Structural and Enzymatic Properties of Adenine 1-Oxide Nucleotides†

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ABSTRACT: We describe the preparation of adenine 1-oxide nucleotides by oxidation of the natural compounds with monopermaleic acid in aqueous solutions at neutral pH, with an overall yield after chromatographic purification between 75 and 80%. If irradiated, the adenine 1-oxide nucleotides undergo a photochemical rearrangement reaction, the main photoproducts in aqueous solution at alkaline pH being the corresponding isoguanine nucleotides. The modified ring vibration pattern of the 1-oxide analogues as well as the ^{13}C chemical shift indicate a loss of aromaticity as compared to the natural compounds. Coupling constant measurements show that the dihedral angle between the ^{31}POC and $OC^{13}C$ planes is around 180°, i.e., trans, as in the natural adenine nucleotides. The modified adenine nucleotides were tested as potential substrates and/or inhibitors of mitochondrial processes, as substrates of various phosphotransferases from mitochondria or cytosol, and as allosteric effectors in the reactions catalyzed by glutamate dehydrogenase and phosphofructokinase. Although the adenine 1-oxide nucleotides are not recognized by the translocase system of the inner mitochondrial membrane, they are good substrates for mitochondrial phosphotransferases located in the intermembrane space. Similarly, they participate in the phosphoryl group transfer reactions catalyzed by pyruvate kinase, phosphofructokinase, and hexokinase. As allosteric effectors, the modified nucleotides are less active than the natural compounds, probably because of a lower binding capacity to the allosteric sites of the regulatory enzymes.

The search for an explanation of the specificity of most enzymatic systems for only particular nucleotide has led to an almost exponential increase of publications dealing with nucleotide analogues with modifications in the base or sugar moiety, in the phosphate side chain, or in the base sugar linkage (Ikehara et al., 1972; Goody and Eckstein, 1971; Hohnadel and Cooper, 1972; Miller et al., 1973; Haley and Yount, 1972; Stütz and Scheit, 1975).

In view of the central role of adenine nucleotides both as energy source and as metabolic control agents, adenine nucleotide analogues represent potentially valuable probes to investigate structure–function relationships (Okazaki et al., 1968; Boswell et al., 1973; Lawrence et al., 1974). We describe here the structural properties of N_1 oxygen substituted adenine nucleotides which were obtained by a mild oxidation of the natural compounds with permaleic acid in aqueous solution at neutral pH and room temperature. The main goal of this investigation was to look for connections between the different biological responses of these adenine nucleotide analogues and modifications at the molecular level as revealed by their spectroscopic properties. We report on the involvement of these analogues in the reactions of the oxidative phosphorylation, their participation as substrates in the phosphotransferase activity of mitochondrial or cytosolic enzymes, and as allosteric effectors of enzymes requiring adenine nucleotides as modifiers.

Experimental Section

Chemicals. AMP, ADP, ATP (free acids), NAD^+, NADH, $NADP^+$, NADPH, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), phosphofructokinase (EC 2.7.1.11), triosephosphate isomerase (EC 5.3.1.1), α-glycerolphosphate dehydrogenase (EC 1.1.1.8), and lactate dehydrogenase (EC 1.1.1.28) were commercial products from Boehringer Mannheim. Other chemicals from commercial suppliers were: rabbit muscle pyruvate kinase (EC 2.7.1.40), dithiothreitol (Sigma Chemical Co., St. Louis Mo.), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), yeast hexokinase (EC 2.7.1.1), bovine serum albumin (Nutritional Biochemicals, Cleveland, Ohio), and beef liver glutamate dehydrogenase (EC 1.4.1.3) (Fluka, Switzerland).

Monopermaleic acid was obtained from maleic anhydride and H_2O_2 by a slightly modified procedure used by Pyne (1959) for the preparation of monoperphthalic acid. To an ice-cold stirred solution of 0.05 mol of sodium carbonate in 50 ml of H_2O were added 0.06 mol (7 ml) of 30% hydrogen peroxide and 0.05 mol (4.9 g) of pulverized maleic anhydride pellets. After the mixture was stirred 30 min the monopermaleic acid was liberated by adding 3 ml of concentrated H_2SO_4 in 15 ml of ice-water. The peracid was extracted with ether (eight times, 50 ml) and stored at 0°C. Monopermaleic acid in aqueous solution was freshly prepared prior to each oxidation by adding water to the required amount of ether solution, followed by removal of the ether with a gentle stream of air.

General Procedure for the Preparation of Adenine 1-Oxide Nucleotides. Adenine nucleotide (free acid) (0.5 mmol) in 1 ml of water, adjusted to pH 7 with 1 *N* NaOH (ice cooling), was reacted with 1.5 mmol of monopermaleic acid in 2 ml of water brought to pH 7 with 1 *N* NaOH and kept in the dark at room temperature for 24 hr in a closed flask. The rate of oxidation by using this threefold excess of reagent proceeded rapidly during the first 4 hr after which it slowly approached the plateau. The initial peak ratio A_{233}/A_{260} of 0.2 increased to 4.8 upon complete oxidation. In order to eliminate the excess maleic acid the solution was brought to pH 4–5 with 1 *N* HCl (ice cooling) and the ade-

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nine 1-oxide nucleotides were precipitated by adding a five-fold excess (by volume) of ethanol. The fine suspension was centrifuged at 6000 rpm, the supernatant containing most of the maleic acid was decanted, and the solid was redissolved in 1 ml of water, the pH adjusted to 4–5, and again precipitated with ethanol. The degree of oxidation was around 90%. The oxidized nucleotides were further purified by ion-exchange chromatography. In a typical run, the whole amount (about 0.45 mmol) was passed through a column (1.1 × 22 cm) of Dowex-1-carbonate (mesh 200–400) and eluted for 24 hr with a linear gradient (0.01–1 *M*) of ammonium bicarbonate at a rate of 0.5 ml/min. The eluted samples were monitored with an LKB Uvicord III (254 and 267 nm). Free acids, if necessary, were obtained by passing the ammonium salts over a cationic column (Dowex-50-H). The overall yield after chromatographic purification was between 75 and 80%. The purity of all compounds was finally verified by their spectroscopic properties and thin-layer chromatography on PEI-cellulose (Randerath and Randerath, 1967).

