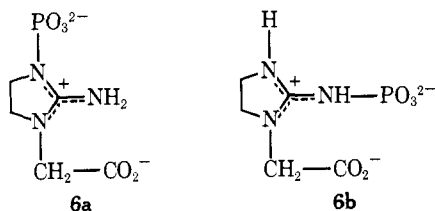


trogen furthest removed from the COO⁻ group, generating **6a** rather than **6b**.^{26,28}



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- (2) Department of Pharmaceutical Chemistry.
- (3) United States Public Health Service Predoctoral Fellow, 1970-1974, Fellowship No. GM 41089.
- (4) Recipient of a Career Development Award, GM-70718, from the National Institute of General Medical Sciences.
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Phosphorus-31 Nuclear Magnetic Resonance Studies on Nucleoside Phosphates in Nonaqueous Media

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Abstract: ³¹P nuclear magnetic resonance chemical shift and coupling constant data obtained from tetra-*n*-butylammonium nucleoside phosphates in water and anhydrous tetramethylurea support an early proposal by Albert Szent-Gyorgi, where it was suggested that the phosphate side chain of the adenosine triphosphate molecule was folded so as to lie upon the adenine ring, the conformation being stabilized by the formation of a hydrogen bond between the β phosphorus and the 6-amino nitrogen of the ring. This conformation, which predominates in anhydrous media, is observed for all of the common nucleoside di- and triphosphates.

The role of adenosine triphosphate (ATP) as the energy mediator in a large number of biological reactions has been known since its discovery in 1929.¹ However, the conformation of the molecule in the context of these reactions, i.e., the nature of the transition state, has not been established with certainty. In 1957, Albert Szent-Gyorgi² speculated that the ability of the aromatic ring of the adenine base of the nucleotide to "quench" long-lived excited states of organic systems by energy absorption through π-π* transitions allows the adenine ring to participate in the activated complex in some biological reactions, e.g., oxidative phosphorylation.

The capture or release of energy in reactions of this type is associated with the formation and cleavage of P-O-P bonds, usually the terminal P-O-P linkage of the triphosphate side chain. However, these two functional groups, the adenine ring and the polyphosphate chain, are separated by the sugar ribose "which has no conjugated double bonds and no π electrons"² through which energy interactions could be effected. Szent-Gyorgi postulated that the ribose might function as a "hinge" to allow the polyphosphate chain to double back over the ring, thus creating a close physical and chemical rela-

tionship between these two components. The structure would be stabilized by the formation of a hydrogen bond between the 6-amino nitrogen of the ring and the β phosphate of the chain, leaving the terminal phosphate group free for cleavage.² Alternate structures proposed¹⁻¹⁰ included mono- or polyvalent cations, particularly Mg²⁺ and Ca²⁺ with these ions forming coordination complexes incorporating the γ phosphate of the chain and the 7 nitrogen of the adenine base.

Several investigations of ATP in aqueous solutions^{1,3-9} have produced varying results, with some observers concluding that little interaction exists between the ring and the chain in the absence of metal ions,⁸ while others^{1,4,5,7} have found such associations when monovalent metal ions such as Na⁺ and K⁺ were used to titrate solutions. Still others have reported the formation of numerous complexes between nucleotides and divalent cations.^{1,5,6,9}

Szent-Gyorgi's original and bold proposal has also inspired a number of mathematical investigations of this nucleotide, and these have lent credence to the concept of "folded" ATP.^{8,10}

Intracellular, and certainly intramitochondrial environ-

Table I. Nucleoside Triphosphate Chemical Shifts and Coupling Constants in Water and in Anhydrous Tetramethylurea at H⁺ Ion Concentrations Corresponding to pH 7.40

Compd	Solvent	Chemical shifts ^a			Coupling constants, Hz	
		α	β	γ	$J_{\alpha-\beta}$	$J_{\beta-\gamma}$
ATP	H ₂ O	11.45	22.66	7.33	19.75	19.75
	TMU	12.25	23.17	10.64	25.6	23.6
dATP	H ₂ O	11.43	22.83	7.71	20.50	20.50
	TMU	13.47	23.46	10.46	28.3	24.6
ITP	H ₂ O	11.49	22.84	7.63	20.75	20.75
	TMU	12.34	23.03	10.87	25.1	23.6
GTP	H ₂ O	11.49	22.85	7.70	19.25	19.25
	TMU	12.04	23.12	10.97	25.6	25.0
CTP	H ₂ O	11.51	22.77	7.25	20.25	20.25
	TMU	12.28	22.74	10.45	25.1	23.1
UTP	H ₂ O	11.50	22.92	7.52	20.53	20.53
	TMU	12.36	22.70	10.45	25.1	23.8

