Conformational Analyses of Phosphocreatine and *N*-Methylcreatine, a Steric Model for Phosphocreatine

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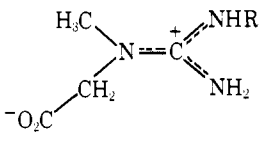
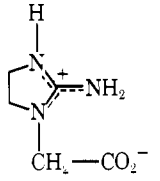
Abstract: The favored conformations of creatine, phosphocreatine, and *N*-methylcreatine (*N*-methylamidino-*N*-methylglycine) were sought using CNDO/2 theoretical calculations. These calculations lead to the prediction that *N*-methylcreatine should exist largely as an approximately 50:50 mixture of two conformers (**3b** and **3c**), both of which have minimal methyl–methyl and methyl–carboxymethyl steric repulsions. Experimental verification of this prediction came from a variable temperature NMR study of *N*-methylcreatine in CD_3OD. Two pairs of *N*-methyl peaks of approximately equal heights were apparent at −85 °C or lower, and these pairs of peaks each coalesced at higher temperatures; the activation parameters for this coalescence were found to be $\Delta H^{\ddagger} = 13.6 \pm 1.9$ kcal/mol and $\Delta S^{\ddagger} = 12.6 \pm 9.3$ eu at −70 °C. In contrast, the calculations lead to the prediction that phosphocreatine in the physiological pH range may prefer to be conformer (**2b**) in order to avoid both unfavorable steric repulsions and unfavorable electrostatic repulsions between the charged COO^- and PO_3^{2-} groups. These results are discussed in terms of the inability of *N*-methylcreatine to function as either a substrate or inhibitor of the enzyme creatine kinase. Also, they are consistent with the postulate that creatine kinase may catalyze a stereospecific phosphorylation of creatine to generate phosphocreatine conformer **2b** at the enzyme's active site.

Despite its great importance as the penultimate energy source for the muscle contractile process in vertebrates,[5] a detailed conformational analysis of phosphocreatine (**2**) has never been reported. The initial impetus for this study arose from the observations that whereas 1-carboxymethyl-2-iminoimidazolidine (**4**) is an excellent substitute for creatine (**1**) as a substrate for the enzyme creatine kinase, *N*-methylcreatine (**3**), the open-chain analogue of **4**, is not.[6,7] In fact, **3** is



1, R = H
2, R = PO_3^{2-}
3, R = CH_3



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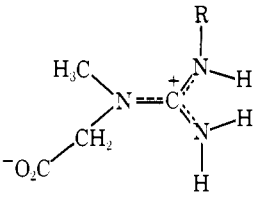
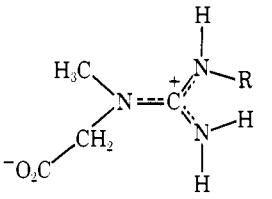
completely devoid of detectable activity as either a substrate or inhibitor.[7] A casual comparison of the structures of **3** and **4** does not reveal any profound steric or electronic differences; the crucial difference between the two structures undoubtedly involves the additional degrees of rotational freedom of **3** relative to **4**.

If one ignores for the moment the different conformers arising from rotation about the -N-CH_2- bond of the carboxymethyl substituent, then there are four major conformers possible for the *N*-methylcreatine molecule (**3a–d**). A similar set may be postulated for the phosphocreatine molecule (**2a–d**). Examination of space-filling models of both **2** and **3** indicated that energetically unfavorable steric repulsions could manifest themselves in certain conformations.[7] This can be readily visualized by considering structure **5** as a composite picture of the possible conformations for the *N*-methylcreatine molecule. The methyl group could in principle occupy any one of the four numbered positions. A space-filling model of the molecule indicates a clear preference for either position 2 or 3. Severe steric repulsions with either the methyl group or the carboxymethyl group come into play when either position 1 or 4 is occupied by a methyl group. Thus conformers **3b** and **3c** are preferred over conformers **3a** and **3d**.