Analytical Methods. Ultraviolet absorption spectra were determined with a Zeiss (Opton) PMQ II spectrophotometer in 0.05 *M* potassium phosphate buffer (pH 7.5). Fluorescence emission spectra were measured with a Perkin-Elmer, MPF-2A Type fluorescence spectrophotometer, equipped with a Hitachi QPD 33 recorder. Infrared spectra were recorded between 400 and 3600 cm^{-1} on a double beam VEB Zeiss (Jena) UR-10 spectrophotometer as KBr pellets. The solid state nucleotides were obtained by precipitation with ethanol from concentrated aqueous solutions at pH 7. ^{13}C nuclear magnetic resonance (NMR) spectra in natural abundance were recorded in 12-mm tubes at 37° on a Varian XL-100-15 spectrometer operating at 25.16 MHz in a pulsed Fourier transform mode with complete proton decoupling. The interferograms with a spectral width of 5000 Hz were accumulated in a 16 K Varian 620 L computer. Chemical shifts are reported in parts per million (ppm) downfield from external Me_4Si. ^1H NMR spectra were taken with a Brucker WP-60 Fourier spectrometer. Polarograms were recorded as 2 m*M* solutions in 0.05 *N* perchloric acid (pH 1.5) on an Orion polarograph Type 7-77-4b using a saturated calomel reference electrode. Apparent formation constants of Mg^{2+} with the adenine 1-oxide nucleotides were determined using the ion-exchange resin method of Schubert (1956) as described by Haley and Yount (1972).

Biochemical Assays. Rat, mouse, guinea pig, and human liver mitochondria were isolated as previously described (Bârzu et al., 1968; Benga et al., 1972). Rat heart mitochondria were prepared by means of the nagarse method of Pande and Blanchaer (1971) as modified by Jacobus and Lehninger (1973). Rat kidney mitochondria were obtained using the procedure of Johnson and Lardy (1967). Only preparations having the respiratory control higher than 4 with succinate and 5 with glutamate and showing an intact structure of both outer and inner membrane, as revealed by electron microscopy, were used in our experiments. The mitochondrial respiration was monitored with a Clark type electrode at 0.5 or 2.5 ml final volume. The basic medium contained 180 m*M* sucrose, 50 m*M* KCl, 25 m*M* Tris-HCl (pH 7.4), 5 m*M* potassium phosphate (pH 7.4), 2.5 m*M* $MgCl_2$, 1 m*M* EDTA, 5 m*M* substrate, 2 mg of bovine serum albumin/ml, mitochondria, and different nucleotide concentrations. $AT^{32}P$ and $o^1AT^{32}P^1$ formation was carried out as follows: after incubation of mitochondria at a final volume of 2.5 ml in the same medium as that used for the respiratory activity with succinate as substrate and 2 m*M* $^{32}P_i$ containing 15 μCi, at the appropriate time 1 ml of 1.5 *N* perchloric acid was added to the reaction medium. The precipitated proteins were removed by centrifugation and the extract (3 ml) was neutralized with KOH. Nucleotides and $^{32}P_i$ 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). The elution was performed during 14 hr at a flow rate of 0.5 ml/min. The recovery of nucleotides from the starting material was higher than 85%. Partially purified rat heart cytosolic nucleosidediphosphate kinase (NDK, EC 2.7.4.6) was obtained following the first three steps described by Colomb et al. (1972) for purification of the beef heart soluble enzyme; our preparations exhibited a specific activity of 10 U/mg of protein at pH 7.4 and 25°C in a coupled assay system with pyruvate kinase and lactate dehydrogenase (Pedersen, 1973) using 0.2 m*M* ATP as phosphate donor and 0.1 m*M* 8-Br-ADP as phosphate acceptor.

Phosphofructokinase activity was measured from the rate of formation of fructose 1,6-bisphosphate in a coupled assay system containing aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase. Auxiliary enzymes as well as phosphofructokinase were desalted prior to the assays by dialysis against 0.1 *M* imidazole buffer (pH 7), 0.2 m*M* EDTA, and 1 m*M* dithiothreitol. The reaction medium contained at 1 ml, 40 m*M* imidazole buffer (pH 7), 50 m*M* KCl, 6 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.05 m*M* NADH, 1 m*M* dithiothreitol, 0.1 mg of bovine serum albumin, 0.8 U of triosephosphate isomerase, 0.8 U of α-glycerolphosphate dehydrogenase, 1.5 U of aldolase, 0.04 m*M* fructose 6-phosphate, and different concentrations of ATP or o^1ATP. The reaction was started with purified rabbit muscle phosphofructokinase. Hexokinase activity was measured by coupling the production of glucose 6-phosphate with glucose-6-phosphate dehydrogenase and following the reduction of $NADP^+$. The reaction medium contained at 0.7 ml, 50 m*M* triethanolamine (pH 8), 6 m*M* $MgCl_2$, 5 m*M* glucose, 0.4 m*M* $NADP^+$, different concentrations of ATP or o^1ATP, and 1.8 U of glucose-6-phosphate dehydrogenase. The reaction was started with purified yeast hexokinase. Pyruvate kinase was measured by coupling the formation of pyruvate with oxidation of NADH in the presence of lactate dehydrogenase. The reaction medium contained at 1 ml, 50 m*M* Tris-HCl (pH 7.4), 80 m*M* KCl, 8 m*M* $MgCl_2$, 0.5 m*M* phosphoenolpyruvate, 0.05 m*M* NADH, 3 U of lactate dehydrogenase, and different concentrations of ADP or o^1ADP. The reaction was started with rabbit muscle pyruvate kinase. When the pH dependence of the activity was studied, the Tris-HCl buffer was replaced by Tris-maleate (pH between 5.5 and 8.5). Adenylate kinase (EC 2.7.4.3) was measured spectrophotometrically by coupling with the assay system of Adelman et al. (1968) as previously described (Jebeleanu et al., 1974). Glutamate dehydrogenase activity was monitored in a reaction medium containing at

[1] Abbreviations used are: o^1ATP, adenosine 1-oxide 5′-triphosphate; o^1ADP, adenosine 1-oxide 5′-diphosphate; o^1AMP, adenosine 1-oxide 5′-monophosphate; iGTP, isoguanosine 5′-triphosphate; iGDP, isoguanosine 5′-diphosphate; iGMP, isoguanosine 5′-monophosphate; 8-BrADP, 8-bromoadenosine 5′-diphosphate; ϵATP, ϵADP, ϵAMP, 1,N^6-etheno derivatives of adenine nucleotides; AOPCP, adenosine 5′-methylenediphosphonate; AMP-PCP, adenylyl methylenediphosphonate; AMP-PNP, adenylyl imidodiphosphate; NDK, nucleosidediphosphate kinase.

