NH_4HCO_3). The result was invariably a single fluorescent spot with the mobility of the original labeled peptide.

Our results thus provide a molecular weight limit for permeation of the junctional membrane channels. The permeating molecules were all short, simple, water-soluble peptide chains and hence were of extended form. Without further physical studies of the molecules themselves, it is not possible to determine from this weight limit a precise bore size of the channels. But the approximate bore size can be bracketed between the sizes of two limiting geometries of the largest permeant molecule, a sphere, representing the largest cross section, and a prolate spheroid with a major diameter of 30 Å, the upper limit of molecular extension. Thus, the effective channel diameter lies approximately between 14 and 10 Å (*13*). This is in satisfying agreement with a coarser estimate of the channel size based on electrical measurements. This estimate, based on the conductance of a minute junctional area (including the conductance component due to electrostatic interaction between channels) and the spacing of intramembranous particles of gap junction (widely assumed to contain the channels), gave a lower limit of conductance of 10^{-10} mho for the junctional channel unit and a lower limit of the channel diameter of the order of 10 Å (*14*).

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9. The pattern of spread of these molecules here in the normal, untreated cells was like that of the smaller molecules (≦ 1158 dalton) in cells whose cytoplasmic Ca^{2+} concentration was elevated—the tracers spread throughout the injected cell, but not beyond its junctional boundaries.
10. On the other hand, the gland lumen, which is open to the exterior through the gland duct and has a depth comparable to the cells, became strongly fluorescent. Furthermore, dead cells became as fluorescent as the duct under these conditions. Dead cells were recognized in bright field by their swollen and granular appearance, and their enlarged nucleus and chromosomes.
11. A small labeled fragment seemed unlikely because, as was already mentioned, the rates of intracellular movement of the tracer varied inversely as their presumed molecular weights.
12. In this condition one would expect maximum lysosome breakage and, hence, maximum peptidase activity.
13. The size ($2r$) for the spherical molecular shape was determined from

$$r = \left(\frac{3 \text{ mol wt} \times \bar{v}}{4\pi N} \right)^{1/3}$$

where N is Avogadro's number and $\bar{v}$ is the specific volume assumed to be 0.7; and the size for the most extended shape, was determined as the small diameter of the corresponding prolate spheroid whose major diameter was obtained with the aid of molecular models. The actual channel bore lies probably closer to the upper bracketing value (14 Å); for the molecules labeled with LBR and FITC, the small diameter of a realistic axiosymmetric equivalent is fixed at about this value by the labels themselves.
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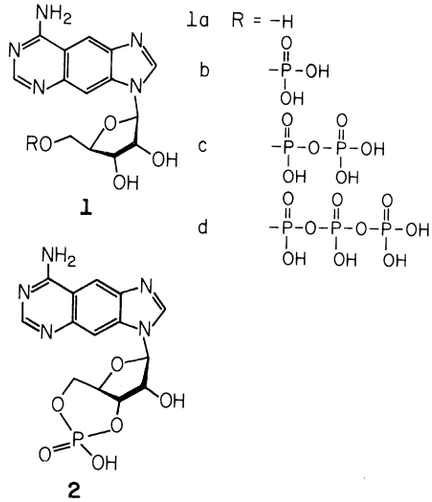
Defined Dimensional Changes in Enzyme Cofactors: Fluorescent "Stretched-Out" Analogs of Adenine Nucleotides

Abstract. *A concept is presented for testing the dimensional restrictions of enzyme-active sites by stretching the substrate or cofactor by known magnitude. These restrictions of enzyme-active sites specific for purine cofactors were tested by the synthesis and evaluation of* lin*-benzoadenosine 5′-triphosphate, 5′-diphosphate, and 3′,5′-monophosphate with respect to enzyme binding and activity. These "stretched-out" (by 2.4 angstroms) versions of the adenine ribonucleotides bind strongly, slow the enzymatic rates, and have useful fluorescence properties.*

Laterally extended adenine nucleotides have been designed to examine the dimensional restrictions of enzyme-active sites specific for purine cofactors. One structural modification of this type involves the formal insertion of a benzene ring (actually four carbons) into the center of the purine ring system. In this way enzyme-binding characteristics at the terminal rings are preserved but are further separated by 2.4 Å while, at the same time, the potential for π interaction is increased. Initial experiments examining the substrate activity of *lin*-benzoadenosine (**1a**) (*1*, *2*) and *lin*-benzoadenine with a range of enzymes (*3*) demonstrated that such defined adjustments in the molecular periphery can help set limitations on the size and flexibility of the enzyme binding sites.



