopes et al., 1977). There was no significant increase in fluorescence when compound 7 was heated in 50 mM NaHCO<sub>3</sub>, pH 9, even up to 80 °C, in further confirmation of this strong intramolecular stacking interaction. The lin-benzoadenine ribonucleotides (2, 3, 4, and 6), like the ribonucleoside (1; Leonard et al., 1976), are fluorescent in aqueous solution, giving a quantum yield in the range 0.40-0.44 upon excitation at 332 nm. The near lack of fluorescence of 7 indicates that lin-benzoadenosine moieties, when connected intramolecularly so that stacking can occur, can form dark complexes and can undergo intramolecular collisional quenching. It will be instructional to synthesize the lin-benzoadenine analogues of FAD and NAD+ and to determine the extent of the fluorescence quenching (Gruber & Leonard, 1975). It is predictable that the intermolecular positioning of a *lin*-benzoadenosine system in close proximity to the aromatic amino acids of an enzyme will result in fluorescence quenching.

Enzyme Activity. Investigation of the biological activity of the lin-benzoadenine ribonucleotides was initiated with enzymes that examined the ability of these nucleotides to serve as donors/acceptors of phosphate. Following the first gratifying discovery that the stretched-out nucleotides showed activity, the degree to which they could substitute for the corresponding adenine nucleotides was determined for a representative series of kinases, comprising pyruvate kinase, adenylate kinase, hexokinase, phosphofructokinase, phosphoglycerate kinase, and acetate kinase. Comparative conditions were chosen to give consistent results for the adenine (natural) and stretched-out adenine nucleotides without seeking to achieve those of maximal activity. Where the enzyme assays were linked to a dehydrogenase-NAD+/NADH system, they were monitored at 370 nm rather than the customary 340-nm wavelength in order to minimize the background absorbance by the lin-benzoadenosine moiety. The very method of synthesis of the lin-benzoadenine nucleotides precludes contamination by the natural adenine nucleotide substrates or cofactors, so that the binding and rate data reported are truly representative of the tricyclic version (as in 3 and 4) examined.

We mentioned earlier that pyruvate kinase (rabbit muscle) was useful to us in the enzymatic conversion of *lin*-benzoadenosine 5'-diphosphate (3) to 5'-triphosphate (4). The  $K_m$ (Michaelis constant) and  $V_{max}$  figures for *lin*-benzoadenosine 5'-diphosphate relative to ADP are given in Table III. These satisfactory figures for binding and maximum velocity for *lin*-benzo-ADP directed our use of the coupled assay for phosphofructokinase (see below) involving pyruvate kinase and

$ATP \cdot Mg^{2+} + AMP \xrightarrow{myokinase} ADP \cdot Mg^{2+} + ADP$	
+	lin-benzo-ATP + AMP
_	lin-benzo-ATP + lin-benzo-AMP
_	ATP + <i>lin</i> -benzo-AMP
-	lin-benzo-ADP
+	lin-benzo-ADP + ADP

lactate dehydrogenase. It has been recognized that pyruvate kinase shows a somewhat broad specificity for the nucleotide substrate of the reaction (Plowman & Krall, 1965), so it is not surprising that a lateral extension of the adenine nucleus is acceptable to this enzyme. Proposed binding models (Mildvan & Cohn, 1966) of pyruvate kinase for nucleotide substrates, together with fluorescence polarization studies with  $\epsilon$ ADP and pyruvate kinase (Barrio et al., 1973a) indicate that the base moiety of the substrate is not strongly associated with the protein. For *lin*-benzo-ADP to be as active as it is, the ADP binding region (Kayne, 1973; Blake & Evans, 1974; Mildvan, 1974; Brevet et al., 1975) at the least must be able to accommodate the extra volume of the extended molecule.

