Enzymatic Properties of 8-Bromoadenine Nucleotides†

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ABSTRACT: 8-Bromoadenine nucleotides were tested as potential substrates and/or inhibitors of mitochondrial processes in intact or disrupted organelles, as substrates of various phosphotransferases, and as allosteric effectors in the reactions catalyzed by phosphofructokinase, isocitrate dehydrogenase, glutamate dehydrogenase, and fructose-1,6-bisphosphatase. 8-BrATP and 8-BrADP are not recognized by the translocase system located in the inner mitochondrial membrane and cannot be used as substrates in oxidative phosphorylation and related reactions catalyzed by beef heart submitochondrial membranes. This confirms the high specificity for adenine nucleotides of the mammalian systems involved in energy-yielding and energy-requiring reactions. However, 8-BrATP and 8-BrADP are able to substitute for the natural adenine nucleotides in reactions catalyzed by many phosphotransferases, although their capacity as phosphate donors and acceptors is generally much reduced. On the other hand, in almost all investigated cases, the 8-bromoadenine nucleotides have lost the capability of the natural adenine nucleotides to act as allosteric effectors, indicating that the structural requirements for allosteric activity are more stringent than those for catalytic activity.

Among the various structural requirements determining the specific interactions of adenine nucleotides with individual enzymatic systems, an important factor is the relative position of the base to the ribose moiety. It is generally agreed that natural adenine nucleotides prefer the anti conformation in aqueous solutions (Ikehara et al., 1972; Sundaralingam, 1975). 8-Substituted purine nucleotides occupy a special place among the nucleotide analogues: (1) because a bulky substituent in this position can seriously alter the normal conformation of the glycosidic linkage (Tavale & Sobell, 1970; Uesugi & Ikehara, 1977), thus a priori affecting the specificity of binding to different enzymatic systems, and (2) because this position is easily accessible for chemical reactions, thus leading to the availability of a large number of synthetic analogues. Recently, Takenaka et al. (1978) studied a large number of syn and anti conformers of 8-substituted ATP analogues, including 8-BrATP,[1] in relation to the hindrance of rotation about the glycosidic bond. All 8-substituted ATP analogues were unable to cause actin activation of myosin NTPase, superprecipitation of actomyocin, or myofibrillar contraction. It is interesting, however, that 8-bromoadenine nucleotides can be quite successful in replacing the natural nucleotides with certain enzymatic systems, such as NDP kinase (Nagel et al., 1976; Kezdi et al., 1976), beef liver glutamate dehydrogenase (Koberstein et al., 1976; Lascu et al., 1977), or muscle phosphorylase *b* (Morange et al., 1976), but are unable to substitute the natural adenine nucleotides as substrates or allosteric effectors with other enzymatic systems, such as rabbit muscle phosphofructokinase (Bârzu et al., 1977), rabbit muscle pyruvate kinase (Koberstein & Sund, 1975; Bârzu et al., 1976a) or leucine–tRNA synthetase from *Escherichia coli* (Marutzky et al., 1976).

In the present investigation we explore the interaction of 8-bromoadenine nucleotides with a large variety of enzymatic systems, extending beyond the commonly studied phosphotransferases. For a better function–structure correlation, the effect of these nucleotide analogues is often studied in comparison with that of other base-modified adenine nucleotides, including 8-azidoadenine nucleotides, which are increasingly used as photoaffinity reagents.

Materials and Methods

Chemicals. The following commercially available biochemicals were obtained from Boehringer, Mannheim: ATP, ADP, AMP, GTP, GDP, dAMP, cAMP, 8-Br-cAMP, AMP-P(NH)P, NAD^+, NADH, Ap_5A, phosphoenolpyruvate, lactate dehydrogenase (EC 1.1.1.27), α-glycerolphosphate dehydrogenase (EC 1.1.1.8), glutamate dehydrogenase (EC 1.4.1.3), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), adenylate kinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), phosphoglycerate kinase (EC 2.7.2.3), aldolase (EC 4.1.2.13), and triosephosphate isomerase (EC 5.3.1.1). The 8-bromoadenine nucleotides 8-BrAMP, 8-BrADP, and 8-BrATP were obtained through bromination of the corresponding adenine nucleotides in aqueous solution (Ikehara et al., 1972). The 8-bromoinosine analogues 8-BrIMP, 8-BrIDP, and 8-BrITP were prepared from the corresponding 8-bromoadenine nucleotides by gentle oxidative deamination (Lascu et al., 1977). The 1,N^6-etheno analogues ϵADP and ϵATP were synthesized according to the method of Secrist et al. (1972) and were a gift of Professor Klingenberg, Munich, Germany. The N^1-oxide analogues o^1ADP and o^1ATP were obtained from ADP and ATP by oxidation with permaleic acid, and the isoguanine analogue iGTP was obtained by a photoisomerization of

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[1] Abbreviations used: 8-BrAMP, 8-BrADP, and 8-BrATP, 8-bromoadenosine 5′-mono-, 5′-di-, and 5′-triphosphate; 8-Br-cAMP, 8-bromoadenosine 3′,5′-monophosphate; 8-N_3AMP, 8-azidoadenosine 5′-monophosphate; 8-BrIMP, 8-BrIDP, and 8-BrITP, 8-bromoinosine 5′-mono-, 5′-di-, and 5′-triphosphate; 8-N_3IMP, 8-azidoinosine 5′-monophosphate; iGMP, iGDP, and iGTP, isoguanosine 5′-mono, 5′-di-, and 5′-triphosphate; ϵADP and ϵATP, 1,N^6-ethenoadenosine 5′-di- and 5′-triphosphate; o^1ADP and o^1ATP, adenosine N^1-oxide 5′-di- and 5′-triphosphate; AMPS, adenosine 5′-phosphorothionate; AMP-P(NH)P, 5′-adenylyl (β,γ-imido)diphosphate; AMP-P(CH_2)P, 5′-adenylyl (β,γ-methylene)diphosphonate; NDP and NTP, nucleoside 5′-di- and 5′-triphosphate; Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate; PEP, phosphoenolpyruvic acid; F6P, fructose 6-phosphate; PGA, 3-phosphoglyceric acid; FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; NAD–ICDH, NAD-linked isocitrate dehydrogenase; FDP, fructose 1,6-diphosphate; FDPase, fructose-1,6-bisphosphatase.

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o[1]ATP (Mantsch et al., 1975). 8-Azido-AMP was obtained by heating 8-BrAMP to 75 °C with tri-*n*-butylamonium azide in anhydrous dimethylformamide (Haley & Hoffman, 1974). The ion chromatographic separation as well as the purity check of the individual nucleotides was similar to that described in previous publications (Mantsch et al., 1975; Bârzu et al., 1976a, 1977; Lascu et al., 1977).