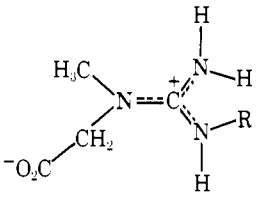
The conformational preferences for *N*-methylcreatine seemed to indicate that consideration of similar phenomena



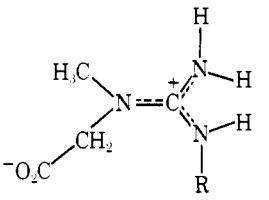
2a, R = PO_3^{2-}
3a, R = CH_3



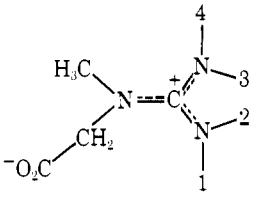
2b, R = PO_3^{2-}
3b, R = CH_3



2c, R = PO_3^{2-}
3c, R = CH_3



2d, R = PO_3^{2-}
3d, R = CH_3



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could be important in understanding the dynamics of the phosphocreatine molecule. In the case of phosphocreatine, electrostatic repulsions with the carboxymethyl group might be expected to play a significant role in addition to the bulk effects of the phosphoryl group. With this in mind it was decided to undertake a detailed conformational analysis of *N*-methylcreatine using variable temperature NMR spectroscopy. It seemed reasonable to assume that the barrier to rotation might be large enough to allow differentiation of the various types of methyl groups in these proton NMR spectra.[8-11] In the present study the results of the variable temperature NMR studies of *N*-methylcreatine and the activation parameters derived from them are discussed and interpreted with respect to theoretical calculations which have been made on the relative conformational stabilities of creatine, *N*-methylcreatine, and phosphocreatine.

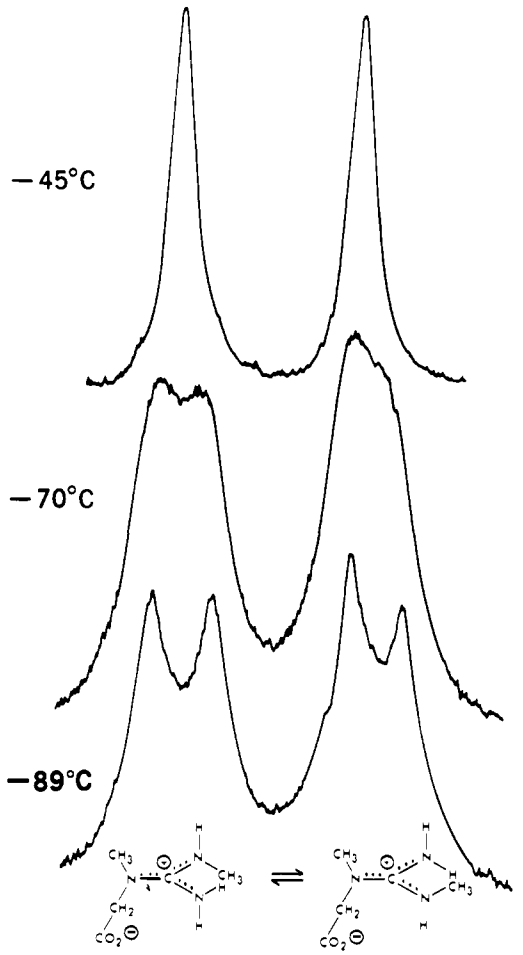


Figure 1. Series of low-temperature proton NMR spectra of *N*-methylcreatine, focusing on the $N-CH_3$ absorptions only.

Experimental Methods and Computational Details

The synthesis of *N*-methylcreatine (*N*-methylamidino-*N*-methylglycine) has been reported previously.[6] Proton NMR spectra at various temperatures were measured using a Varian HA-100 spectrometer. Owing to the zwitterionic nature of *N*-methylcreatine, a polar solvent was required for these studies. Water was unsuitable as a solvent, as it froze before the rotation slowed down sufficiently to be discernible on the NMR time scale. Mixtures of water–glycol were attempted, but viscosity and interference from glycol sidebands rendered this system unsatisfactory. *N*-Methylcreatine was found to be slightly soluble in methanol. This fact, along with the low freezing point of methanol (−100 °C), led to the use of CD_3OD (Bio-Rad) for these measurements. Rate constants for the restricted rotation of *N*-methylcreatine were determined by line-shape analysis.[12,13] Computer simulations of NMR spectra were generated using Program DNMR 3, Quantum Chemistry Program Exchange No. 165, written by Klein and Binsch.[14]