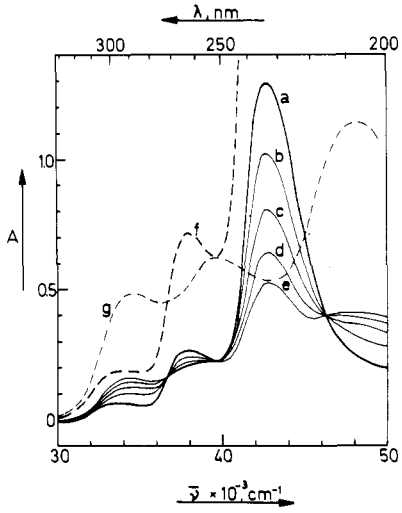


FIGURE 1: Photochemical changes induced by uv radiation on a 1.5×10^{-4} *M* solution of o^{1}ATP in 0.01 *M* NH_4OH using a 120-W Hanau Q 400 high-pressure mercury lamp. The decomposition is followed with a 2-mm quartz cell at 0 (a), 2 (b), 4 (c), 6 (d), and 8 (e) min intervals and with 5-mm cell at 0 (f) and 30 (g) min.

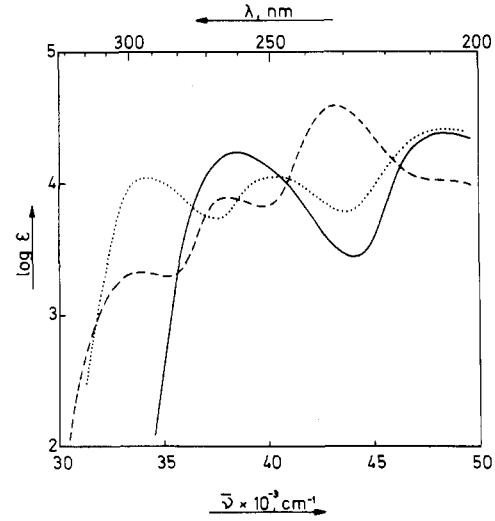


FIGURE 2: Electronic absorption spectra of ATP (—), o^{1}ATP (- - -), and iGTP (• • •) in aqueous buffer solutions (0.05 *M* potassium phosphate) at pH 7.5.

1 ml, 80 m*M* Tris-acetate (pH 8), 100 m*M* NH_4Cl, 0.01 m*M* EDTA, 50 m*M* α-oxoglutarate, 0.08 m*M* NAD(P)H, and different concentrations of nucleotides. The reaction was started with purified beef liver glutamate dehydrogenase. All enzymatic reaction rates were determined at 366 nm and 25°C, except the hexokinase assay which was done at 30°C, using an Eppendorf 1101 M Type photometer equipped with a W+W Type 4410 recorder (full scale deflection 0.25 absorbance unit).

Results and Discussion

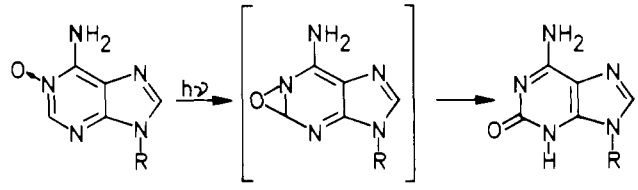
Synthesis and Chemical Properties. The most basic nitrogen atom in the adenine moiety is the N_1 atom which is not only the site of protonation but also of N-oxidation. Adenine and adenosine can be oxidized by hydrogen peroxide in acetic acid (Stevens et al., 1958). In order to oxidize the corresponding nucleotides, organic peracids like *m*-chlorobenzoic or perphthalic acid have to be used (Haar, von der et al., 1971). We have been using most successfully permaleic acid which under very mild conditions, i.e., aqueous solutions at pH 7 and room temperature, is a much stronger oxidant than most other organic peracids (White and Emmons, 1962) with a rate of oxidation nearly as great as that of trifluoroperacetic acid. The nucleotide 1-oxides are very soluble in water and insoluble in ethanol and organic solvents.

The 1-oxygen reduces the basicity of the neighboring amino group, which in contrast to the natural nucleotides can be deprotonated already at p*K* = 12.5 (Sigel and Brintzinger, 1964). The conversion to the corresponding 1-oxides results in a labilization of the pyrimidine moiety and an increased chemical and photochemical reactivity at the C_2 carbon atom revealed by the cleavage of the pyrimidine ring at C_2 by acid and alkaline hydrolysis (Stevens et al., 1959) and the photochemical behavior of adenine 1-oxide nucleotides. The oxidation does not affect the rate of hydrolysis of phosphate groups with 0.1 *N* H_2SO_4 at 100°C as compared to that of the natural adenine nucleotides.

Photochemical Behavior. If irradiated the adenine 1-oxide nucleotides undergo an interesting photochemical rearrangement reaction, the main photoproducts in aqueous solutions at alkaline pH (0.01 *M* NH_4OH) being the corresponding isoguanine nucleotides. It had been shown previously (Cramer and Schlingloff, 1964; Brown et al., 1964) that adenine 1-oxide and adenosine 1-oxide are sensitive to uv irradiation yielding two main products, namely adenine (adenosine) and isoguanine (isoguanosine). More recently Kazimierczuk and Shugar (1973) did obtain by such a rearrangement reaction also isoguanosine 5′-monophosphate and cyclic isoguanosine 3′,5′-monophosphate. So far, however, we were not able to find any data on the two other products we obtained, i.e., iGDP and iGTP.

Two systems of irradiation were used. For analytical purposes (Figure 1) the source was a 120-W Hanau Q 400 high-pressure mercury lamp. The kinetics of the decomposition was followed in 5- and 2-mm quartz cells using a Vycor (UG2 Zeiss) filter. For preparative purposes a 70-W Hanau TQ 81 high-pressure mercury immersion lamp was employed. The reaction mechanism involves a transposition of the heterocyclic *N*-oxygen to the neighboring carbon atom via an oxazirane intermediate (Mantsch et al., 1969; Kazimierczuk and Shugar, 1973) (Chart I).

Chart I: Photoisomerization of Adenine 1-Oxide Nucleotides to the Isoguanine Nucleotides.[a]



[a] R = ribosyl mono-, di-, or triphosphate.