^a Chemical shifts in parts per million relative to 85% orthophosphoric acid.

ments, where most biochemical reactions occur, are not simple aqueous solutions, however. Indeed, the surfaces of enzyme proteins or biomembranes are best considered as nonaqueous, and in certain instances aprotic as well. In such environments even water molecules, when present, participate in ordered structures.^{2,11}

Data on such nonaqueous systems are scant and, thus, the present ³¹P nuclear magnetic resonance (NMR) investigation into the properties of ATP in an anhydrous, aprotic medium was undertaken, and the data were interpreted in terms of the conformation of ATP and of other nucleotides. In vivo ³¹P spectra of ATP in intact frog gastrocnemius muscle were also obtained and the data parallel to a considerable degree those observed in the anhydrous system.

Materials and Methods

Instrumentation. Samples were examined by the method of ³¹P nuclear magnetic resonance.^{12,13} The spectrometer employed was a Bruker HFX-5 operating at 36.43 MHz for ³¹P and 90 MHz for ¹H, and containing Fourier transform and broad-band proton decoupling capabilities. In this work, protons were routinely decoupled from phosphorus by broad-band irradiation techniques, to permit precise measurement of the α -phosphate resonances. The proton-coupled spectra were not sufficiently resolved to permit precise measurements of the POCH couplings, although it was clear that these parameters also reflected the same differences observed with greater precision through the POP couplings.

Chemical shift (δ) data are reported in parts per million (ppm) relative to the usual standard of 85% H₃PO₄¹² with chemical shifts increasing with increasing field intensity, as is customary in ³¹P NMR. The primary standard, however, was a capillary (1-mm) containing either acetone-*d*₆, or 1.0 M methylenediphosphonic acid [(HO)₂OPCH₂PO(OH)₂] in D₂O (pD 9.5, Na⁺ counter-cation in muscle studies) coaxially mounted in the sample tube.^{12,14,15}

Preparation of Solutions. The solvent chosen was *N,N,N',N'*-tetramethylurea (TMU),¹⁶ which contributes no free protons, and whose monoprotonated species exhibits a pK value of <2 in H₂O.¹⁷ The range of pH studied was 3–10 (pH here defined as $-\log [H^+]$, regardless of whether the medium is water or tetramethylurea). The cation employed was tetra-*n*-butylammonium, which is sufficiently bulky so that it does not form association complexes with the polyphosphate chain;¹⁵ the various salts were prepared as has been described.¹⁴ Each sample was diluted to a concentration of 0.001 M to eliminate the phenomenon of base stacking.^{18–21} Spot checks of the data with nucleotide concentrations in the range of 10⁻⁴ to 10⁻² M showed that the ³¹P data were independent of nucleotide concentration within this range.⁴¹

The background electrolyte was 0.1 M tetra-*n*-butylammonium chloride. After spectroscopic analysis in water, each sample was dried

by rotary evaporation at 35 °C, redissolved in anhydrous acetone–benzene (1:1) and evaporated at least three times to render the preparation anhydrous. After dissolution of this preparation in a volume of anhydrous tetramethylurea equivalent to the volume of the original aqueous solution, the sample was again analyzed by ³¹P spectroscopy.

Evaporations from anhydrous acetone–benzene were repeated until the ³¹P chemical shifts no longer changed upon further treatment. Such final solutions showed no H₂O signal in the ¹H NMR spectrum, and also proved to be nonreactive to CaH₂.

In these anhydrous preparations, the concentrations of nucleotide and protons were the same as in the original aqueous solutions. To check for changes in the proton concentration (pH) resulting from the evaporative procedure, the tetramethylurea solutions were lyophilized and redissolved in water, and the ³¹P shifts and pH values were remeasured. It was found that, using reasonable care, the ³¹P shifts and pH values of the solutions were reproducible within experimental precision. The above outlined procedure ensures that the ratio of acid protons to nucleotide was identical in both the aqueous and nonaqueous solutions; however, the nature of the solvation state of the protons in the anhydrous system is not known, and it is not meant to be implied by the use of the term “pH” that this state is analogous to that in water.