Biochemical Assays. Heavy beef heart mitochondria were prepared as described by Löw & Vallin (1963), stored for 2–7 days at −10 °C, and thawed only shortly before use. EDTA particles were prepared as described by Lee & Ernster (1967), and MgATP particles were prepared according to Löw & Vallin (1963). Oxidative phosphorylation, the ATP-linked reduction of NAD^+ by succinate, and the ATPase activity of beef heart submitochondrial particles were assayed essentially as described by Penefsky (1974). The isolation of rat liver mitochondria, the measurement of mitochondrial respiration, and the exchange of intramitochondrial ^{14}C-labeled adenine nucleotides with externally added nucleotide analogues were performed as described in previous publications (Jebeleanu et al., 1974; Mantsch et al., 1975). Rat kidney nucleoside diphosphate kinase was obtained according to Lascu and Bârzu (unpublished), pig kidney FDPase was obtained as described by Colombo et al. (1972), rabbit muscle phosphoglycerate kinase was obtained according to Krietsch & Bücher (1970), and yeast pyruvate kinase was obtained as described by Yun et al. (1976).

For the following enzymes, adenylate kinase, hexokinase, pyruvate kinase, nucleoside diphosphate kinase, phosphofructokinase, phosphoglycerate kinase, glutamate dehydrogenase, and NAD–ICDH, the activity was measured in systems involving the interconversion of pyridine nucleotides. All enzymatic rates were determined at 25 °C in 1-mL final volume either at 366 nm by using an Eppendorf 1101 M photometer equipped with a W+W 4410 type recorder (full-scale deflection 0.25 absorbance unit) or at 340 nm with a Gilford 2400 spectrophotometer equipped with a 2400 S type recorder (full-scale deflection 0.1 absorbance unit). The reaction was triggered by addition of the pure or crude enzymes to be assayed, in an amount sufficient to yield ΔA values between 0.02 and 0.1 per min at 366 or 340 nm. For the various assays, the reaction medium is as follows (final concentrations). (a) Adenylate kinase assay: 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.15 mM NADH, 6 units of pyruvate kinase, 3 units of lactate dehydrogenase, 0.2 mM AMP, different concentrations of ATP or 8-BrATP, and rabbit muscle adenylate kinase. (b) Hexokinase assay: 50 mM triethanolamine (pH 8.0), 5 mM glucose, 6 mM $MgCl_2$, 0.4 mM $NADP^+$, 1.8 units of glucose-6-phosphate dehydrogenase, different ATP and 8-BrATP concentrations, and yeast hexokinase. (c) Pyruvate kinase assay: 50 mM Tris-HCl (pH 7.4) or imidazole (pH 8.0), 80 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH, different phosphoenolpyruvate concentrations (0.03–10 mM), 10 units of lactate dehydrogenase, different ADP or 8-BrADP concentrations, and rabbit muscle or yeast pyruvate kinase [when necessary, fructose 1,6-diphosphate (1 mM) was included in the reaction medium]. (d) Phosphoglycerate kinase assay: 50 mM triethanolamine (pH 7.6), 10 mM $MgCl_2$, 0.15 mM NADH, 10 mM PGA, 0.1 mM EDTA, 3 units of glyceraldehyde-3-phosphate dehydrogenase, different ATP and 8-BrATP concentrations, and rabbit muscle or yeast phosphoglycerate kinase. (e) Nucleosidediphosphate kinase assay: 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.1 mM NADH, 0.5 mM ATP, 1.5 units of pyruvate kinase, 1.5 units of lactate dehydrogenase, different 8-BrADP or 8-BrIDP concentrations, and rat liver or kidney NDP-kinase. (f) Phosphofructokinase assay: 40 mM imidazole buffer (pH 7.0), 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.05 mM NADH, 0.1 mg of bovine serum albumin, 0.8 unit of triosephosphate isomerase, 0.8 unit of α-glycerolphosphate dehydrogenase, 1.5 units of aldolase, 0.2 mM F6P, different ATP and 8-BrATP concentrations, and rabbit muscle phosphofructokinase. (g) Glutamate dehydrogenase assay: 80 mM Tris–acetate buffer (pH 8.0), 0.08 mM NADH, 0.01 mM EDTA, 100 mM NH_4Cl, 50 mM α-oxoglutarate, different ADP and 8-BrADP concentrations, and beef liver glutamate dehydrogenase. (h) NAD–ICDH assay: 50 mM Tris-HCl (pH 7.4), 200 mM sucrose, 1 mM $MnCl_2$, 1 μg of rotenone, 0.25 mM NAD^+, 1–10 mM sodium isocitrate, different ADP and 8-BrADP concentrations, and 0.1–0.2 mg of rat liver mitochondrial protein (mitochondria were previously lysed with Lubrol WX by using 0.5 mg/mg protein). The formation of NADH was followed with an Eppendorf fluorometer with the appropriate filter combinations (excitation filter 366 nm; emission filter 470–3000 nm). (i) FDPase assay: The activity was followed by the rate of inorganic phosphate liberation and measured according to the method of Lowry & Lopez (1946). The reaction medium contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2.5 mM $MgCl_2$, 133 mM KCl, 0.4 mM FDP, different nucleotide concentrations, and purified pig kidney enzyme.

Experimental details, if different from those given here, are mentioned in the legends to the figures and the tables.

Results and Discussion

Effect of 8-Bromoadenine Nucleotides on Mitochondrial Oxidative Phosphorylation and Related Reactions. While only ADP is able to stimulate mitochondrial respiration, 8-BrADP, similar to other nucleotide diphosphate analogues or to the natural purine diphosphates GDP, IDP, and XDP, does not stimulate mitochondrial respiration of intact organelles. However, if 8-BrADP is added to mitochondria after the state-3 to state-4 transition, state-3 being induced by 0.15 mM ADP, an immediate stimulation of respiration can be observed. The rate of respiration stimulation by 8-BrADP is strongly dependent on the Mg^{2+} concentration in the medium as well as on the 8-BrADP concentration itself (Figure 1). In the absence of exogenous Mg^{2+}, 8-BrADP, similar to the previously investigated nucleotide analogues o[1]ADP and ϵADP (Kezdi et al., 1973; Bârzu et al., 1976b), has no effect at all on mitochondrial respiration. However, unlike the situation with o[1]ADP and ϵADP, increasing concentrations of 8-BrADP lead to a progressive inhibition of mitochondrial respiration. This effect is analogous to that observed with GDP (Pedersen, 1973).

These experimental results can be explained as follows: the stimulation of mitochondrial respiration through addition of 8-BrADP after the state-3 to state-4 transition must be due to the coupling of the respiratory chain linked ADP phosphorylation on the inner side of the inner mitochondrial membrane to the reaction catalyzed by nucleoside diphosphate kinase on the outer side of the inner mitochondrial membrane. In the absence of exogenous Mg^{2+}, the phosphorylation of ADP is not affected, but the transphosphorylation of 8-BrADP via the nucleoside diphosphate kinase reaction is blocked. Since high concentrations of 8-BrADP do not affect the phosphorylation of ADP, the effect of the analogue shown in Figure 1 can be considered as a consequence of the nucleoside diphosphate kinase inhibition through excess of substrate.