These new purine nucleotides (uv spectra, dotted trace in Figure 2) can be considered structural analogues of adenine nucleotides with the same functional groups as guanine nucleotides but in different positions. The biological properties of isoguanine nucleotides shall be reported elsewhere (O. Bârzu et al., in preparation).

Polarographic Studies. The polarographic reduction of the adenine 1-oxide base was reported to yield two polarographic waves, one below 1 V arising from a two-electron reduction of the N_1-oxygen, and a second wave above 1 V due to the reduction of the heterocyclic adenine ring itself

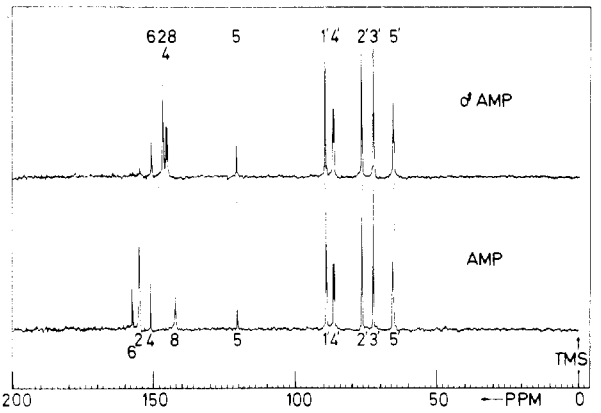


FIGURE 3: Proton decoupled ^{13}C NMR spectra of o^{1}AMP (upper trace) and AMP (lower trace) as 0.4 *M* aqueous solutions (17 K transients).

(McGinn and Brown, 1960; Warner and Elving, 1965). The polarographic behavior of adenine 1-oxide does not change drastically on attachment of the sugar or the sugar-phosphate moiety.

Electronic Absorption Spectra. The conversion of adenine nucleotides to the corresponding 1-oxides is accompanied by a very characteristic change of the ultraviolet spectrum at neutral and alkaline pH. As exemplified by o^{1}ATP in Figure 2 all 1-oxides possess a very strong absorption maximum (log ϵ = 4.6) at 42900 cm^{-1} (233 nm) and one at 33900 cm^{-1} (295 nm) with a much lower molar extinction coefficient (log ϵ = 3.3), however, with the advantage of being in a region where the natural compounds have no absorption at all. The abscissa scale in Figures 1 and 2 is linear in wavenumbers (cm^{-1}) which relates directly to the energy scale, and the ordinate gives the logarithm of the molar extinction coefficient which allows a better presentation of very strong bands along with weak absorption maxima. The characteristic adenine absorption maximum at 260 nm in ATP (log ϵ = 4.2) appears only slightly shifted in o^{1}ATP to 261 nm and somewhat less intense (log ϵ = 3.9). This for adenine nucleotides unusual ultraviolet spectrum greatly facilitates the detection of 1-oxides among other nucleotides. With decreasing pH both absorption bands at 233 and 295 nm are loosing intensity and disappear completely in strong acids, indicating the protonation must involve the N_1-oxygen, thus restoring the uv pattern of normal nucleotides.

Fluorescence Spectra. o^{1}AMP, o^{1}ADP, and o^{1}ATP exhibit interesting and useful fluorescence properties with a large Stokes shift which compares to that of the 1,N^6-ethenoadenosine derivatives described by Secrist et al. (1972). The fluorescent emission of an aqueous solution of o^{1}ATP (at neutral pH and room temperature) by excitation at 300 nm yields a single unresolved band at 402 nm with a half-bandwidth of 4.1 × 10^3 cm^{-1}. The fluorescence intensity decreases with decreasing pH and is lost upon complete protonation of the N_1-oxide group. The nucleotide 1-oxides have identical fluorescence spectra, this being an intrinsic property of the adenine 1-oxide moiety. It has been shown (Sigel and Brintzinger, 1964) that in o^{1}AMP the *o*-amino-*N*-oxide group is competing with the phosphate group for binding certain metals (Cu^{2+}). If the *o*-amino-*N*-oxide ligand group in o^{1}ATP would participate in such a binding or be engaged in a backbinding to the polyphosphate chain, this should affect (i.e., reduce) fluorescence. Preliminary data with different concentrations of Mg^{2+} showed no marked influence on the fluorescence properties of o^{1}ATP indicating that the phosphate group has a much higher affinity for Mg^{2+} and that the 1-oxide is not implicated in the binding of Mg^{2+}.

Vibrational Spectra. The ir spectrum of adenine 1-oxide shows two absorption bands at 484 and 576 cm^{-1}, which are missing in adenine and can be assigned to the out-of-plane and in-plane bending vibrations of the N–O group (Ionescu et al., 1966). These bands are observed also in the adenosine 1-oxide but are obscured in the spectra of nucleotide 1-oxides due to overlapping P–O absorption bands. The strong absorption band at 1671 cm^{-1} which in adenine and adenosine is due to the NH_2 deformation vibration is shifted to 1700–1712 cm^{-1} in the natural adenine nucleotides. To explain this shift it has been suggested (Angell, 1961) that in the solid state spectra the adenine phosphates exist in a zwitterion form with a proton at the N_1 nitrogen atom. In the corresponding nucleotide 1-oxides the NH_2 deformation vibration of adenosine 1-oxide at 1680 cm^{-1}, still the strongest band of the spectrum, is not shifted to higher wavenumbers, suggesting that in solid state the amino group forms a very stable system with the adjacent *N*-oxide group, perhaps stabilized by an intramolecular hydrogen bond, rendering it insensitive to the influence of the phosphate side chain. The ring vibration which in the natural adenine nucleotides gives rise to a strong band around 1615 cm^{-1} is shifted to 1580 cm^{-1} in the 1-oxides. The direction of this shift along with the decrease in intensity indicates a loss of aromaticity in the system. The whole vibrational pattern of the heterocyclic ring modes shows profound changes in the corresponding 1-oxides, both with regard to the position and intensity of these vibrations, suggesting that the N–O group produces far reaching modifications in the molecular structure of the heterocyclic ring system.