In view of these results it was anticipated that the enzymatic evaluation of the *lin*-benzoadenine nucleotides would also be informative since many enzymes utilize adenine nucleotides as substrates, cofactors, or allosteric effectors. In addition, it can be foreseen that the concept of defined dimensional changes is applicable to the construction and study of inhibitors. Furthermore, *lin*-benzoadenosine and its derivatives exhibit satisfactory fluorescence properties (a quantum yield of 0.44; a fluorescence lifetime of 3.7 nsec), and the nucleotides show sensitivity of the fluorophore to environmental conditions, such as divalent metal ions and stacking.

lin-Benzoadenosine (**1a**) was converted to its 5′-monophosphate derivative (**1b**) by reaction with pyrophosphoryl chloride according to the procedure described by Imai *et al.* (*4*). The integrity of the 5′-phosphorylation was established (i) by observing complete conversion of the *lin*-benzoadenosine 5′-monophosphate to the nucleoside (**1a**) on incubation with 5′-nucleotidase (*5*) and (ii) by ^{31}P NMR (nuclear magnetic resonance) spectroscopy.

lin-Benzoadenosine diphosphate (**1c**)

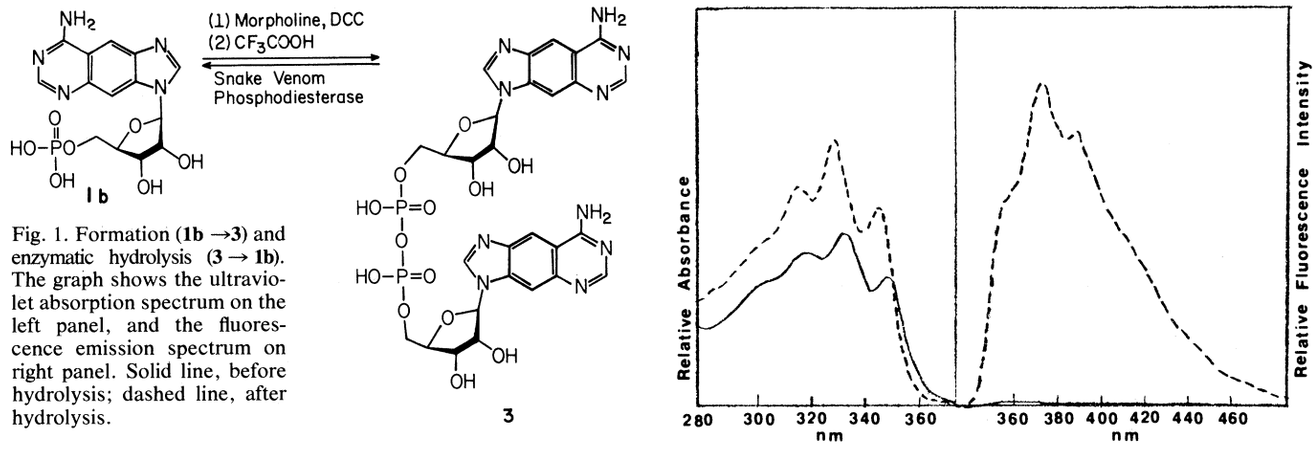


Fig. 1. Formation (**1b** →**3**) and enzymatic hydrolysis (**3** → **1b**). The graph shows the ultraviolet absorption spectrum on the left panel, and the fluorescence emission spectrum on right panel. Solid line, before hydrolysis; dashed line, after hydrolysis.

and triphosphate (**1d**) were prepared from *lin*-benzoadenosine 5′-monophosphate by the phosphoromorpholidate method (*6*). Essentially quantitative conversion of the diphosphate to the triphosphate can be achieved enzymatically with pyruvate kinase. *lin*-Benzoadenosine 3′,5′-monophosphate (**2**) was synthesized from *lin*-benzoadenosine via the trichloromethylphosphonate derivative (*7*). The identity and purity of the new *lin*-benzoadenine nucleotides (**1b** to **1d** and **2**) were established by ^{31}P NMR spectroscopy, high performance liquid chromatography, and electrophoresis. The structure of the 3′,5′-monophosphate was further confirmed by its conversion to **1b** on incubation with beef heart 3′,5′-nucleotide phosphodiesterase (*5*), an enzyme that plays an important role in regulating intracellular cyclic adenosine monophosphate (cAMP). With this enzyme, the initial rate of hydrolysis of *lin*-benzoadenosine 3′,5′-monophosphate (at 0.5 m*M* concentration) was approximately 5 percent of that for cyclic AMP.

To initiate our studies on the biological activity of the "stretched-out" nucleotides we have selected a representative group of kinases comprising pyruvate kinase, phosphofructokinase, phosphoglycerate kinase, and hexokinase. These enzymes, which exhibit broad to moderate nucleotide specificity, serve as representatives for measuring the degree to which *lin*-benzoadenosine 5′-diphosphate and *lin*-benzoadenosine 5′-triphosphate can function in enzyme systems. Conditions were selected to give consistent results for both adenosine triphosphate (ATP) and *lin*-benzoadenosine triphosphate and adenosine diphosphate (ADP) and *lin*-benzoadenosine diphosphate without seeking to achieve those of maximal activity for **1d** or **1c** (*8*).