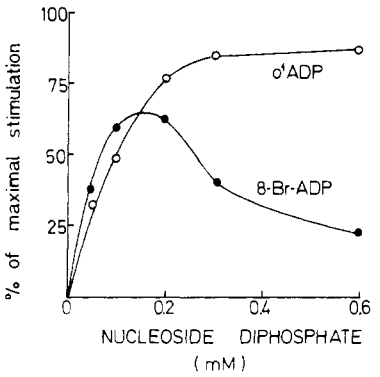

FIGURE 1: Effect of 8-BrADP on the respiratory rate of rat liver mitochondria with glutamate as the substrate. The respiratory medium contained the following at 0.5-mL final volume and 24 °C: 180 mM sucrose, 50 mM KCl, 25 mM Tris-HCl (pH 7.4), 5 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 1 mg of bovine serum albumin, and 5 mM glutamate. The respiration was initiated by addition of 0.9–1.2 mg of mitochondrial protein, and the state-4 respiration was followed for about 2 min; thereafter, 0.15 mM ADP was added to initiate state-3 respiration. After the state-3 to state-4 transition, different concentrations of 8-BrADP or o¹ADP were added, and the stimulation of respiration was recorded. The percentage rate of stimulation was calculated from the expression (8-BrADP rate − state-4 rate)/(ADP rate − state-4 rate) × 100, where the state-4 rate refers to the rate of respiration after the consumption of added ADP.

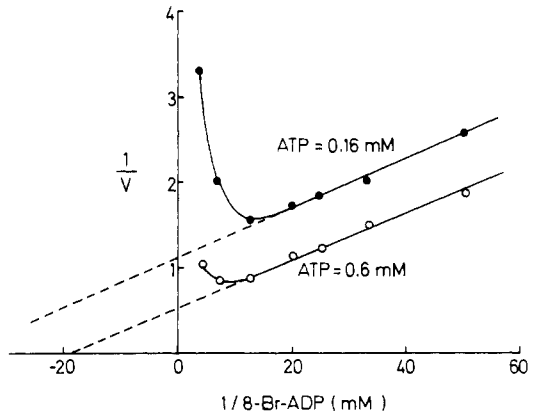

FIGURE 2: Kinetic properties of rat liver mitochondrial NDP kinase with ATP as the donor and 8-BrADP as the acceptor. Experimental conditions for the biochemical assay are described in the text. While varying the 8-BrADP concentration, we kept the ATP concentration constant at 0.6 mM (○) or at 0.16 mM (●). The reaction was started with 10–20 μg of protein of partially purified mitochondrial NDP kinase [for details see Jebeleanu et al. (1974)].

In order to verify this supposition, we have investigated the reaction kinetics of 8-BrADP phosphorylation at various donor (ATP) and acceptor concentrations, using partially purified nucleoside diphosphate kinase obtained from rat liver mitochondrial preparations. From the double-reciprocal plots in Figure 2, a value of 3.1 μmol/(min·mg of protein) was calculated for V_{max} (at infinite ATP and 8-BrADP concentrations) along with a K_m^{ATP} of 0.38 mM and a $K_m^{8\text{-BrADP}}$ of 0.09 mM. As clearly shown by the 0.16 mM ATP sample in Figure 2, 8-BrADP concentrations in excess of 0.1 mM show a strong inhibition of the enzyme activity. This inhibition can be reversed by high concentrations of ATP.

The lack of 8-BrADP phosphorylation by the respiratory chain enzymes can either be due to the fact that this analogue is not translocated across the inner mitochondrial membrane or be due to the fact that it is not a substrate for the oxidative phosphorylation, or both. The results in Table I demonstrate that the exchange between internal [^{14}C]ADP and externally added 8-BrADP is negligible compared to the exchange of

Table I: Exchange of Intramitochondrial ^{14}C-Labeled ADP with Extramitochondrial 8-BrADP[a]

additions	[^{14}C]ADP exchanged (%)
50 μM ADP	100
50 μM 8-BrADP	<1
200 μM 8-BrADP	<1
50 μM ADP + 50 μM 8-BrADP	96
50 μM ADP + 200 μM 8-BrADP	82
50 μM ADP + 1000 μM 8-BrADP	60

[a] Rat liver mitochondria (4–5 mg of protein) which had been previously loaded with [^{14}C]ADP were incubated for 10 min at 2 °C in 110 mM KCl, 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA at a final volume of 0.4 mL. The reaction was triggered by addition of ADP or 8-BrADP. When the competition between ADP and 8-BrADP was investigated, the 8-bromo analogue was added to the mitochondria 2 min prior to the addition of ADP. The incubation was stopped by addition of 100 μM atractyloside; after centrifugation at 20000*g* for 5 min, 0.2-mL aliquots of the supernatant were used for liquid scintillation counting.

Table II: Hydrolysis of Base-Modified ATP Analogues by Different Mitochondrial Preparations[a]

	rat liver lubrol particles		beef heart EDTA particles	
nucleotide (0.1 mM)	μmol/(min·mg of protein)	ATPase act. (%)	μmol/(min·mg of protein)	ATPase act. (%)
ATP	0.360	100[b]	0.432	100[b]
8-BrATP	0.162	45.0	0.037	8.6
ϵATP	0.237	65.8	0.068	15.7
o¹ATP	0.188	52.2	0.025	5.8
iGTP	0.305	84.7	0.168	38.9

[a] The test medium contained the following: 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4), 0.1 mM NADH, 1 mM PEP, 1 mM KCN, 1 unit of pyruvate kinase, and 0.1 mM ATP or 0.1 mM of the corresponding ATP analogues. The reaction was initiated by addition of 5–50 μg of submitochondrial particles. Since 8-BrADP and o¹ADP have low reaction rates with pyruvate kinase, when these analogues were used as substrates, the reaction medium contained an additional 30 units of the coupling enzyme (previously desalted by extensive dialysis), in order to make this not a rate-limiting step. [b] Relative values, the ATPase activity of the natural nucleotide being considered as 100%

ADP. These results are in agreement with data obtained by Schlimme & Stahl (1974) and Schlimme et al. (1977), which show that 8-BrATP and 8-BrADP undergo both specific (atractyloside-sensitive) and unspecific binding to rat liver mitochondria, but there was no detectable exchange across the inner mitochondrial membrane. As shown in Table I, a high concentration of 8-BrADP (1 mM) reduces the exchange of external ADP by about 40%, indicating that the analogue, which is unable to penetrate the inner mitochondrial membrane, can engage in a more or less specific binding to the site of the ADP translocase; this effect apparently has no limiting consequences on the rate of exogenous ADP phosphorylation, since the stimulated ADP respiration is not reduced, even by high concentrations of the 8-BrADP analogue. It is interesting that the phosphate-modified ATP analogue, AMP-P(NH)P, which is a very potent inhibitor of the exchange between extra- and intramitochondrial ADP or ATP (Melnick & Donohue, 1976), also does not affect the oxidative phosphorylation of ADP (Penefsky, 1974). Such effects could possibly be evidenced at much lower ADP concentrations (10 μM) if the oxygraphic or radioisotopic method would be used in the presence of a trapping system containing glucose and hexokinase, as described by Schuster et al. (1977) for the case of AMP-P(CH_2)P.