^{13}C NMR Spectra. Most successful for probing the solution structure of natural nucleotides and nucleotide analogues has become the multinuclear magnetic resonance spectroscopy and particularly the ^{13}C NMR spectroscopy which allows detailed studies of their molecular framework in aqueous solutions, the most relevant solvent for such biomolecules (Stothers, 1973; Gray, 1973; Smith et al., 1973; Barry et al., 1974). The introduction of an oxygen in the heterocyclic ring system induces only small changes in the ribose carbon resonances (less than 0.4 ppm); however, considerable modifications occur with the ^{13}C chemical shifts of the base carbons (Figure 3). The assignment of the individual carbon atoms was aided by off resonance spectra and pH-induced shifts. In comparison to the natural nucleotides the C_2 and C_6 resonances are shifted to higher field in the 1-oxides by 8.3 and 6.7 ppm, respectively. The ^{13}C chemical shift of C_5 which is in a position meta to the oxidized nitrogen atom is not affected upon N-oxidation, while the para standing C_4 also experiences an upfield shift of 5.1 ppm. The C_8 in the imidazole ring is the only deshielded position in the *N*-oxide series (3 ppm).

Aromatic *N*-oxides are interesting in that their *N*-oxide group can act both as an electron donor and an electron acceptor (Lagowski and Katritzky, 1970). Consequently, the considerable upfield shift experienced by the C_2 and C_6 resonances indicates that in aqueous solutions the *N*-oxide group in adenine 1-oxide nucleotides is acting as electron donor leading to an increased electron density in the whole pyrimidine moiety of the adenine molecule. This is revealed also by its increased chemical and photochemical reactivity

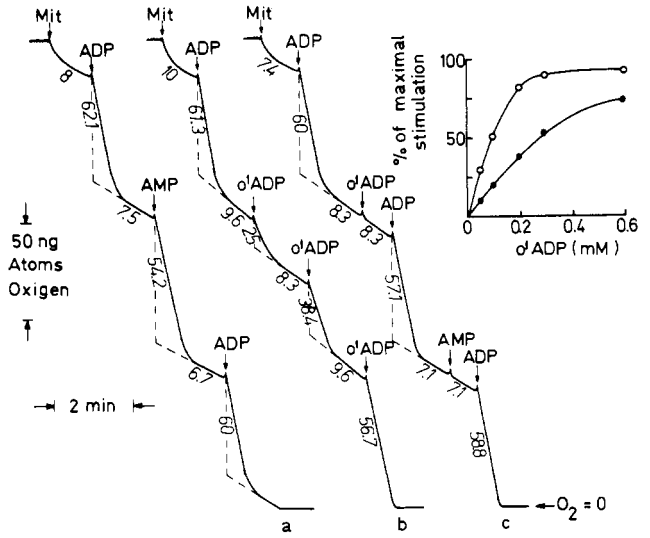


FIGURE 4: Effect of o¹ADP on the respiratory rate of rat liver mitochondria. The basic respiratory medium contained at 0.5 ml final volume and 24° the reagents indicated in the Experimental Section. For each experiment 1.9 mg of mitochondrial protein with glutamate as respiratory substrate was used. Additions were as follows: trace a, 0.25 m*M* ADP, 0.16 m*M* AMP, 0.25 m*M* ADP; trace b, 0.25 m*M* ADP, 0.07 m*M* o¹ADP, 0.15 m*M* o¹ADP, 0.45 m*M* o¹ADP; trace c, 0.25 m*M* ADP, 0.3 m*M* o¹ADP, 0.25 m*M* ADP, 0.16 m*M* AMP, 0.25 m*M* ADP. No Mg^{2+} was present in this experiment. The numbers beside the traces indicate the oxygen consumption as ng-atoms per min per mg of protein. Inset: Stimulation of the rate of respiration by different o¹ADP concentrations following the state 3 to state 4 transition in the presence of two respiratory substrates, pyruvate (○) and succinate (●).

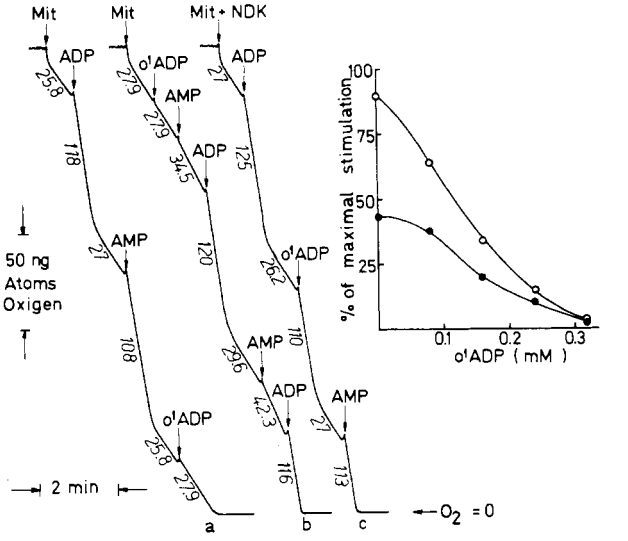


FIGURE 5: Effect of o¹ADP on the respiratory rate of rat kidney and heart mitochondria; 1.2 mg of rat kidney mitochondrial protein with succinate as respiratory substrate was used. Additions were as follows: trace a, 0.18 m*M* ADP, 0.1 m*M* AMP, 0.3 m*M* o¹ADP; trace b, 0.3 m*M* o¹ADP, 0.1 m*M* AMP, 0.18 m*M* ADP, 0.1 m*M* AMP, 0.18 m*M* ADP; trace c, 0.2 U of NDK, 0.18 m*M* ADP, 0.15 m*M* o¹ADP, 0.1 m*M* AMP. The numbers beside the traces indicate the oxygen consumption expressed as ng-atoms per min per mg of protein. Inset: Inhibition by o¹ADP of AMP-released respiratory activity of rat kidney (○) and heart (●) mitochondria.

at C_2, while the downfield shift of the C_8 atom in the imidazole moiety indicates a loss of aromaticity of this part of the molecule and suggests a more difficult electrophilic substitution at this position. Indeed we were not able to brominate the adenine 1-oxides at C_8 under conditions which easily lead to 8-bromoadenine nucleotides.

In the proton decoupled spectra of adenine nucleotide oxides the two bond phosphorus–carbon coupling $^2J_{^{31}PO^{13}C}$ is 4.5 Hz while the long range coupling $^3J_{^{31}POC^{13}C}$ to C_4 is 9 Hz. The large value of this vicinal coupling indicates that the dihedral angle between the ^{31}POC and $OC^{13}C$ planes is around 180°, i.e., trans as in the natural adenine nucleotides (Smith et al., 1973).