Results and Discussion

³¹P Chemical Shifts and POP Coupling Constants. Triphosphates. The shifts and coupling constants (J) of several common nucleoside triphosphates in water and tetramethylurea (pH 7.40) are presented in Table I. In all cases the γ phosphate experiences a pronounced upfield shift of ca. 3 ppm in tetramethylurea relative to that in water. Similarly, the α phosphate is shifted upfield, but to a lesser degree. For the ribose nucleotides, this shift is about 0.8 ppm; for deoxy-ATP this shift is more pronounced (2.04 ppm). The β groups undergo the smallest shift changes between aqueous and nonaqueous media (ca. 0.3 ppm). This behavior is not due solely to a solvent effect on the NMR parameters, for when different anhydrous solvents such as dimethylformamide or pyridine were employed, the ATP spectra were virtually identical with those in tetramethylurea. Furthermore, this effect is not due to intrinsic behavior of the tripolyphosphate chain, because chemical shift changes observed for inorganic polyphosphates²³ do not parallel those found for nucleoside di- and triphosphates. It should be noted in Table I that, while there are essentially no differences in β group chemical shifts between the purine and pyrimidine phosphates in water, a shift difference for this grouping is observed in the anhydrous solvent. On the average, the pyrimidine β groups lie 0.37 ppm to lower field than those of the corresponding purines.

The coupling constants between the two pairs of phosphates, α - β and β - γ , are always equal in aqueous solution, and smaller than their corresponding values in tetramethylurea. Furthermore, in tetramethylurea the α - β coupling is larger than the β - γ coupling by about 2.0 Hz.

Diphosphates. The chemical shifts and coupling constants of some nucleoside diphosphates in water and in tetramethylurea (pH 7.40) are given in Table II. Again, the chain terminal (now the β) group experiences an upfield shift in the anhydrous solvent relative to that in water (ca. 2 ppm). In contrast to the triphosphate data, the α group of the diphosphate is shifted downfield (ca. -1.5 ppm); an exception is the deoxy compound TDP, which experiences a 2.35-ppm upfield shift in tetramethylurea.

The shift changes observed in both the nucleoside di- and triphosphate are interpreted to indicate that in the anhydrous system the phosphate side chain is brought into juxtaposition (folded) with the base of the nucleotide. Such a configuration would give rise to at least three phenomena which can alter the ³¹P chemical shifts. (1) The conformation of the polyphosphate chain, which is constantly changing in aqueous solution, would

Table II. Nucleoside Diphosphate Chemical Shifts and Coupling Constants in Water and in Anhydrous Tetramethylurea at H⁺ Ion Concentrations Corresponding to pH 7.40

Compd	Solvent	Chemical shifts ^a		Coupling constants, Hz <i>J</i> _{α-β}
		α	β	
ADP	H ₂ O	11.13	6.96	23.1
	TMU	10.84	8.54	22.6
TDP	H ₂ O	11.26	7.02	23.6
	TMU	13.61	13.61	<i>b</i>
UDP	H ₂ O	11.18	6.85	23.6
	TMU	9.40	8.80	20.8
IDP	H ₂ O	11.03	7.11	22.1
	TMU	9.40	9.40	<i>b</i>
CDP	H ₂ O	11.13	6.94	22.6
	TMU	9.66	8.98	23.3
GDP	H ₂ O	11.10	6.69	23.1
	TMU	9.45	9.45	<i>b</i>

^a Chemical shifts in parts per million relative to 85% orthophosphoric acid. ^b Not measurable because of the equivalence of the chemical shifts of the α and β groups.

be locked into the folded position, thus altering the P–O–P bond angle distribution: Gorenstein²⁴ has obtained data showing that ³¹P chemical shifts of phosphate esters may depend in large measure on P–O–P bond angle. (2) The proximity of the phosphate chain to the aromatic ring would expose the phosphates to ring-current shielding and deshielding, depending on the aromatic character of the nucleotide base. (3) Hydrogen-bonding interactions between the phosphate and the base would give rise to chemical-shift changes depending on the π character of the P–O bonds, the electronegativity of the PO₄ oxygens,¹³ and depending on whether the phosphate is the proton donor or acceptor.²⁵

It should be noted that parallel chemical-shift changes are not observed for orthophosphate monoesters of nucleoside monophosphates, for unesterified (inorganic) ortho- and condensed phosphates, or for small alkyl ortho- or condensed phosphates. These characteristic changes are only observed for the nucleoside di- and triphosphates, suggesting that the β phosphate group plays a key role in the interaction of the polyphosphate side chain with the aromatic ring.