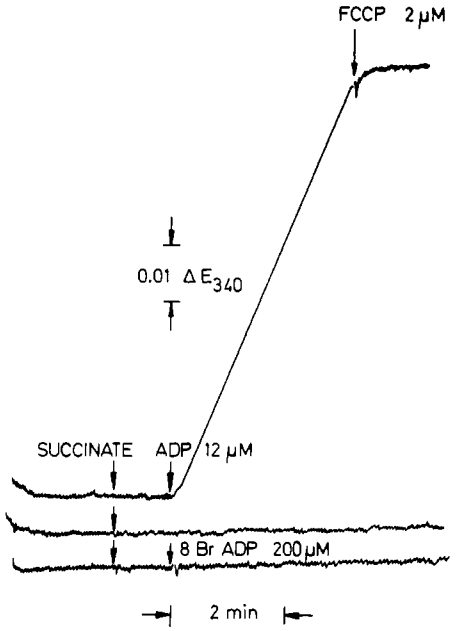

FIGURE 3: Participation of ADP and 8-BrADP in the reactions of oxidative phosphorylation, catalyzed by beef heart MgATP particles. The reaction medium contained the following at 24 °C in 1 mL: 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 5 mM potassium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 2 mg of bovine serum albumin, 0.5 mM EDTA, 5 mM glucose, 0.4 mM $NADP^+$, 5 units of hexokinase, 1.8 units of glucose-6-phosphate dehydrogenase, 0.05 mM Ap_2A, 1 μg of rotenone, and 0.091 mg of protein of MgATP particles. The submitochondrial particles were incubated for 3 min, and the reaction was initiated by addition of 5 mM succinate and the indicated amounts of ADP and 8-BrADP, respectively.

The hydrolysis of 8-BrATP by different mitochondrial ATPases is compared in Table II with the hydrolysis of ATP and that of other base-modified nucleotides. Lubrol particles obtained from rat liver mitochondria (which resemble in many respects sonicated mitochondria) and beef heart EDTA particles were used to test the analogues. While submitochondrial rat liver preparations were able to hydrolyze all investigated nucleoside triphosphates with a similar rate, beef heart submitochondrial preparations showed large differences in the rates of hydrolysis.

Finally, we turned our attention to a quite controversial subject, the substrate specificity of oxidative phosphorylation with ADP as the phosphate acceptor. It is known that, in the absence of nucleoside diphosphate phosphorylation other than ADP, the decisive step is dictated by the translocating system which has an almost absolute specificity for the adenine moiety. While Hohnadel & Cooper (1972) could show that sonicated rat liver mitochondrial membranes are able to make use of other natural or synthetic ATP analogues as acceptors in the mechanism of oxidative phosphorylation, we have shown in previous experiments (Bârzu et al., 1976b) that o^1ADP and ϵADP cannot act as phosphate acceptors in the reactions of oxidative phosphorylation catalyzed by lubrol particles obtained from rat liver mitochondria. Therefore, along with 8-BrADP, we also investigated the above-mentioned analogues in their behavior toward MgATP particles obtained from beef heart mitochondria (Figure 3). With the assay system we were using ($NADP^+$ reduction in the presence of excess glucose, hexokinase, and glucose-6-phosphate dehydrogenase), the presence of contaminating mitochondrial adenylate kinase (V_{max} = 70.4 nmol/(min·mg of protein) and K_m^{ADP} = 27 μM) had to be eliminated. This was achieved by addition of Ap_5A to the reaction medium; 50 μM Ap_5A reduced the adenylate kinase reaction rate already under 5% at an ADP concentration of 40 μM. Due to the high affinity of the system of oxidative phosphorylation for ADP (K_m = 5 μM), there is no need for high nucleoside diphosphate concentrations. Therefore, at an ADP concentration of 12 μM, the $NADP^+$ reduction is entirely due to the process of oxidative phosphorylation. As shown in Figure 3, the reduction of $NADP^+$ is completely inhibited by 2 μM FCCP. Under identical conditions, a much higher concentration of 8-BrADP (and of ϵADP or o^1ADP, not shown in this figure), does not affect the rate of $NADP^+$ reduction at all, thus indicating that these base-modified ADP analogues cannot act as phosphate acceptors in the reactions of oxidative phosphorylation catalyzed by beef heart submitochondrial particles. An additional argument in favor of this conclusion, supported by the results in Table III, is that none of the investigated analogues is able to support the energy-dependent NAD^+ reduction by succinate with beef heart MgATP particles. The above data confirm and extend our previous results regarding the high specificity of the oxidative phosphorylation system for ADP.

Table III: Effect of Base-Modified ATP Analogues on the Energy-Dependent NAD^+ Reduction by Succinate with Beef Heart Submitochondrial Particles[a]

nucleotides	nmol of NAD^+ reduced per min per mg of protein
0.06 mM ATP	13.6
0.60 mM ATP	40.9
0.50 mM 8-BrATP	0.0
0.50 mM ϵATP	0.0
0.50 mM o^1ATP	0.0
0.06 mM ATP + 0.50 mM 8-BrATP	7.7
0.06 ATP mM + 0.50 mM ϵATP	5.2
0.06 ATP mM + 0.50 mM o^1ATP	7.7

[a] The reaction medium contained the following at 24 °C in 1 mL: 200 mM sucrose, 50 mM KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM NAD^+, 1 mM KCN, 10 mM succinate, and 0.15–0.25 mg of protein of MgATP particles. The submitochondrial particles were incubated for 3 min, and the reaction was started by addition of nucleotides at the indicated concentrations.

8-Bromoadenine Nucleotides as Substrates for Soluble Phosphotransferases. From the kinetic constants of different phosphotransferases by using 8-BrATP and 8-BrADP as substrate substitutes (Table IV), it results that these analogues can mimic the natural adenine nucleotides, although their capacity as phosphate group donors or acceptors is much reduced. Most mammalian soluble phosphotransferases use 8-BrATP and 8-BrADP with an efficiency of 1–5% compared to that of ATP and ADP, respectively. An exception is nucleoside diphosphate kinase, which has a very low specificity for nucleotides as acceptors or donors, and can use the 8-bromo analogues with almost the same efficiency as the natural substrates. It is also interesting to note that the yeast enzymes hexokinase and phosphoglycerate kinase are better "adapted" to use 8-bromoadenine nucleotides (with 8–22% efficiency) than the mammalian enzymes. We shall take now a more detailed look at some of these enzymatic systems.