Adenylate kinase (rabbit muscle) catalyzes the transfer of phosphate between MgATP and AMP and, for the reverse reaction, between MgADP and ADP. The accumulated evidence with regard to the functioning of this enzyme supports a two-site model in which the first site is specific for AMP or ADP and the second, less demanding, is specific for ATP or ADP (Rhoads & Lowenstein, 1968; Secrist et al., 1972; Noda, 1973; Price et al., 1973, McDonald et al., 1975, for pig adenylate kinase). Our findings with lin-benzo-AMP and linbenzo-ATP support this concept. The results are shown in abbreviated form in Table IV. In the adenylate kinase (myokinase) system, when lin-benzo-ATP replaced ATP, reaction proceeded as evidenced by the formation of lin-benzo-ADP and ADP, monitored by HPLC (see Materials and Methods). However, the reaction was sufficiently slow compared with that using the natural AMP and ATP that accurate measurement of kinetic data was not possible. by contrast, the combination ATP and lin-benzo-AMP provided no diphosphates and the use of lin-benzo-AMP and lin-benzo-ATP likewise produced no diphosphate. The AMP binding site is highly selective. Its relation to an ADP site was then shown unequivocally by using a mixture of lin-benzo-ADP and ADP with the adenylate kinase. The stretched-out analogue, linbenzo-ADP, with the enzyme alone remained unchanged, while the mixture came to equilibrium with five species present: AMP, ADP, ATP, lin-benzo-ADP, and lin-benzo-ATP. Thus, it is clear that one of the ADP binding sites is highly specific and corresponds to the AMP binding site. Neither lin-benzo-ADP nor lin-benzo-AMP can react at that site.

As a probe of the conformation of enzyme-bound AMP with porcine adenylate kinase, Hampton et al. (1972) found 8,5'cycloadenosine 5'-phosphate to be a good substrate and concluded that the phosphate group is closer to 8-H than to 2-H. Since the preferred conformation of *lin*-benzo-AMP (2) also appears to be more anti than syn (Table II), the sugar/base conformation does not account for its failure as a substrate for adenylate kinase. Since *lin*-benzo-AMP possesses the requisite NH<sub>2</sub> group (Noda, 1973), a contributing reason probably lies in the extension of the heteroaromatic ring system. We showed earlier (Leonard & Laursen, 1965) that 3-isoadenosine 5'- monophosphate, which has roughly equivalent bulk to AMP and which serves as a kind of foreshortened dimensional probe since it has the amino and ribosyl groups on the same (pyrimidine) ring, is active with adenylate kinase. Accordingly, the AMP-binding site of the enzyme will accept 3-iso-AMP and AMP, but not lin-benzo-AMP, stated in the order of increasing distance between the positions, or rings, of attachment of the amino and ribosyl groups. In pig adenylate kinase, NMR results suggest that there is no direct interaction of the adenine of AMP with an aromatic residue (McDonald et al., 1975), and X-ray results suggest that the adenine may lie in a hydrophobic pocket between Gly-20 and Val-179 (Schulz et al., 1974). Our stretched-out model 3 helps define the limiting size of such a pocket, at least for the rabbit muscle enzyme in solution. The ATP hydrophobic pocket (McDonald et al., 1975), by contrast offers sufficient volume for the laterally extended lin-benzo-ATP to fit. The topology of the ATP binding site has been stated to be very similar to that of the corresponding sites of dehydrogenases and phosphoglycerate kinase (see below).

The nucleotide binding domains in monomeric and dimeric yeast hexokinases (Fletterick et al., 1975; Steitz et al., 1976) exhibit similarities and differences in relation to the domains (for NAD<sup>+</sup>) in lactic dehydrogenase. Nucleotide binding is promoted by glucose, leading to an ordered mechanism of reaction (Colowick, 1973). lin-Benzo-ATP (4) can replace ATP for the phosphorylation of glucose catalyzed by yeast hexokinase, as assayed by the standard procedure of coupling to glucose-6-phosphate dehydrogenase. The  $K_{\rm m}$  values are in the same range (Table III), so that the stretched-out analogue of ATP binds satisfactorily, indicating that the site has at least an extra 2.4-Å dimensional tolerance. There was, however, a substantial reduction in the rate of phosphate transfer to the substrate to approximately one-fortieth the rate from ATP. It is likely that the juxtaposition of bound glucose and bound lin-benzo-ATP is not as favorable for this transfer. The combination of the efficient binding of lin-benzo-ATP and the slow rate of reaction may be turned to advantage in facilitating fluorescence polarization measurements.