In examining the ³¹P spectra of the nucleoside diphosphates, one striking feature is immediately apparent: with TDP, UDP, IDP, CDP, and GDP nucleotides in tetramethylurea, the spectra show that for each molecule the α-group shift is nearly identical with that of the β. However, ADP is unique in that the two phosphates experience different shielding effects, and a well-resolved ab multiplet is observed (Δδ 2.3 ppm). These findings suggest that the β group is interacting with a functional group of the adenine ring, most probably the amino group at the 6 position. It can be seen in Table II that the β group of ADP is relatively deshielded in tetramethylurea with respect to those of the other nucleoside diphosphates, and such a deshielding is consistent with participation in hydrogen bonds when the phosphate group is the proton acceptor.²⁵

Triphosphate Titrations. Figure 1 presents the chemical-shift behavior of the ATP phosphorus atoms as a function of pH in both water and tetramethylurea. The titration curves of ATP in H₂O with the tetra-*n*-butylammonium cation are similar to those seen by other investigators using other cations.^{26–28} Characteristically, the γ group, and to a lesser extent the β group, undergoes a downfield shift with increasing pH, with both curves having an inflection point at pH 6.7, corresponding to the p*K*_a of the weak acid deprotonation of the chain terminal (γ) phosphate group. The α group, being remotely removed from this reaction, undergoes almost no shift change throughout the pH range.

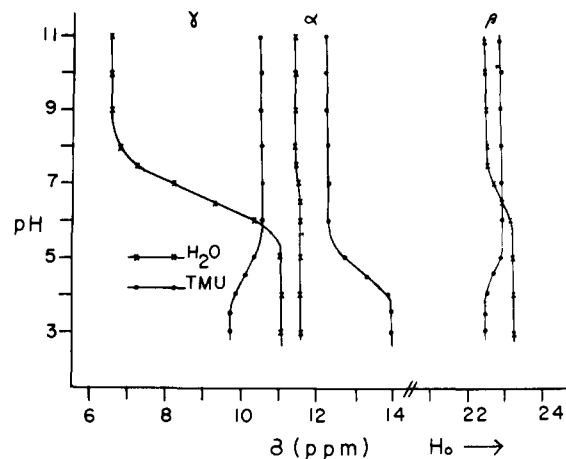


Figure 1. pH dependence of ATP phosphates in H₂O and in anhydrous tetramethylurea (TMU).

In tetramethylurea, however, the inflection at pH 6.7 is no longer observed. Instead, a new transition is seen at pH 4.7, involving all three phosphate groups. The α group, which is shifted far upfield at low pH when compared with its water curve, shows a downfield transition as the pH is raised, while the β and γ curves show a transition in the opposite direction with respect to their counterparts in water.

In view of the fact that there is no known protonation reaction intrinsic to the phosphate chain at pH 4.7, that the observed transitions at this pH are not consistent with protonation of the chain, and that the adenine ring itself undergoes protonation at the 1(N) position at about this pH in water,^{3,5,8,29} the observed effect most likely can be attributed to close contact between the ring and the chain, and the participation of the polyphosphate chain in the protonation.⁴² At low pH, such a protonation would add a positive charge to the ring, providing additional stabilization of the folded conformation. Moreover, participation of the phosphates may account for the slight elevation of the p*K*_a value of the ring from pH 4.1 in water to pH 4.7 in tetramethylurea. However, in water, where the chain is extensively hydrated and undoubtedly removed from the ring,¹ no such shift change effect is observed in the phosphorus spectrum.

That the transition at pH 4.7 is, in fact, a reflection of protonation of the adenine ring, and not protonation of the phosphate chain, in this nonaqueous system is supported by the corresponding data obtained from the pyrimidine, uridine triphosphate (UTP), shown in Figure 2. In water, the UTP and ATP titration curves are virtually identical, with only small (ca. 0.1 ppm) shift differences being detected between the corresponding phosphate groups. In tetramethylurea, the α phosphate of UTP, unlike that of ATP, shows no variation whatsoever in its chemical shift over the entire range of proton concentrations examined. The β and γ groups again, unlike those of ATP, show no transition on the acid side of neutrality. These phosphate groups, however, do exhibit a transition at low proton concentrations corresponding to deprotonation of the 4-OH groups of the pyrimidine base in aqueous solution. This p*K*_a value for UTP in water is 9.6;³ the corresponding transition in anhydrous tetramethylurea has the same value ± 0.2 pH units. In addition, the γ group of UTP shows a slight change in chemical shift at pH 6.5, which is similar to the p*K*_a value of the weak acid dissociation of the triphosphate chain in water.