(*1*) *Adenylate Kinase.* 8-BrATP and 8-BrADP are easily interconverted via the reaction catalyzed by rabbit muscle adenylate kinase; however, under no experimental condition could we observe the phosphorylation of 8-BrAMP, regardless whether ATP or other triphosphates were used as phosphate donors. This is in disagreement with the observation of Lee & Kaplan (1975), who found 8-BrAMP to be a substrate for adenylate kinase, with an acceptor capacity of approximately 8% that of AMP. Since their conclusion was based on kinetic results, we performed the following experiment to check this

Table IV: Kinetic Constants of Different Phosphotransferases for 8-Bromoadenine Nucleotides

enzyme	variable substrate	fixed substrate	K_m (mM)[a]	V_{max}[b] [μmol/(min·mg of protein)]	k^*/k[c]
rabbit muscle pyruvate kinase	8-BrADP	PEP (1 mM)	1.18 (0.28)	0.04 (283)	0.010
rabbit muscle phosphofructokinase	8-BrATP	F6P (0.2 mM)	0.45 (0.02)	0.57 (48)	0.019
yeast pyruvate kinase	8-BrADP	PEP (5 mM)	0.73 (0.12)	0.21 (52)	0.035
rabbit muscle adenylate kinase	8-BrATP	AMP (0.2 mM)	0.67 (0.16)	0.17 (357)	0.041
rabbit muscle phosphoglycerate kinase	8-BrATP	PGA (10 mM)	1.00 (0.40)	0.13 (387)	0.052
yeast hexokinase	8-BrATP	glucose (5 mM)	0.68 (0.14)	0.40 (140)	0.082
yeast phosphoglycerate kinase	8-BrATP	PGA (10 mM)	0.25 (0.36)	0.15 (671)	0.216
beef liver nucleoside diphosphate kinase[d]	8-BrATP	dGDP (0.6 mM)	1.07 (0.59)	1.58 (53)	0.871

[a] Values in parentheses refer to the apparent K_m for normal substrates. [b] These maximal velocities are relative to that of the normal substrate. In parentheses are given the actual V_{max} values for normal substrates as micromoles per minute per milligram of protein. [c] k and k^* represent the relative V_{max}/K_m ratios for ATP (or ADP) and its 8-bromo analogue. The k^*/k ratio is a better measure than the corresponding K_m value for defining the efficiency of substrate analogues. [d] Taken from Nagel et al. (1976).

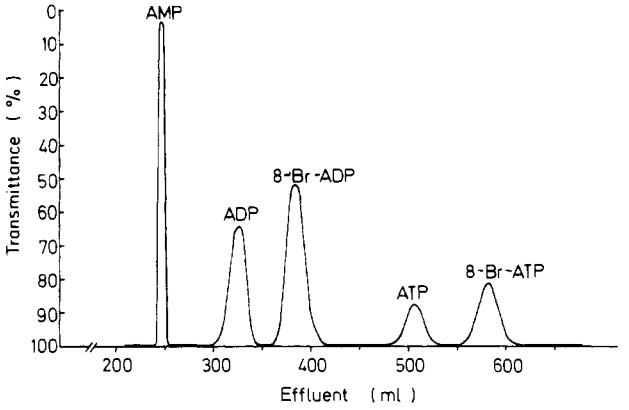

FIGURE 4: Interconversion of adenine nucleotides and 8-bromoadenine nucleotides catalyzed by rabbit muscle adenylate kinase. The reaction medium contained the following in a final volume of 1 mL: 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 2 mM 8-BrATP, and 2 mM AMP. After incubation for 30 min at 25 °C with 10 units of purified rabbit muscle adenylate kinase, the individual nucleotides were separated by column chromatography (1 × 50 cm) on Sephadex A-50, using a linear gradient from 0.1 to 0.5 M of NaCl in 0.1 M acetate–acetic acid buffer (pH 4.1) at a flow rate of 0.5 mL/min.

discrepancy. In three separate test experiments, equimolecular concentrations (2 mM) of ATP plus 8-BrAMP, AMP plus 8-BrATP, and ADP plus 8-BrADP were incubated in the presence of excess rabbit muscle adenylate kinase, sufficient to reach equilibrium in 30 min at 25 °C. The reaction products were then subjected to chromatographic separation in order to identify and quantify the individual products. In all three cases, over 90% of the added nucleotides were recovered. After the equilibrium was established, in the first case we could identify only the initial nucleotides ATP and 8-BrAMP. In the second and third case, at equilibrium we always could identify five individual peaks. These are shown in Figure 4 and correspond to the nucleotides ATP, ADP, AMP, 8-BrATP, and 8-BrADP. Neither in the second case nor in the third case could we detect 8-BrAMP.

The following possible equilibria have to be considered:

$$\text{ADP} + \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \quad (1)$$

$$\text{8-BrADP} + \text{ADP} \rightleftharpoons \text{8-BrATP} + \text{AMP} \quad (2)$$

$$\text{8-BrADP} + \text{ADP} \rightleftharpoons \text{ATP} + \text{8-BrAMP} \quad (3)$$

$$\text{8-BrADP} + \text{8-BrADP} \rightleftharpoons \text{8-BrATP} + \text{8-BrAMP} \quad (4)$$

The corresponding equilibrium constants are as follows: $K_1 = [\text{ATP}][\text{AMP}]/[\text{ADP}]^2$; $K_2 = [\text{8-BrATP}][\text{AMP}]/([\text{8-BrADP}][\text{ADP}])$; $K_3 = [\text{ATP}][\text{8-BrAMP}]/([\text{8-BrADP}][\text{ADP}])$; $K_4 = [\text{8-BrATP}][\text{8-BrAMP}]/[\text{8-BrADP}]^2$. It is immediately evident that out of the four possible equilibria, only eq 1 and 2 are compatible with the results in Figure 4. Following the identification of the individual five nucleotides in Figure 4, their concentration was determined by using the following millimolar extinction coefficients: $\epsilon_{260} = 15.4$ for the natural adenine nucleotides and $\epsilon_{265} = 14.5$ for the 8-bromoadenine nucleotides. The equilibrium concentrations for the natural nucleotides were 0.24 mM ATP, 0.60 mM ADP, and 1.19 mM AMP, leading to an equilibrium constant of $K_1 = 0.74$ for eq 1. The corresponding equilibrium concentrations for the two 8-bromoadenine nucleotides were 0.52 mM 8-BrATP and 1.31 mM 8-BrADP, leading to $K_2 = 0.75$ for the equilibrium in eq 2.

The results of the above test experiments support our conclusion that 8-BrAMP is not a substrate for rabbit muscle adenylate kinase. Actually, with the exception of tubercidine 5′-monophosphate (Bloch et al., 1967) and formycin 5′-monophosphate (Ward et al., 1969), all base-modified adenine nucleotides which we tested so far were unable to bind to the AMP site of this enzyme. All our experimental results indicate that the integrity of the adenine moiety is a critical requirement for nucleoside monophosphates to function as phosphate acceptors in the reactions catalyzed by rabbit muscle adenylate kinase. Also, to avoid false conclusions regarding the substrate capacity of synthetic nucleotide analogues, we consider it absolutely necessary to perform preliminary tests under equilibrium conditions; an analogue containing less than 1% of the natural compounds as impurity, an amount which is found often in commercial samples, can lead to false conclusions in the kinetic measurements.