The molecular orbital calculations were carried out using the CNDO/2 semiempirical method,[15] QCPE No. 91. No d orbitals were used for the phosphorus atomic orbitals, and standard parameters were employed.[16] For methyl and phosphoryl substitution on the guanidinium group, standard bond lengths and angles were used with the exception of the geometry of the guanidinium group and the P–O bond lengths; these were taken from the x-ray diffraction studies of Herriott and Love.[17] The pK_a's for ionization of the second P–OH group in phosphocreatine[18,19] and in simple phosphoguanidines[20] are all ca. 4.5. Hence, all theoretical calculations on phosphocreatine were carried out using the PO_3^{2-} group, which is the dominant form for this substituent in the physiological pH range.

Results

The variable-temperature NMR measurements on *N*-methylcreatine focused mainly on the peaks arising from the $N-CH_3$ absorptions. At room temperature and considerably below the spectrum of the *N*-methylcreatine showed two sharp bands in the $N-CH_3$ region corresponding to a time-averaged peak for each of the different types of methyl groups. At low temperatures, the existence of the two favored steric conformations, presumably those with the amidino methyl group in either positions 2 or 3 in structure **5**, manifested itself by the resolution of each of the high-temperature methyl singlets into doublets. The conformational equilibrium which was observed is presumably the **3b** ⇌ **3c** interconversion. In Figure 1 a series of selected temperature-dependent spectra are shown. The temperature range where the most noticeable changes in the shape of the spectra occurred was ca. −60 to −85 °C. It was the data in this region which proved most useful for matching with the computer simulations (see below). From −85 °C down to the freezing point of the solvent, no significant changes in the spectra could be discerned. Complete resolution of the peaks never occurred. The line widths at half-height remained consistently broad relative to the difference in chemical shifts.

Attempts were also made to monitor the restricted rotation by observing the peak from the $-CH_2-$ group adjacent to the carboxyl function. This group should be subjected to different magnetic environments in each conformation, and this, in principle, should be expressed by differences in chemical shifts analogous to those experienced by the methyl groups. While some line broadening was observed in the peak for these protons, they were never completely resolved. The difference in chemical shift for the two conformations was apparently too small relative to the peak widths to allow complete resolution.

At each temperature where a spectrum of *N*-methylcreatine was recorded, an external Me_4Si peak was also recorded. This served as a check on possible mechanical broadening of the peaks. Over the range −60 to −90 °C no change in the width at half-height of the Me_4Si peak could be observed.

Computer Simulations. Rate constants for the rotational process taking place in the *N*-methylcreatine molecule were ascertained by matching the experimental spectra with spectra derived from the DNMR3 program. The changes which took place in the spectra, both experimental and simulated, were not large. Nevertheless, the experimental spectra at the low-temperature end of the range could be matched with a reasonable degree of accuracy. In this region, where resolution was beginning to take place, the peak-to-valley ratio of the spectra offered a reasonable parameter of comparison. Near the coalescence temperature and above, the only criteria available for matching was the width and flatness of the peaks. In this region a large deviation in rate constants in the simulated spectra would provide a satisfactory fit for a given experimental spectrum. Thus, the uncertainty in the rate constants for the high-temperature spectra, i.e., those in the range of ca. −68 to −58 °C is quite large. While the uncertainty of the rate constants at lower temperatures was much smaller in terms of absolute values, the deviation on a percentage basis was also large due to their much smaller numerical values. The most satisfactory agreements occurred in the range of −63.74 to −77.84 °C. These values were consequently used for the calculation of the activation parameters. Figure 2 shows an example of a typical match of a simulated and experimental spectrum. It should be noted that the dissymmetry in the doublet at higher field was never resolved. The simulated spectra for smaller values of k exhibited a slight amount, although smaller magnitude, of similar dissymmetry. The integration under both doublets in the experimental spectrum, determined by cutting out both peaks and comparing their weights, showed that they differed by less than 1%.