Diphosphate Titrations. In the case of the diphosphates, the chain terminal protonation would perturb the hydrogen bond to the β phosphate. This is observed in the titration curve of ADP, depicted in Figure 3, which compares the ADP phosphates in water and tetramethylurea as a function of pH. Once

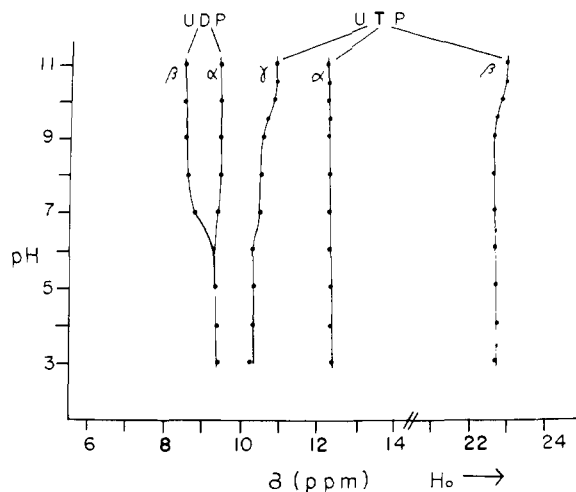


Figure 2. pH dependence of UTP and UDP phosphates in anhydrous tetramethylurea.

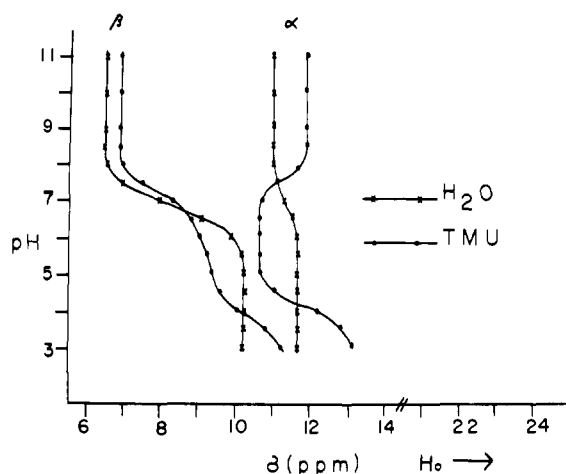


Figure 3. pH dependence of ADP phosphates in H_2O and in anhydrous tetramethylurea (TMU).

again, in water, a transition is observed at pH 6.7, corresponding to protonation of the chain terminal phosphate, while no transition is observed below this value. In anhydrous tetramethylurea, both phosphates experience strong shielding effects at pH 4.1, corresponding to protonation of the aromatic ring. In addition, a transition is also observed at pH 7.5, corresponding to the protonation of the β phosphate which is apparently a weaker acid in tetramethylurea than in water. Both the shift changes and the pH values of the transitions are consistent with a folded conformation of the diphosphate having a hydrogen bond between the β group phosphate and the 6-amino nitrogen. It is understandable that, in ATP, deprotonation of the chain terminal phosphate, which is not the site of the hydrogen bond as in the diphosphates, will not directly perturb the proposed folded conformation. If the observed shift in the anhydrous solvent is primarily a function of the conformation of the molecule, which appears to be the case, then the absence of a transition in the neutral pH range for ATP in tetramethylurea is not surprising.

Again, the titration of uridine diphosphate (UDP) in tetramethylurea (Figure 2) supports the interpretation that the transition for ADP at proton concentrations corresponding to pH 4.1 arises as a result of interaction with the purine ring and is not a simple protonation of the phosphate chain. In the data of Figure 2, no transition is observed in the UDP spectrum in the region of high proton concentrations, as was observed with the corresponding triphosphate, UTP. The α group undergoes

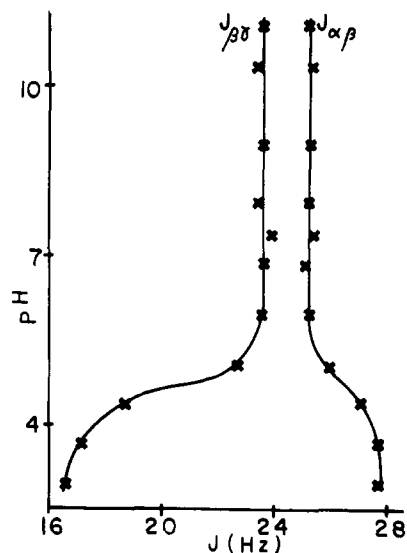


Figure 4. pH dependence of ATP coupling constants in anhydrous tetramethylurea.

virtually no chemical-shift change over the entire range studied. The β group exhibits a transition at proton concentrations corresponding to pH 6.6, a value which is consistent with the dissociation of the phosphate weak acid proton. For the diphosphate UDP, no transition is observed which corresponds to deprotonation of the uridine base.