Interconversion of Adenine 1-Oxide Nucleotides in Mitochondria. The addition of o¹ADP to intact mitochondria was shown to have no effect on respiration (Kezdi et al., 1973). On the other hand, o¹ATP hydrolysis by intact mitochondria was insensitive to stimulation by 2,4-dinitrophenol and ATP-requiring reactions were not activated by o¹ATP. These results were explained by the fact that o¹ADP and o¹ATP are not translocated across the inner mitochondrial membrane. However, Schlimme and Schäfer (1972) using radioactive o¹ADP and o¹ATP obtained by oxidation of [^{14}C]ADP and [^{14}C]ATP with monoperphthalic acid described an atractyloside-sensitive translocation of these nucleotides. Jebeleanu et al. (1974) found that the exchange between intramitochondrial ^{14}C-labeled adenine nucleotides and added o¹ADP or o¹ATP is negligible compared to the exchange of ADP or ATP. We believe that the "translocation" of the analogues observed by Schlimme and Schäfer (1972) are due to traces of nonoxidized nucleotides present in their preparations, which become important if we keep in mind the extremely high affinity of the translocase system for ADP and ATP (Klingenberg and Pfaff, 1968; Vignais et al., 1970).

When o¹ADP was added to respiring rat, mouse, or human liver mitochondria after the state 3 to state 4 transition, an immediate stimulation of the respiration is observed (Figure 4, trace b). The percentage rate of respiration stimulation as calculated from the expression (o¹ADP rate − state 4 rate)/(ADP rate − state 4 rate) × 100, where the state 4 refers to the rate of respiration after the consumption of added ADP or o¹ADP, depends on the concentrations of o¹ADP and Mg^{2+} and on the rate of substrate oxidation in mitochondria (Figure 4, traces b and c and inset). If pyruvate is used as substrate (oxygen uptake 40 ng-atoms per min per mg of protein) then 0.2 m*M* o¹ADP stimulates the respiration to about 85% as compared to ADP stimulation, whereas in the presence of succinate (oxygen uptake 128 ng-atoms per min mg of protein) at the same o¹ADP concentration, the stimulation is only 40% that of ADP. In the absence of exogenous Mg^{2+} and at EDTA concentration higher than 0.1 m*M*, o¹ADP as well as AMP have no effect at all on the mitochondrial respiration. A somewhat different picture is shown by rat kidney, rat heart, or guinea pig liver mitochondria. As shown in Figure 5 traces a and b) o¹ADP does not stimulate the respiration of kidney mitochondria neither before nor after ADP addition. It is interesting, however, that in this case the stimulatory effect of AMP is completely inhibited at o¹ADP concentrations higher than 0.3 m*M* (Figure 5, trace b and inset). Nevertheless o¹ADP is able to stimulate respiration even with heart or kidney mitochondria if previously various amounts of NDK are added and provided there is sufficient ATP in the mitochondria (Figure 5, trace c). o¹AMP does not affect mitochondrial respiration in any way.

These experiments lead us to the following conclusions. (1) The stimulation of mitochondrial respiration through addition of o¹ADP to mitochondria after the state 3 to state 4 transition is due to the coupling of the respiratory chain-linked ADP phosphorylation on the inner side of the inner

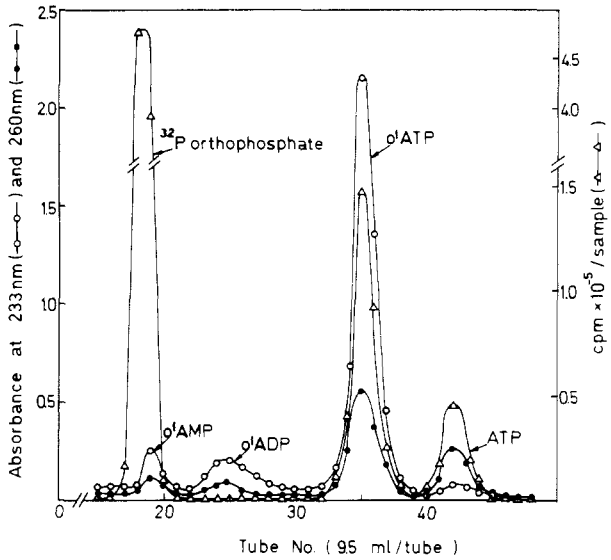


FIGURE 6: Chromatographic separation of nucleotides after incubation of rat liver mitochondria with ADP, o¹ADP, and ^{32}P-labeled orthophosphate. The experimental conditions are as described in Table I, corresponding to line one. Other details are given in the Experimental Section.

mitochondrial membranes, to the reaction catalyzed by NDK on the outer side of the inner mitochondrial membrane, according to the following steps:

$$ADP_{out} \xrightarrow{\text{translocase}} ADP_{in} \quad (a)$$

$$ADP_{in} + P_i \xrightarrow{\text{respiratory chain}} ATP_{in} \quad (b)$$

$$ATP_{in} \xrightarrow{\text{translocase}} ATP_{out} \quad (c)$$

$$ATP_{out} + o^1ADP_{out} \overset{\text{NDK}}{\rightleftharpoons} ADP_{out} + o^1ATP_{out} \quad (d)$$

By admitting that the transport steps a and c are much faster as compared to the phosphorylation steps (Klingenberg and Pfaff, 1968) the rate-limiting step is the NDK catalyzed reaction (step d).

(2) EDTA which cannot penetrate the inner mitochondrial membrane does not affect the phosphorylation of ADP (the matrix contains sufficient Mg^{2+} to support the phosphorylation of ADP through the respiratory chain enzymes (Colli and Pullman, 1969)) but it does block the transphosphorylation reaction d which takes place at the outer side of the inner mitochondrial membrane.

(3) The inhibitory effect of o¹ADP on the AMP-released respiration of rat kidney and rat heart mitochondria is a consequence of the inhibition of mitochondrial adenylate kinase through a competition of o¹ADP with mitochondrial ATP. Indeed, IDP, GDP, or CDP which cannot serve as substrates for adenylate kinase does not inhibit AMP-released respiration of rat kidney or heart mitochondria.