(2) *Pyruvate Kinase.* While studying the inhibition of mammalian pyruvate kinase by 8-BrATP, we also included in this investigation iGTP, a 3-hydroxy analogue of ATP, in order to possibly distinguish between the effect of base modification (iGTP) and that of changing the conformation of the glycosidic linkage (8-BrATP). 8-BrATP and iGTP are both potent inhibitors of pyruvate kinase from rabbit muscle. The inhibitory effect of ATP first demonstrated by Reynard et al. (1961) is due primarily to the preferential complexation by ATP of Mg^{2+} ions which are required to "activate" the substrate ($MgADP^-$). Yet, according to Wood (1968), an increased Mg^{2+} concentration can reduce the inhibitory effect of ATP quite drastically. Therefore, we also investigated the effect of varying Mg^{2+} concentrations on the rabbit muscle pyruvate kinase inhibition by 8-BrATP and iGTP (Figure 5). As in the case of ATP, the inhibition by 8-BrATP of pyruvate kinase depends on the concentration of Mg^{2+}, with the difference that above a certain Mg^{2+} concentration no further decrease of the inhibition could be observed. In the case of the iGTP analogue, a nucleotide concentration of 1 mM

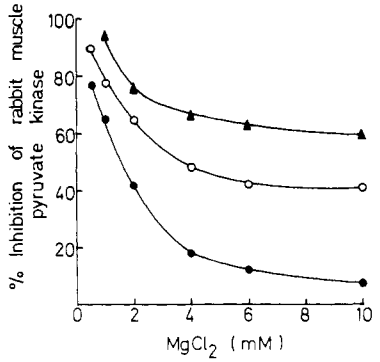

FIGURE 5: Inhibition of rabbit muscle pyruvate kinase by 5 mM ATP (●), 2 mM 8-BrATP (○), and 1 mM iGTP (▲) as a function of $MgCl_2$ concentration. Phosphoenolpyruvate and ADP concentrations were kept constant at 1 mM each. The enzyme assay is described under Materials and Methods.

Table V: Inhibition Constants for Rabbit Muscle Pyruvate Kinase[a]

	PEP fixed substrate (0.5 mM), ADP variable substrate			ADP fixed substrate (0.5 mM), PEP variable substrate
nucleotide	K_i^{NTP} (mM)	K_m^{NDP} (mM)	K_i^{NTP}/K_m^{NDP}	K_i^{NTP} (mM)
ATP	1.81	0.28	6.46	1.16
8-BrATP	0.49	1.18	0.42	1.04
iGTP	0.17	0.41	0.41	0.19

[a] Experimental conditions are described in the text. The K_i^{NTP} values were obtained from a plot of $1/v$ vs. $1/[PEP]$ (or $1/[ADP]$) in the presence (0.1–2 mM) or absence of the corresponding nucleotide triphosphates. The $MgCl_2$ concentration in the medium was varied such that the free Mg^{2+} concentration was kept constant at 4 ± 0.1 mM.

produces an inhibition of 60% which is practically independent of Mg^{2+} concentrations above 4 mM.

The inhibitory constants for 8-BrATP and iGTP in Table V reveal a competitive effect with respect to ADP and to phosphoenolpyruvate. It is surprising to find that 8-BrATP as well as iGTP has a higher affinity for pyruvate kinase than the corresponding nucleoside diphosphates, which is not the case for the natural nucleotides ATP and ADP, as shown by the K_i^{NTP}/K_m^{NDP} ratio in Table V. Accepting the mechanism proposed by Reynard et al. (1961), a concurrent superposition of the ATP molecule with the ADP site and the PEP site, and bearing in mind that the polyphosphate chain and the ribose moiety are identical for all three investigated nucleotides, we find that the K_i^{NTP}/K_m^{NDP} ratio should be constant, regardless of the nature of the individual base modification. Two possibilities could be taken into consideration. The first possibility is that the corresponding NTP's are acting not only as simple competitive inhibitors but also as inhibitors which decrease the K_m values for the substrates via conformational changes of the enzyme. This is a weak argument in the case of rabbit muscle pyruvate kinase which really has no typical allosteric properties (Kayne, 1973). A second possibility is that the conformation and consequently the properties of individual NDP's do not suffer identical modifications through addition of the third phosphate to yield the corresponding NTP's. Althought the data in Table VI do not provide conclusive evidence for this argument, they reveal that 8-BrADP binds Mg^{2+} 3 times tighter than ADP, while 8-BrATP binds Mg^{2+} only twice as tight as the natural nucleotide ATP. Regardless of the exact inhibitory mechanism and of the structural effects which cause the inhibitory capacity of NTP,

Table VI: Formation Constants (K_f) for Adenine and 8-Bromoadenine Nucleotides with Mg^{2+}

nucleotide	K_f (M^{-1})[a]
ADP	1 600 ± 100
8-BrADP	4 700 ± 120
ATP	10 200 ± 350
8-BrATP	21 400 ± 380

[a] Measurements were made at pH 8.0 and 24 °C at a final volume of 10 mL in a medium containing 50 mM KCl, 50 mM Tris-HCl, 0.05–0.3 mM nucleotide, from 0 to 2 mM $MgCl_2$, and 50 mg of Dowex 1-X4. The results are the mean of three separate experiments.

this phenomenon is real and the competitive effect is manifest both with regard to NDP and with regard to phosphoenolpyruvate.

We also investigated the inhibitory effect of 8-BrATP and iGTP with pyruvate kinase from different rat tissues, since possible differences in reactivity could lead to the identification of isoenzymes, based on a substrate specificity. We found, however, no significant differences in the inhibitory effect of these analogues with heart, brain, liver, and kidney rat pyruvate kinase. Yet it should be mentioned that we found yeast pyruvate kinase to be considerable less sensitive to inhibition by iGTP than was pyruvate kinase from mammalian tissues.

8-Bromoinosine Nucleotides. Many phosphotransferases which are nonspecific for nucleotides as substrates, such as pyruvate kinase, phosphofructokinase, or phosphoglycerate kinase, can use inosine nucleotides instead of adenine nucleotides; compared to the natural adenine nucleotides, the inosine derivatives are between 2 and 6 times weaker substrates (Scopes, 1973; Hohnadel & Cooper, 1973; Bloxham et al., 1973). Considering that the substitution of bromine in position 8 of the adenine moiety leads to a considerable decrease in the substrate capacity of 8-bromoadenine nucleotides, one would anticipate that in the case of 8-bromoinosine nucleotides the combination of two structural factors, namely, the modification of the C_6–N_1 substructure and the change in the conformation of the glycosidic linkage, should lead to analogues whose capacity to act as ADP or ATP substitutes for phosphotransferases should be absolutely negligible. This "cumulative" effect indeed seems to hold for the mammalian phosphotransferases pyruvate kinase and phosphofructokinase. For instance, 8-BrIDP is about 15 000 times weaker than ADP, 2200 times weaker than IDP, and 164 times weaker than 8-BrADP as a phosphate acceptor with rabbit muscle pyruvate kinase. We have shown recently (Kezdi et al., 1976) that these special properties of 8-BrIDP make it an extremely useful substrate for measuring the activity of NDP kinase as a phosphate acceptor in coupled systems involving pyruvate kinase and lactate dehydrogenase.

Interestingly, however, a number of yeast enzymes (hexokinase, pyruvate kinase, and phosphoglycerate kinase) do not show this cumulative effect. ITP has 1.5% of the ATP capacity as a substrate for yeast hexokinase, while 8-BrITP has 15%, which, instead of being less, is 10 times more than that of ITP and even more than that of 8-BrATP (8.2%, Table IV). These observations strengthen the supposition that the structural requirements of these phosphotransferases are complex and can differ from one species to another.