ATP POP Couplings. Plotted in Figure 4 are the POP couplings $J_{\alpha-\beta}$ and $J_{\beta-\gamma}$ of ATP as a function of pH in anhydrous tetramethylurea. The coupling constants do not vary with pH in the neutral and alkaline range. At pH 4.7 a transition is observed for both coupling constants, and the respective values diverge, until at low pH values their magnitudes differ by almost 12 Hz. This divergence markedly alters the appearance of the ATP spectrum. The most striking change is observed in the β -group multiplet which is transformed from a simple 1:2:1 triplet to a pattern of four well-resolved signals of nearly equal intensities. This spectroscopic observation is unusual, and to our knowledge no similar divergence in the values of the coupling constants of ATP has ever been observed.

In water (with ethylenediaminetetraacetic acid (EDTA) to chelate interfering polyvalent metal cations) and in the presence of the tetra-*n*-butylammonium ion, both of the ATP POP couplings are equal ($J = 21.0$ Hz) and do not change with pH. A similar observation was made by Cohn and Hughes,²⁶ in their aqueous studies using the tetramethylammonium cation, and with EDTA present ($J = 19.3 \pm 0.5$ Hz²⁶). In another study Ellenberger et al.²⁷ did observe a slight decrease in the ATP POP couplings with decreasing pH (19.0 Hz, pH >8.0; 17.4 Hz, pH <5.0²⁷); however, this observation was clouded by the fact that no strong chelating agent was used to preclude interference by polyvalent cations. Furthermore, while sodium phosphates were the starting materials, the pH values of the solutions were adjusted with potassium hydroxide, thus introducing two counter-cations into the same medium. Crutchfield and Irani³¹ have observed ATP coupling constant changes associated with a change in the counter-cation ($J = 18.8$ Hz, Na^+ ; $J = 20.4$ Hz, K^+ ; both measurements at pH 7³¹); however, the separate $J_{\alpha-\beta}$ and $J_{\beta-\gamma}$ were again equal.^{13,32}

Apparently, protonation of a phosphate, which occurs around pH 7 for ATP, has little or no effect on the POP couplings in either aqueous or anhydrous media. Therefore, some other interaction must be invoked to rationalize the transition observed in the couplings in the anhydrous system at pH 4.7. Since the proton concentration associated with the transition is that which is also associated with the protonation of the

adenine ring, it is reasonable to suggest that the altered electronic environment of the protonated adenine ring gives rise to the change and does so through the association of the polyphosphate with the base in a folded ATP molecule. The pronounced J value changes associated with the pH 4.7 transition indicate that the extended arrangement of atoms in the hydrated P–O–P chain is being altered in the anhydrous system. At this time it cannot be said whether the bond lengths or the bond angles are being altered, or whether the changing J values reflect rotational alterations. Giessner-Prettre and Pullman,³³ in quantum mechanical calculations of ^{31}P – ^{13}C and ^{31}P – ^1H couplings of ethyl phosphate have found that coupling constants involving phosphorus depend primarily on the rotational angles about the P–O– bond. We are currently involved in a similar analysis of the ^{31}P –O– ^{31}P couplings in inorganic tri- and tetrapolyphosphates.³⁴

Folded ATP. Figure 5 shows the arrangement of atoms in a folded ATP molecule which could give rise to the observed spectroscopic behavior of ATP in anhydrous tetramethylurea. In this conformation, the α and β groups of the triphosphate chain lie upon the aromatic ring system. After the manner of Szent-Gyorgi,² the ribose sugar acts as a "hinge" holding the α phosphate end of the chain in place. The β phosphate is held in position by a hydrogen bond to the functional group at the 6 position of the purine, and the entire structure is stabilized to some degree by overlap of the π -electronic cloud of the aromatic ring system with the delocalized electrons of the condensed phosphate.³⁵ Measurements on molecular models show that the α and β phosphorus atoms may lie within 3.1 Å of the adenine ring (measured from the plane of the adenine ring to the center of the P atoms).