Since the oxygen consumption offers only indirect evidence with regard to the participation of modified nucleotides in the mitochondrial transphosphorylation reaction, in a number of separate investigations we have been following the $^{32}P_i$ incorporation into the o¹AT^{32}P and AT^{32}P molecules by incubating mitochondria under different experimental conditions. Rat liver mitochondria are able to form o¹AT^{32}P both in the presence or the absence of exogenous ADP (Figure 6 and Table I). In other words the rat liver

Table I: ^{32}P-Labeled Orthophosphate Incorporation in o¹ATP Molecules Catalyzed by Rat Liver and Kidney Mitochondria Incubated under Different Experimental Conditions.[a]

Mitochondria	Assay Conditions	Time of Incubation (min)	o¹ATP Formed (μmol)	cpm/μmol of o¹ATP
Liver	Complete	5	1.34	289000
	−ADP	10	0.17	294000
	+ oligomycin	10	0.22	34600
	+ atractyloside	10	0.20	29200
Kidney	Complete	5	0.09	249000
	+ NDK	5	1.18	270000

[a] The assay system contained in a total volume of 2.5 ml the basic respiratory medium described in the Experimental Section. To this medium 0.22 m*M* ADP, 0.65 m*M* o¹ADP, 15 μCi of labeled orthophosphate, and 3 mg of liver or kidney mitochondrial protein were added. When indicated 10 μg of oligomycin, 50 μ*M* atractyloside, or 0.5 U of NDK were included in the assay. Radioactive o¹ATP was separated from radioactive ATP and orthophosphate by column chromatography.

Table II: K_m and V_m Values of Phosphotransferases for ATP, ADP, and Their N_1-Oxide Derivatives.

Enzyme	Substrate	K_m (m*M*)[a]	V_m[b]	k^*/k[c]
Hexokinase	o¹ATP	0.59 (0.21)	0.27	0.098 (0.023)
Phosphofructokinase	o¹ATP	0.012 (0.012)	0.83	0.830 (0.412)
Pyruvate kinase	o¹ADP	1.76 (0.28)	0.28	0.045 (0.800)
Adenylate kinase	o¹ATP	0.90 (0.40)	0.55	0.240 (0.031)

[a] K_m for normal substrates in parentheses. [b] Relative to normal substrate. [c] k and k^* are the relative V_m/K_m ratios for natural nucleotides and their N_1-oxide analogues, respectively. In parentheses are done the same parameters for 1,N^6-ethenoadenine nucleotides (Secrist et al., 1972) in order to compare the efficiency of two classes of modified adenine nucleotides as substrates for the phosphotransferases.

mitochondrial adenylic pool is sufficient in order to synthesize about 5 nmol of o¹ATP per min per mg of protein. This "basal" level can be increased 30–40 times by increasing the ADP availability. As expected, atractyloside and oligomycin, which respectively inhibit the adenine nucleotide transport in mitochondria and the reactions of the oxidative phosphorylation, both cut down the o¹AT^{32}P formation almost completely. However, nonradioactive o¹ATP was formed under these conditions from o¹ADP and ADP by way of the adenylate kinase reaction. So, the "specific" radioactivity of o¹ATP is an index for the contribution of different mitochondrial reactions to the phosphorylation of o¹ADP. Rat kidney and rat heart mitochondria are able to form much less o¹AT^{32}P under identical incubation conditions as rat liver mitochondria. However, when partially purified NDK was added to these mitochondria, o¹AT^{32}P was formed at a rate closely similar to that obtained with rat liver mitochondria, in agreement with oxygraphic experiments.

Adenine 1-Oxide Nucleotides as Substrates of Cytoplasmatic Phosphotransferases. As shown in Table II o¹ATP and o¹ADP act as substrates for various other phosphotransferases than mitochondrial or cytosolic NDK. In order to compare our data with those obtained with other purine nucleotide analogues which can replace ATP or ADP in phosphoryl group transfer reactions we calculated

Table III: Release by AMP and o^{1}AMP of ATP-Induced Inhibition of Rabbit Muscle Phosphofructokinase.[a]

ATP (mM)	AMP (mM)	o^{1}AMP (mM)	Relative Rate
0.17			100
1.25			9
1.25	0.25		45
1.25	1.25		63
1.25		0.25	30
1.25		1.25	44

[a] Assays were carried out in imidazole buffer at pH 7 as described in the Experimental Section. The fructose 6-phosphate concentration was 0.04 mM. The maximal activity obtained at 0.17 mM ATP was considered as 100%.

the relative V_m/K_m ratio for each nucleotide as compared to the same ratio for ATP or ADP. It results that o^{1}ATP is a better substrate for hexokinase than ϵATP, GTP, or ITP (Secrist et al., 1972; Hohnadel and Cooper, 1972) which confers this nucleotide a special place to be used as fluorescent substrate for hexokinase. o^{1}ADP, however, is a poor substrate for pyruvate kinase as compared to other purine nucleotides with modifications in the heterocyclic ring (Secrist et al., 1972; Hohnadel and Copper, 1973). Although it was not our purpose to undertake a detailed kinetic investigation, in a separate experiment we explored the effects of the pH and Mg^{2+} concentration on the pyruvate kinase activity with ADP or o^{1}ADP as substrate. The ratio of the reaction velocity with the natural and modified substrate remains constant at different pH values and Mg^{2+} concentrations. This fact suggests that neither the ionization degree of nucleotide nor Mg^{2+} complexes formation has any involvement in the observed differences between kinetic parameters of pyruvate kinase acting on ADP or o^{1}ADP. In fact, we found very close values for the apparent dissociation constants of the Mg-ADP$^-$ and Mg-o^{1}ADP$^-$ complexes at pH 8. The same is true for the Mg-ATP^{2-} and Mg-o^{1}ATP^{2-} pairs.

Phosphofructokinase is known to use a wide variety of nucleoside triphosphates as phosphoryl group donors, preferentially GTP, ITP, UTP, CTP, and ϵATP (Uyeda and Racker, 1965; Bloxham and Lardy, 1972; Secrist et al., 1972). o^{1}ATP was shown to be very similar to ATP as substrate for rabbit muscle enzyme, the only difference being that the allosteric inhibition of phosphofructokinase is induced at a much higher concentration of o^{1}ATP than ATP. At 1.25 mM ATP the phosphofructokinase inhibition is 90% while a twofold concentration of o^{1}ATP determines only a 40% inhibition, which signifies that the structural requirement for the inhibitory phenomenon appears to be somewhat more stringent than for catalytic activity. Since the ATP-induced inhibition of phosphofructokinase is counteracted by AMP, in another experiment we also tested the comparative effects of o^{1}AMP and AMP. As shown in Table III, o^{1}AMP like AMP is able to reverse the ATP-induced inhibition of the enzyme, but at higher concentrations than the natural nucleotide. It has to be mentioned that natural or modified nucleoside monophosphates, other than AMP or cAMP, were shown to be unable to reverse the inhibition of phosphofructokinase by ATP (Uyeda and Racker, 1965; Bloxham, 1973).