We have found that *lin*-benzoadenosine diphosphate serves as substrate for pyruvate kinase (rabbit muscle) with a K_m (Michaelis constant) of 0.74 m*M* compared to 0.30 m*M* for adenosine diphosphate (ADP) and a V_{max} equal to 20 percent of that for ADP. These data directed the use of the coupled assay for phosphofructokinase involving pyruvate kinase and lactate dehydrogenase. Proposed binding models (*9*) of pyruvate kinase for nucleotide substrates and fluorescence polarization studies with ϵADP (*10*) indicate that the base moiety of the substrate is not strongly associated with the protein. In view of this and the known broad specificity (*11*) of pyruvate kinase, it is not surprising that a lateral extension of the adenine nucleus is acceptable to this enzyme.

The next enzyme examined was phosphofructokinase (PFK, rabbit muscle) which requires ATP to phosphorylate fructose 6-phosphate. At low concentrations of *lin*-benzoadenosine triphosphate and ATP the K_m values for the cofactors were determined to be 0.16 m*M* and 0.04 m*M*, respectively, while the V_{max} values were of comparable magnitude. PFK is able to utilize several nucleoside triphosphates as phosphoryl donors (*5*). While these mainly comprise purine nucleotides, uridine triphosphate (UTP) and ϵATP (*12*) have also been shown to serve as cofactors. Acceptance of *lin*-benzoadenosine triphosphate by PFK represents the largest dimensional deviation known from the natural cofactor. At high concentrations of ATP (and UTP or ϵATP) PFK is significantly inhibited (*13*); *lin*-benzoadenosine triphosphate exhibits allosteric inhibition of this enzyme to approximately the same degree as ATP.

Yeast hexokinase, which exhibits more stringent nucleotide specificity, was assayed by the standard procedure of coupling to glucose-6-phosphate dehydrogenase. *lin*-Benzoadenosine 5′-triphosphate (**1d**) replaces ATP with this enzyme; but, while the K_m values are of the same order (0.18 m*M* and 0.09 m*M*, respectively), the reaction rate with the "stretched-out" cofactor is approximately 40 times slower. Our kinetic data suggest that the "stretched-out" analog of ATP can be accommodated at the coenzyme binding site, but that there is a reduction in the efficiency of phosphoryl transfer to the substrate, possibly because of greater steric restrictions within the glucose and coenzyme binding regions. Hexokinase has an intrinsic hydrolytic activity that is low compared with the glucose phosphorylating activity (*14*). The occurrence of adenosine triphosphatase (ATPase) activity makes it essential to carry out binding measurements rapidly, a problem that has yet to be satisfactorily solved. *lin*-Benzoadenosine triphosphate may provide a means of studying the interaction of cofactor and hexokinase since it can be accommodated at the coenzyme binding site, while its slow reactivity makes it eminently suitable for fluorescence polarization measurements.

Finally, we examined the ability of *lin*-benzoadenosine triphosphate to phosphorylate 3-phosphoglyceric acid, catalyzed by yeast 3-phosphoglycerate kinase (PGK), in comparison with ATP. The phosphorylation was assayed according to the standard procedure of coupling the reaction to glyceraldehyde-3-phosphate dehydrogenase. *lin*-Benzoadenosine triphosphate functioned in the system with a K_m of 0.4 m*M*, while under identical conditions the K_m observed for ATP was 0.5 m*M*. The V_{max} value for the "stretched-out" analog was approximately 1 percent of that of ATP. This activity of the *lin*-benzoadenosine triphosphate in the PGK system has permitted the enzymatic synthesis of *lin*-benzoadenosine 5′-[γ-^{32}P]triphosphate (*15*), the availability of which facilitates further enzymatic studies.

A considerable body of evidence has now been presented that the nucleotide-binding sites of PGK and several dehydrogenases (lactate, malate, alcohol, and glyceraldehyde-3-phosphate dehydrogenases) are alike (*16*) and it is thus attractive to speculate from our data that the *lin*-benzo analog of NAD^+ (nicotinamide adenine dinucleotide) will bind to these dehydrogenases.