8-Bromoadenine Nucleotides as Allosteric Effectors. The specificity of various enzymatic systems for nucleotides as allosteric effectors has been much less investigated than their specificity as substrates in phosphoryl-group transfer reactions. (*1*) *Phosphofructokinase.* With phosphofructokinase, the natural nucleotide ATP is both a substrate and an allosteric

Table VII: Effect of 8-Azido-AMP on ATP-Inhibited Rabbit Muscle Phosphofructokinase[a]

ATP (mM)	cAMP (mM)	8-N_3AMP (mM)	enzyme act.[b] (%)
0.15	0	0	100
1.50	0	0	19
1.50	0.2	0	63
1.50	1.0	0	70
1.50	0	0.2	30
1.50	0	1.0	34
1.50	1.0	1.0	52

[a] Assays were carried out in imidazole buffer (pH 7.0) with 0.04 mM F6P, 6 mM $MgCl_2$, and nucleotides as indicated. [b] The activity at optimal ATP concentration (0.15 mM), corresponding to 37.8 μmol/(min·mg of protein), was considered as 100%.

inhibitor, while AMP and cAMP reactivate the ATP-inhibited enzyme. We have shown (Bârzu et al., 1977) that 8-BrATP is a weak substrate of phosphofructokinase (K_m = 450 μM), compared to the natural substrate ATP (K_m = 15 μM). This nucleotide analogue shows no effects in the inhibitory concentration range (1–10 mM), but high 8-BrATP concentrations produce an enzyme inhibition by decreasing the free Mg^{2+} concentration, necessary for optimal activity. If, however, the 8-BrATP concentration is increased simultaneously with the $MgCl_2$ concentration in the reaction medium, such that the free Mg^{2+} concentration is always kept between 1 and 2 mM, this analogue has no inhibitory effect at all on rabbit muscle phosphofructokinase. Similarly, 8-BrAMP and 8-Br-cAMP are lacking the allosteric properties of the corresponding natural nucleotides to reactive phosphofructokinase, once the enzyme is inhibited by an excess of ATP. In order to establish whether the lack of allosteric activity is connected to the unusual glycosidic conformation of 8-bromoadenine nucleotides, we have synthesized and tested the 8-azido-AMP analogue, which has the same conformation of the glycosidic bond. Interestingly, we found that 8-N_3AMP, unlike 8-BrAMP, is able to reactivate the ATP-inhibited enzyme, although to a smaller extent than AMP or cAMP (Table VII). The binding of 8-N_3AMP to phosphofructokinase seems to be of a competitive nature with respect to cAMP and also AMP; therefore, it could be considered as a possible photoaffinity reagent for the allosteric site of this enzyme. To check whether this is the case, we have incubated rabbit muscle phosphofructokinase with 8-N_3AMP and then irradiated the mixture at 2 °C for 5–30 min. The results are presented in Table VIII. A progressive reduction of the enzymatic activity was observed, but the "residual enzyme" maintained its sensitivity for inhibition by excessive ATP and for reactivation by AMP or cAMP. Since the nucleoside triphosphates show a strong protective effect against inactivation by 8-N_3AMP, while AMP and cAMP show practically no protective effect at all, we conclude that 8-N_3AMP, when irradiated, binds to the active center of phosphofructokinase, most likely via the cysteine residues present in the active center, and not to the allosteric center. This conclusion is indirectly supported by the effect of the 8-N_3IMP analogue, which in the dark does not affect the phosphofructokinase activity at all but which, if irradiated, behaves similar to 8-N_3AMP (Table VIII).

(*2*) *Fructose-1,6-bisphosphatase and NAD-Linked Isocitrate Dehydrogenase.* Adenine nucleotides are known to act as allosteric effectors in modulating the activity of these two enzymes. FDPase is inhibited by AMP (Pontremoli et al., 1968), while NAD–ICDH is activated by ADP at suboptimal concentrations of isocitrate (Atkinson, 1966). The results obtained with 8-BrAMP and 8-BrADP are summarized in Table IX. Neither with pig kidney FDPase nor with rat liver NAD–ICDH were the 8-bromoadenine nucleotides able to mimic the natural adenine derivatives as allosteric effectors. The inhibition of the FDPase activity by 12% at 1 mM 8-BrAMP is not a specific effect, since similar concentrations of IDP also produce a 10% inhibition of this enzyme. Other base-modified AMP analogues, such as o^1AMP and iGMP were also unable to inhibit the pig kidney FDPase activity at concentrations below 200 μM where the natural allosteric modifiers AMP and dAMP show an inhibitory effect of over 95%. Only the phosphate-modified analogue AMPS (Table IX) had a noticeable inhibitory effect (I_{50} = 80 μM). Very recently Marcus & Haley (1979) showed that 8-azido-AMP, like AMP, is a noncompetitive inhibitor of pig kidney FDPase and possibly a photoreactive allosteric-site-directed irreversible inhibitor of this enzyme.

(*3*) *Glutamate Dehydrogenase.* In contrast to the three allosteric enzymes discussed so far, the behavior of 8-BrADP

Table VIII: Irradiation of Rabbit Muscle Phosphofructokinase in the Presence of 8-Azido-AMP or 8-Azido-IMP and Various Natural Adenine Nucleotides[a]

nucleotides and substrates in the irradiation mixture[b]	μmol/(min·mg of protein)[c] I	II	III
none	34.4	8.4	24.4
50 μM 8-N_3AMP	6.7	2.5	6.0
50 μM 8-N_3AMP + 0.4 mM ATP	28.9	6.8	17.0
50 μM 8-N_3AMP + 0.4 mM ADP	20.2	4.2	14.9
50 μM 8-N_3AMP + 0.4 mM AMP	6.6	2.2	5.6
50 μM 8-N_3AMP + 0.4 mM cAMP	7.0	2.1	5.3
50 μM 8-N_3IMP	4.4	0.9	2.9
50 μM 8-N_3IMP + 0.4 mM ATP	29.6	8.4	24.4

[a] Rabbit muscle phosphofructokinase (0.2 mg) in 0.8 mL of 0.05 M phosphate buffer (pH 7.0) and 0.5 mM EDTA was irradiated for 10 min in the presence and absence of nucleotides at the indicated concentrations. A total of 5 μL of enzyme solution containing 1.25 μg of protein was withdrawn for the enzymatic assay. [b] Irradiation was done at 2 °C using a 120-W Hanau Q 400 high-pressure mercury lamp equipped with a glass filter to cut off the radiation below 300 nm. The samples in 0.5-cm path quartz cells were placed at 9 cm from the lamp. [c] The enzymatic assay was performed in a medium buffered with imidazole (pH 7.0), containing 6 mM $MgCl_2$, 0.04 mM F6P, and different amounts of ATP. The ATP concentration was optimal (0.15 mM) in the assay system I and inhibitory (1.5 mM) in II. The assay system III contained both 1.5 mM ATP (for inhibition) and 1.0 mM cAMP (for reactivation).