The exact disposition of each functional group in ATP with respect to its neighbors cannot be determined with certainty from NMR data alone, and verification of such a proposed structure must depend on the results of other physical–chemical measurements. Nonetheless, this structure is reasonable; it provides a working model to explain the ^{31}P NMR data, and it may in fact suggest explanations for other established properties, e.g., the ATP coordination of Mg^{2+} involving the γ group and the 7(N) of ATP. These two functional sites are suitably positioned in the model for such an interaction. The model also suggests reason for the facile hydrolysis of the γ phosphate since it is the one functional group left dangling in the medium. Also, the 1(N) position and three of the oxygens of the condensed phosphate chain form an electronegative nest for the binding of the pK_a 4 dissociable proton.^{2,5,8,29}

The model predicts that there, in fact, could be two such folded conformations of ATP which would differ depending on which face of the adenine ring is presented to the condensed phosphate. Either arrangement is possible. We have observed that at pH values above the pH 4.7 transition point in several samples the ^{31}P β and γ multiplets are clearly split into two nearly equivalent sets of resonances, suggesting two nearly identical populations of phosphates. The ratio of the low-field multiplet to the high-field multiplet is 1:1.5 for each group. The chemical-shift difference between the sets of resonances of each group is: 10.5 Hz for the γ groups and 6.7 Hz for the β groups. At pH values below this transition point, splitting is observed in the α and β groups, and again the ratio of the low-field to the high-field resonances is 1:1.5; the chemical-shift difference between the sets of resonances of each group is: 6.3 Hz for the α groups, and 6.7 Hz for the β groups. Observation of this multiplicity seems to depend on the relative moisture content of the sample. Merely exposing the sample to air for a few moments was often sufficient to cause coalescence of the set of peaks.

Investigations of ATP in the Intact Muscle. Recent studies in these laboratories involving biological systems^{35–37} have shown that ^{31}P NMR spectra of high information content can

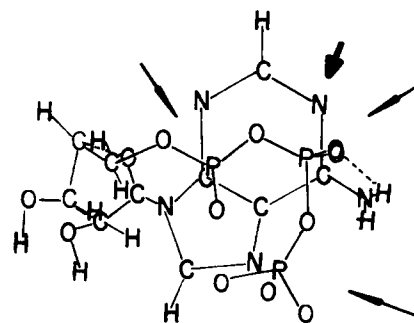


Figure 5. Projection of a model of folded, ATP, showing a hydrogen bond (dotted line) between the α phosphate and the 6-amino nitrogen. Slender arrows point to the phosphorus atoms. Wide arrow indicates the 1 (nitrogen) position.

be obtained from large macromolecular structures^{12,36,37} and intact cells³⁸ or tissues.³⁹ In such spectra, it is possible to identify ^{31}P resonances arising from discrete molecular species, and to observe changes in these signals which can be correlated with the metabolic state of the sample. Information regarding the environments of these molecules in these intact systems can also be obtained from the spectra.

Spectra obtained from *in vivo* ^{31}P studies on intact frog (*Rana pipiens*) gastrocnemius muscle (with M. Bárány et al.³⁹) were sufficiently resolved so that the ATP coupling constants could be determined. The couplings $J_{\alpha-\beta}$ and $J_{\beta-\gamma}$ in freshly prepared muscle are equal and have the value of 14.8 Hz. The corresponding value in our aqueous system with tetra-*n*-butylammonium cation is 21.0 Hz at pH 7, while in our anhydrous system $J_{\beta-\gamma}$ is 23.6 Hz and $J_{\alpha-\beta}$ is 25.3 Hz. The POP couplings in resting muscle were observed to change with the lowering of the pH upon aging so that a sample aged 10 h at 28 °C exhibited a $J_{\alpha-\beta}$ of 22.8 and a $J_{\beta-\gamma}$ of 14.8 Hz.

To date, we have no firm interpretation for the marked lowering of the coupling constants in fresh muscle relative to the couplings in free aqueous solution, or the divergence of the POP couplings in aged (acid) muscle. But markedly altered coupling constants relative to those obtained from sodium ATP in neutral aqueous solution have only been observed when the phosphate is dissolved in anhydrous media (this work and reference 40). For all cases in aqueous solution thus far examined, $J_{\alpha-\beta}$ and $J_{\beta-\gamma}$ are always equal and tightly clustered about the value of 19 Hz;^{13,26,27,31} therefore, we propose that the very altered couplings in muscle arise because the ATP is in either a structured aqueous and/or a hydrophobic environment which may or may not include interactions with metal cations. The specific coupling constants in this system would then reflect particular conformations of the ATP molecule, and the changing values of the coupling constants with changing metabolic state would reflect transitions between conformations as the local environment is altered.