The lateral extension of the purine ring system has provided a heterocycle with interesting spectroscopic properties. For example, evidence for an intramolecular interaction of the 5′-phosphate substituent with the chromophore comes from the spectroscopically determined pK_a values (base protonation) for the "stretched-out" analogs of adenine nucleotides in aqueous solution: *lin*-benzoadenosine triphosphate, pK_a, 7.1 (6.6 in presence of 5 m*M* Mg^{2+}); *lin*-benzoadenosine diphosphate, pK_a 7.3 (6.9 in presence of 5 m*M* Mg^{2+}); *lin*-benzoadenosine monophosphate, pK_a, 7.6 (unchanged in 5 m*M* Mg^{2+}); *lin*-benzoadenosine 3′,5′-phosphate, pK_a, 5.6 (unchanged in 5 m*M* Mg^{2+}); and *lin*-benzoadenosine, pK_a, 5.6 (unchanged in 5 m*M* Mg^{2+}). When intramolecular interaction of the phosphate and the base is not possible, as in *lin*-benzoadenosine 3′,5′-monophosphate, no change in pK_a is observed with respect to that of *lin*-benzoadenosine. These data suggest participation of the phosphates in base protonation. The lowering of the pK_a values in the presence of 5 m*M* Mg^{2+} indicates the formation of magnesium chelate complexes with the polyphosphate residues of *lin*-benzoadenosine di- and triphosphates. At the *p*H at which all our enzyme studies have been conducted (*p*H 7.5, 5 m*M* Mg^{2+}) the "stretched-out" base moiety of the di- and triphosphates can be considered mainly in the unprotonated form.

In acidic aqueous solutions of *lin*-benzoadenosine triphosphate, the fluorescence emission shifted to longer wavelength—385 nm compared to 372 nm (corrected values) for the unprotonated form, pK_a* being close to the ground state pK_a—with little change in quantum yield. This makes the usefulness of the compound equally satisfactory over a wide *p*H range. The second protonation of the system takes place at approximately *p*H 1. The presence of the long wavelength absorption band permits excitation of the fluorophore without interference from any other ultraviolet-absorbing species in proteins and nucleic acids. The fluorescence emission spectra are also sensitive to the presence of divalent cations. The addition of 5 m*M* Mg^{2+} alters the pK_a* of *lin*-benzoadenosine triphosphate, as judged by the fluorescence emission spectra, without incurring significant change in fluorescence intensity. Fluorescence quenching by Co^{2+} in the case of *lin*-benzoadenosine di- and triphosphates is also indicative of divalent metal ion complexes with the polyphosphate residues and their interaction with the fluorophore.

We have been interested in intramolecular base-stacking interactions of the heteroaromatic bases of nucleic acids (*17*). With a variety of *lin*-benzoadenine derivatives on hand, we therefore investigated the magnitude of the interaction between two of the tricyclic ring systems. The percentages of hypochromism for P^1,P^2-di-*lin*-benzoadenosine-5′-pyrophosphate (**3**) and P^1, P^2-diadenosine-5′-pyrophosphate were compared by means of hydrolytic cleavage with snake venom phosphodiesterase and were found to be 23 and 9 percent, respectively, at *p*H 8.5 and 25°C. The hydrolytic cleavage of P^1,P^2-di-*lin*-benzoadenosine-5′-pyrophosphate also results in an increase in fluorescence intensity of approximately two orders of magnitude (Fig. 1). This dramatic change in fluorescence yield indicates that the *lin*-benzoadenosine moieties, when connected intramolecularly, can form dark complexes and can undergo intramolecular collisional quenching. It is therefore predictable that intramolecular positioning of a *lin*-benzoadenosine system in close proximity to other moieties—for example, nicotinamide and isoalloxazine—will result in fluorescence quenching.

Pilot studies indicate that *lin*-benzoadenosine 5′-diphosphate acts as a substrate for primer-independent polynucleotide phosphorylase (*Micrococcus luteus*) in the presence of Mn^{2+}. The polymeric material isolated by gel chromatography was essentially nonfluorescent, and its long-wavelength band in the ultraviolet spectrum lacked the characteristic fine structure of the monomeric species (Fig. 1) and was broadened. The fluorescence and the fine structure of the long-wavelength absorption band returned on treatment with 0.1*M* KOH at 100°C or as a result of enzymatic cleavage (*18*), showing again the strong stacking interaction between tricyclic base units. It should now be possible to study polynucleotide binding for the purpose of testing new complementarity relationships.

For the enzymes investigated, the formal insertion of a benzene ring in the center of the adenine nucleotides does not greatly diminish their binding properties with respect to those of the normal nucleotides, but usually decreases the rate of reaction. Overall, the series **1a** to **1d** and **2** exhibit significant biological activity, varying with different enzymes. In addition, the useful fluorescence properties of *lin*-benzoadenine nucleotides and their increased π interactions can be directed to many studies of static and dynamic interactions with different moieties, complexation, the nature of enzyme binding sites, and conformational changes induced by surrogate coenzyme-enzyme binding.

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