By contrast,  $V_{max}$  values of comparable magnitude were observed for ATP and lin-benzo-ATP at low concentrations in the phosphorylation of fructose 6-phosphate catalyzed by phosphofructokinase (PFK, rabbit muscle; Table III). PFK is able to utilize several nucleoside triphosphates as phosphoryl donors (Bloxham & Lardy, 1973). The most efficient are purine or modified purine ribonucleoside triphosphates including  $\epsilon$ ATP (Secrist et al., 1972). Utilization of *lin*-benzo-ATP by PFK represents the largest dimensional deviation known from the natural cofactor ATP. The structural requirements for inhibitory activity of yeast PFK by higher concentrations of a nucleoside triphosphate are more stringent than for catalytic activity. Significant allosteric inhibition has been observed at high concentrations of ATP, UTP, and eATP, and now linbenzo-ATP is observed to exhibit inhibition of the enzyme to approximately the same degree as ATP. Thus, the stretchedout version of ATP is more effective than  $\epsilon$ ATP in the concentration range for inhibition, and its size is completely acceptable for this function.

The ability of *lin*-benzoadenosine 5'-triphosphate to phosphorylate 3-phosphoglyceric acid, catalyzed by yeast 3-phosphoglycerate kinase (PGK), has also been examined (Table III). Several purine nucleoside triphosphates, in addition to ATP and including ITP, GTP, dGTP, and dATP, can serve as phosphoryl donors (Scopes, 1973). The phosphorylation was assayed according to the standard procedure of coupling the reaction to glyceraldehyde 3-phosphate dehydrogenase. The binding of ATP and *lin*-benzo-ATP to the enzyme was com-

parable, while the  $V_{\text{max}}$  value for the stretched-out analogue was approximately 1% of that of ATP, i.e., in the same range as UTP. The ability of lin-benzo-ATP to phosphorylate 3phosphoglyceric acid catalyzed by yeast 3-phosphoglycerate kinase permitted equilibration of the  $\gamma$  (terminal) phosphate in the nucleoside triphosphate with  ${}^{32}PO_4{}^{3-}$ . Thus,  $[\gamma {}^{-32}P]$ lin-benzo-ATP was prepared by procedures previously used for  $[\gamma^{-32}P]ATP$  (Glynn & Chappell, 1964),  $[\gamma^{-32}P]$ - $\epsilon ATP$ , and  $[\gamma^{-32}P]$ - $\epsilon$ CTP (Barrio et al., 1973b). The structures of yeast phosphoglycerate kinase (Bryant et al., 1974) and horse muscle phosphoglycerate kinase (Blake & Evans, 1974) are similar, and the binding site for Mg-ADP and Mg-ATP has been located on domain A, consisting of a slot for the adenine ring at the edge of the  $\beta$  sheet. The dimensions of this "slot" are thus subject to sufficient variability to accommodate the specific active analogues (Scopes, 1973). The similarity in the nucleotide binding sites of PGK and several dehydrogenases (LDH, MDH, ADH, and GPDH) (Buehner et al., 1973; Blake & Evans, 1974; Öhlsson et al., 1974) suggests that the linbenzo analogue of NAD<sup>+</sup>, when synthesized, should be a good candidate for binding to these dehydrogenases.

Acetate kinase from *E. coli* catalyzes the phosphorylation of acetate by ATP. The activity of *lin*-benzo-ATP, as determined by a coupled enzyme assay, was somewhat less than that of ATP (and ITP; Rose, 1962; Table III).

Polynucleotide phosphorylase from *Micrococcus luteus*, which is primer independent, catalyzes the polymerization of *lin*-benzoadenosine 5'-diphosphate in the presence of  $Mn^{2+}$ at 37 °C. The polymeric material isolated by gel chromatography showed almost no fluorescence. We were prepared for this observation attributable to stacking interactions since  $P^1,P^2$ -lin-benzoadenosine 5'-pyrophosphate (7) exhibited only slight fluorescence. The structural integrity of the polymer was shown by its hydrolysis with the aid of a mixture of enzymes or alkali to *lin*-benzoadenosine (1), with the return of

$$n3 \xrightarrow{Mn^{2+}} poly(lin-benzoadenylic acid) + nP_i$$
  
micrococcal nuclease  
snake venom phosphodiesterase  
alkaline phosphatase  
$$1$$