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 (41) Hanlon and Major²² have shown that, even at pH values corresponding to protonation of the adenine ring, ethylene glycol effectively prevents the stacking of 5'-AMP when the nucleotide concentration is in the ranges used in this study. At neutral or alkaline pH values this glycol effectively prevents stacking within polyriboadenylic acid (and AMP). Stacking involving the nucleoside polyphosphates ought to be even less likely. Corresponding data with tetramethylurea as the solvent are not available because of the absorbance of this and similar molecules at key regions in the ultraviolet spectrum.
 (42) A possible mechanism for these shift changes may be the alteration of the torsional conformation resulting from the protonation of the base. Gorenstein and Kar³⁰ have demonstrated that such shifts in phosphoric diesters can arise from conformational changes.

Phosphorus-31 Chemical Shift Anisotropy in Unsonicated Phospholipid Bilayers

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Abstract: Phosphorus-31 NMR spectra of unsonicated bilayer dispersions of dipalmitoyl-3-*sn*-phosphatidylcholine are recorded at temperatures above and below the gel-to-liquid crystalline phase transition. Proton decoupling yields spectra characteristic of axially symmetric chemical shift anisotropy. A quantitative interpretation of proton decoupled and nondecoupled spectra yields accurate numbers for the chemical shift anisotropy and the proton-phosphorus dipole-dipole interactions.

Normal phosphorus-31 NMR spectra of unsonicated phospholipid bilayers at resonance frequencies up to 90 MHz show one broad line of approximately 50 ppm line width.^{1,2} The line shape of the powder-type spectra is caused by (a) the chemical shift anisotropy of the phosphorus nuclei and (b) the proton-phosphorus dipole-dipole interactions. One possibility to distinguish between the two contributions is to perform the experiments at higher resonance frequencies, because only the chemical shift is dependent on the magnetic field strength.³ An alternative method is to suppress the proton-phosphorus dipole-dipole interactions by means of a proton decoupling field. A first successful application of this method to phospholipid bilayers has been published.⁴ Recently the static chemical shift tensors of some phospholipids and phospholipid constituents have also become available.⁵ Since phosphorus-31 NMR may thus prove to be of increasing importance for the elucidation of the polar region of membranous structures, the purpose of this report is to give a quantitative interpretation of phosphorus-31 NMR spectra of unsonicated lipid bilayers under proton decoupled and nondecoupled conditions.

Experiment and Results

The experimental conditions are the same as described earlier.⁴ Dipalmitoyl-3-*sn*-phosphatidylcholine (50 wt%) was thoroughly mixed with water (50 wt %) in a sealed ampule.

About 500 mg of material was placed in a 10-mm NMR sample tube. ³¹P-NMR measurements were made above and below the gel-to-liquid crystal phase transition at 41 °C. All spectra were recorded on a Bruker-Spectrospin HX-90 pulse-fourier-transform spectrometer at a resonance frequency of 36.4 MHz. Proton decoupling experiments were performed with a Bruker-Spectrospin B-FS-100 frequency synthesizer and modulation unit.

Figure 1 shows phosphorus-31 NMR spectra of coarse dispersions of phospholipid bilayers above and below the phase transition. The proton decoupled spectra (Figures 1A and 1C) have the line shapes anticipated for powder-type spectra with axially symmetric chemical shift anisotropy,⁶ while the non-decoupled spectra are dominated by dipole-dipole interactions. Sonication of the lipid dispersion produces a sharp resonance line at frequency ν_i (cf. also ref 4, Figure 5). Also shown in Figure 1 are theoretical spectra which were calculated using the following assumptions. Let us denote with σ_{kk} ($k = 1, 2, 3$) the principal components of the static chemical shift tensor of the phosphorus nucleus in the choline group. In solution, due to the rapid tumbling of the molecule, only the average value σ_i (corresponding resonance frequency ν_i) is observed

$$\sigma_i = \frac{1}{3}(\sigma_{11} + \sigma_{22} + \sigma_{33}) \quad (1)$$

The motion of the phosphate group in a lipid bilayer is however