Effect of Adenine 1-Oxide Nucleotides on Glutamate Dehydrogenase. It is interesting to note that the enzyme specificity for adenine nucleotides not as substrates but as allosteric effectors has been investigated to a lesser extent,

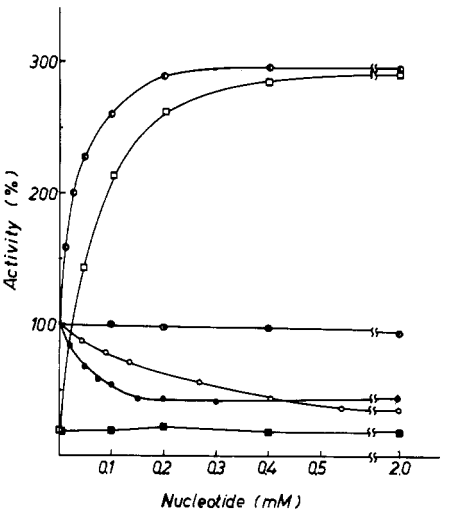


FIGURE 7: Effect of natural and modified purine nucleotides on beef liver glutamate dehydrogenase. The activity in the absence of any purine nucleotide (72 μmol per min per mg of protein) is considered as 100%. (●) ATP; (○) o^{1}ATP; (◐) ADP; (⊖) o^{1}ADP; (□) 0.4 μM of GTP + ADP; (■) 0.4 μM GTP + o^{1}ADP.

although the exploration of this aspect could lead to a better understanding of the in vivo effect of many structural analogues. Since the activity of glutamate dehydrogenase is known to be strongly dependent on the presence of purine nucleotides as allosteric effectors (Wolff, 1962; Frieden, 1965; Dalziel and Egan, 1972) we examined the comparative effects of adenine nucleotides and their N_1-oxides on purified beef liver glutamate dehydrogenase. As shown in Figure 7, o^{1}ATP as well as ATP inhibit the enzyme. The I_{50} values calculated as the nucleotide concentration required for half-maximal inhibition were 0.046 mM for ATP and 0.164 mM for o^{1}ATP. At high concentrations o^{1}ATP was slightly more active (66% maximal inhibition) than ATP (57% maximal inhibition). While ADP is able to activate the purified beef liver enzyme by a factor of three, the half-maximal activation being reached at 0.025 mM ADP, its 1-oxide analogue shows a very weak inhibition. Since ADP is known to reverse the inhibitory effect of GTP, we further investigated the effect of o^{1}ADP on the GTP-inhibited glutamate dehydrogenase. o^{1}ADP, like o^{1}AMP or o^{1}ATP, has no effect at all on the GTP inhibition of beef, rat, or human liver glutamate dehydrogenase. Moreover o^{1}ADP does not even interfere with the activation of glutamate dehydrogenase by ADP. Since by N_1-oxidation, which involves the nitrogen in the immediate vicinity of the C_6 substituent, the activatory effect of ADP is lost and the inhibition by ATP is much attenuated it seems that the intact C_6 amino group is essential for binding to an allosteric site of glutamate dehydrogenase. Indeed, iGTP and iGDP, obtained by a photoisomerization of o^{1}ATP and o^{1}ADP, where the C_6 amino group remains intact, are able to bind strongly to glutamate dehydrogenase, being very powerful inhibitors of this enzyme (the I_{50} value was 2.6 μM for iGTP).

Concluding Remarks

Although the number of synthetic nucleotide analogues has been increasing permanently during the last years there are only few and scattered data available concerning the direct relationship between chemical structure and their biological activity. Since also the number of tested systems is rather limited, it would be premature at this stage of our knowledge to try to establish a quantitative relationship be-

tween the chemical structure of the investigated compounds and their role as substrate or regulatory agents of different enzymatic systems.

The attribute of an individual purine nucleotide to act either as substrate or as allosteric effector seems to be specifically expressed by different parts of the molecule. Nucleotide analogues with modifications at the heterocyclic nucleus, like the adenine 1-oxide derivatives used in our experiments, are not translocated across the inner mitochondrial membrane. This permeability barrier makes them unable to participate in the reactions of the oxidative phosphorylation or other energy requiring processes (Kezdi et al., 1973). This absolute requirement of the translocase system for an intact adenine ring does not apply to the pentose moiety or the polyphosphate chain. Nucleotides like dATP, dADP, AOPCP, AMP-PCP, and AMP-PNP are recognized by the mitochondrial translocase system (Klingenberg and Pfaff, 1968; Duée and Vignais, 1968; Klingenberg, quoted by Yount et al., 1971). Modifications in the structure of the pentose or the polyphosphate chain however lead to an important decrease and even a loss of the property of such nucleotides to participate in the phosphate group transfer reactions (Duée and Vignais, 1968; Yount et al., 1971; Remy et al., 1970). With only a few exceptions most modifications in the base moiety do not seriously affect this phosphate group donor-acceptor function of the nucleotides. There are much less data on modified nucleotides as allosteric effectors; however, it seems that the specificity as effectors is localized primarily in the base structure (Atkinson and Murray, 1967; Haschke et al., 1972; Steiner, 1972; Bloxham, 1973).

What are then the essential structural features which determine the specific behavior of adenine 1-oxide nucleotides? At the first sight it seems to be a local effect, i.e., the structural modification at the N_1 atom affects the adjacent 6 amino residue which plays an important role in the substrate specificity and binding properties of adenine nucleotides. However, the strong new absorption at 233 nm, due to the π-π^* transition of the extended π electron system of the 1-oxides, as well as the ^{13}C chemical shift pattern, indicate that the N_1 oxygen is not acting just as a substituent but has a far reaching contribution to the whole heterocyclic π electron system. Such a redistribution of the whole electron density is expected to affect strongly the stacking properties of the adenine moiety of the modified nucleotides and therefore their binding characteristics to different biological systems. Indeed, as shown by our preliminary ^1H NMR experiments the stacking properties of adenine nucleotides are completely lost in the corresponding N_1-oxides.

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