Table IX: Allosteric Effects of 8-Bromoadenine Nucleotides

enzyme	nucleotide	effect
rabbit muscle phosphofructokinase	ATP	50% inhibition at 0.4 mM
	8-BrATP	no effect on inhibition
	AMP	reactivation of ATP-inhibited phosphofructokinase
	8-BrAMP	no reactivation effect
pig kidney FDPase	AMP	50% inhibition at 4.2 μM 90% inhibition at 20 μM
	dAMP	50% inhibition at 5.0 μM 82% inhibition at 20 μM
	AMPS	50% inhibition at 80 μM
	8-BrAMP	12% inhibition at 1 mM
rat liver mitochondrial NAD–ICDH	ADP	fourfold activation at 1 mM and suboptimal (2 mM) isocitrate concentration
	8-BrADP	no effect
beef liver glutamate dehydrogenase	ATP	inhibition at pH 8
	8-BrATP	inhibition at pH 8
	ADP	activation at pH 7 and 8
	8-BrADP	activation at pH 7 and 8

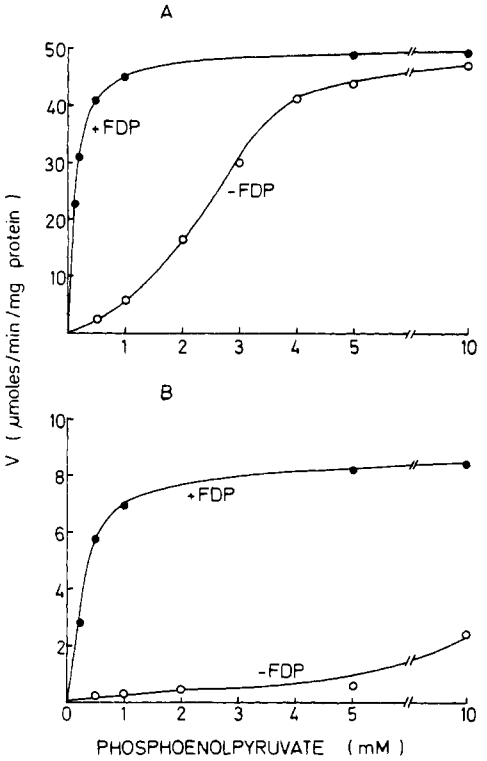

FIGURE 6: Kinetic properties of yeast pyruvate kinase as a function of the phosphoenolpyruvate concentration, with 2 mM ADP (A) or 2 mM 8-BrADP (B) as the nucleotide substrate. The experiments were performed at pH 8.0 in the presence or absence, respectively, of 1 mM FDP.

and 8-BrATP toward beef liver glutamate dehydrogenase is much more like that of the corresponding natural nucleotides (Koberstein et al., 1976; Lascu et al., 1977). Identical (0.5 mM) concentrations of ATP and 8-BrATP, for instance, produce a maximal inhibition of glutamate dehydrogenase of 57 and 60%, respectively, the half-maximal inhibition being reached at 46 μM, both for ATP and 8-BrATP. Also, from a large number of investigated structural ADP analogues, we found 8-BrADP to be the only one to activate beef liver glutamate dehydrogenase, besides the natural activator, ADP.

(*4*) *Yeast Pyruvate Kinase.* We shall also discuss the effect of 8-BrADP on yeast pyruvate kinase, although strictly speaking, this comes only indirectly under the category of "allosteric effects". Yeast pyruvate kinase, as well as pyruvate kinase from the erythrocyte, is known to have allosteric properties for phosphoenolypyruvate (Hunsley & Suelter, 1969; Wieker & Hess, 1971; Staal et al., 1971; Badwey & Westhead, 1976). Through addition of FDP, the sigmoidal kinetics for PEP can be changed into a hyperbolic one, reducing at the same time the apparent K_m for PEP. The kinetics for ADP, on the other hand, is hyperbolic, both in the presence and in the absence of FDP. We have performed our experiments at pH 8.0, where ADP is completely deprotonated and $MgADP^-$ is the only species in the presence of excess Mg^{2+}. The curves in Figure 6 demonstrate that, in the presence of FDP and of a fixed 8-BrADP concentration, the pyruvate kinase affinity for PEP is about 3 times higher than that in the case where ADP is used as the phosphate acceptor. However, in the absence of FDP, the enzyme activity is negligible with 8-BrADP as the acceptor, even at PEP concentrations where the enzyme is substrate saturated in the presence of ADP as the acceptor. This observation can be explained only by assuming that 8-BrADP induces major conformational changes in the structure of pyruvate kinase, thus leading to a reduced affinity for PEP. Such a phenomenon must not be necessarily associated with an allosteric effect, since a similar behavior has been observed with creatine kinase. ESR experiments have shown that the binding of ATP and/or metal–nucleotide complexes to creatine kinase produces a conformational change which can alter the enzyme affinity for the second substrate (Reed & Cohn, 1972).

In order to check whether this phenomenon is characteristic for 8-BrADP, we have also investigated the extent to which ϵADP, o^1ADP, and GDP can affect the affinity of yeast pyruvate kinase for PEP. With ϵADP, the enzyme behavior is similar to that with ADP as acceptor, both in the presence and in the absence of FDP. o^1ADP and GDP, on the other hand, resemble 8-BrADP and both reduce the enzyme affinity for PEP.

Although at this stage it is difficult to decide exactly which structural features determine the different behavior of individual nucleotides for yeast pyruvate kinase, the significance of this effect can have far-reaching consequences regarding the activity of allosteric enzymes. This would imply that an unnatural nucleotide, or a nucleotide which is not characteristic for a particular metabolic pathway, could be eliminated from the metabolic circuit not only by its weak substrate capacity but also indirectly via a conformational change of the enzymes binding the second substrate.

Concluding Remarks. From the results of this investigation, in corroboration with recent literature data (Nagel et al., 1976; Koberstein et al., 1976; Morange et al., 1976; Wagenvoord et al., 1977; Verheijen et al., 1978; Takenaka et al., 1978; Harris et al., 1979; Marcus & Haley, 1979), we can establish three categories of enzymatic systems with regard to their behavior to 8-substituted purine nucleotides. (1) One category is enzymatic systems with a very high specificity for the purine moiety which practically tolerate no modifications without fully loosing the capacity to recognize such nucleotide analogues. This category includes enzymes such as FDPase, the AMP center of adenylate kinase, phosphoenolpyruvate carboxykinase, the allosteric center of phosphofructokinase, or the mitochondrial adenine nucleotide translocase. (2) A second category is enzymatic systems with a fairly low substrate specificity which, although with a reduced efficiency, can use most purine nucleotide analogues and even the natural pyrimidine nucleotides. This category includes many common phosphotransferases such as pyruvate kinase, the ATP center of adenylate kinase, hexokinase, phosphofructokinase, and various ATPases. (3) The third category is enzymatic systems which preferentially use nucleotide analogues or use these at the same level with the natural derivatives. The latter is the smallest group, partly because of the limited number of enzymatic systems so far investigated. This category includes enzymes such as NDP kinase, yeast phosphoglycerate kinase, or beef liver glutamate dehydrogenase, but it is difficult to draw a clear separation line between the last two categories.

The above classification, derived primarily from the behavior of 8-bromonucleotides, could be used for evaluating a priori the success rates of labeling the active or allosteric sites of a particular enzyme with photoreactive 8-azidopurine nucleotides.

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