fluorescence. The long-wavelength band in the ultraviolet absorption spectrum of the polymer lacked the characteristic fine structure of the monomeric species (Leonard et al., 1975; Scopes et al., 1977) and was broadened. Upon enzymatic or alkaline hydrolysis, the fine structure of the long-wavelength absorption band reappeared. The molecular distribution of the polymer, as determined by gel electrophoresis and monitored by ultraviolet absorbance at 260 and 331 nm, indicated that the major polymeric product comprised ca.  $80 \pm 5$  nucleotide units. The rate of polymerization was slower than the formation of poly(adenylic acid) from ADP under the same conditions, but it is interesting that the related tricyclic compound, lin-benzo-ADP (3), served as a substrate. We intend to test the complementarity or lack of complementarity of the poly-(*lin*-benzoadenylic acid) with poly(U) for an abnormal double-helical cross section and with poly(N-acetylaminoribotide) or poly(N-ureidoribotide) for a perfectly proportioned double-helical cross section (Leonard et al., 1976).

We are able to report preliminary results with the stretched-out cofactors and several additional enzymes at this time. For example, it was of interest to establish whether *lin*-benzo-ATP (4) is an activator of *E. coli* aspartate transcar-bamylase (ATCase) (Gerhart & Holoubek, 1967; Evans et al., 1973) like ATP or an inhibitor like  $\epsilon$ ATP (Chien & Weber,

1973). The curves for percent activation of ATCase vs. concentration of ATP or *lin*-benzo-ATP were practically identical, thereby indicating that the fluorescent *lin*-benzo-ATP is an efficient allosteric activator of this enzyme.

Brain protein kinase and protein kinase from skeletal muscle are maximally activated by *lin*-benzo-cAMP (6), and *lin*benzoadenosine (1) inhibits kinase activity slightly less than does adenosine (Schmidt et al., 1978). Thus the 2.4 Å wider versions of cAMP and of adenosine interact with protein kinase in a manner similar to that of the natural compounds.

We have also examined the interaction of *lin*-benzo-ADP (3) and *lin*-benzo-ATP (4) with mitochondrial ATP synthetase, purified ATPase, and the adenine nucleotide carrier (Kauffman et al., 1978). The lateral extension of the adenine ring by 2.4 Å modifies the kinetics of ATP synthesis by submitochondrial particles but has little effect upon the kinetics of ATP hydrolysis by the purified mitochondrial ATPase. *lin*-Benzo-ADP (3) inhibits the latter more potently than does ADP but displays little if any activity as a substrate for the adenine nucleotide carrier.

The failure of some enzymes to function with the stretched-out versions of the adenine nucleotides is to be expected. Even lack of activity will provide a positive indication of the discrimination of the particular enzyme with respect to the dimensional requirements, inter alia, of the cofactor or substrate binding site (Leonard et al., 1976). As an example, current experiments carried out on T7 phage DNA-directed RNA synthesis in vitro show, not too surprisingly, that *lin*-benzo-ATP does not replace ATP for RNA synthesis and does not inhibit ATP incorporation into RNA (D. Nakada, University of Pittsburgh, private communication). The apparent slight stimulatory effect of *lin*-benzo-ATP on RNA synthesis observed when ATP supply is limiting is under study to determine whether any incorporation of the analogue or chain termination by the analogue is taking place.

*lin*-Benzo-ATP does not get incorporated by primase (*dna*G protein), a rifampicin-resistant RNA polymerase (Bouché et al., 1975), into a polynucleotide chain, the synthesis of which is template-directed (Rowen & Kornberg, 1978). This experiment and the one described above indicate that normal Watson-Crick base pairing is exacting in template-directed polymerizations. Strand separation of duplex superhelical replicative form I DNA of phage  $\phi X$  174 is absolutely dependent on cistron A protein, rep protein, single-strand binding protein, ATP, and Mg<sup>2+</sup> and is accompanied by the cleavage of ATP (Scott et al., 1977). The rep protein, in this role, uses lin-benzo-ATP in place of rATP but less well. The rep protein also acts as an ATPase when supplied with single-stranded DNA regions and, in this role, with single-stranded DNA as an effector, it uses *lin*-benzo-ATP in place of rATP (Kornberg et al., 1978). The fact that lin-benzo-ATP works in strand separation suggests that its fluorescence may provide a useful reportorial function.

In sequels, we shall describe the spectroscopic sensitivity of *lin*-benzoadenine nucleotides to divalent metal ions and conformational changes in the phosphate side chain, fluorescence and NMR studies relating to their folding and interactions, and details of their activity with specific enzymes such as mitochondrial ATPase, ATCase, and luciferase.

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