Activation Parameters for Rotation about the Guanidinium Bond. Previous studies investigating activation parameters for hindered rotation of substituted guanidines[8-10] have determined the free energy of activation at the coalescence tem-

Table I. First-Order Rate Constants for the Internal Rotation of *N*-Methylcreatine at Various Temperatures

T, °C	k, s^{-1}	T, °C	k, s^{-1}
−63.74	13.00 ± 2.00	−72.91	3.25 ± 0.25
−68.66	8.00 ± 1.00	−74.72	2.25 ± 0.25
−70.45	4.40 ± 0.40	−75.66	1.80 ± 0.10
−70.97	4.30 ± 0.20	−77.84	1.35 ± 0.15

perature. This activation parameter is readily determined using eq 1 and 2:

$$k_c = \pi(\nu_a - \nu_b)/\sqrt{2} \quad (1)$$

where k_c is the rate of exchange that results in coalescence and ν_a and ν_b are the frequencies of absorption of the two signals and where

$$k_c = \kappa T/h \cdot e(\Delta S^{\ddagger}/R) \cdot e(-\Delta H^{\ddagger}/RT) \quad (2)$$

with κ = Boltzmann's constant, and h = Planck's constant.

Such an analysis could not be applied to the *N*-methylcreatine problem for two reasons. An accurate value for $\nu_a - \nu_b$ is necessary, and this was not available from the study due to the incomplete resolution of the peaks at low temperature. The second reason is the uncertainty of the coalescence point. The coalescence point is defined as the temperature where the rate of interconversion of given signals A and B results in a rather flat-topped, broad absorption.[12] Even with ideal experimental data, the definition of the coalescence point seems to impart a rather subjective quality to this parameter.

An equally valid form of analysis, and one which allows for the determination of the enthalpy of activation for the restricted rotation, is a temperature-dependent line-shape study. The computer simulations described above were used to this end. The data used in the determination of the activation parameters were taken from the temperature range −63.74 to −77.84 °C. The rate constants for the experimental spectra at each individual temperature in this range were determined from the appropriately matched simulated spectra. Table I shows the data used. The data were plotted in the form of ln k vs. $10^3(1/T)$ and analyzed using a linear least-squares method. Using Arrhenius transition-state theory $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were determined using the Eyring equation [eq 2]. Values of $\Delta H^{\ddagger}$ = 13.6 ± 1.9 kcal/mol and $\Delta S^{\ddagger}$ = 12.6 ± 9.3 eu were calculated, with a confidence level of 95%.

Discussion

The variable temperature NMR studies have verified the fact that two conformers of *N*-methylcreatine, presumably those corresponding to a methyl group in either position 2 or 3 in structure **5**, exist in a dynamic equilibrium with each other, i.e., **3b** and **3c**. Furthermore, the $\Delta H^{\ddagger}$ for the interconversion between the two forms indicate that *N*-methylcreatine is behaving in a manner typical of other substituted guanidines.[8-11]

Table II shows the results of CNDO/2 calculations of barriers to rotation in some guanidines and guanidinium ions. The experimentally determined barrier to rotation of 13.6 ± 1.9 kcal/mol for *N*-methylcreatine is lower than that calculated using the CNDO/2 method for either the guanidinium ion itself, the *N,N*-dimethylguanidinium ion, or *N*-methylcreatine. However, more accurate ab initio calculations[21] find a barrier of 14.1 kcal/mol for the guanidinium ion, with a basis set of "double ζ" quality being required to reproduce the magnitude of the experimentally determined barriers typical of this type of ion.[8-10]

All of the compounds listed in Table II are calculated (via CNDO/2) to have a planar guanidinium group in their conformation of minimal energy. In addition, the ab initio calculations, x-ray crystallographic studies,[17,22,23] and NMR experiments[8-10] all support a minimum energy, planar guanidinium group.

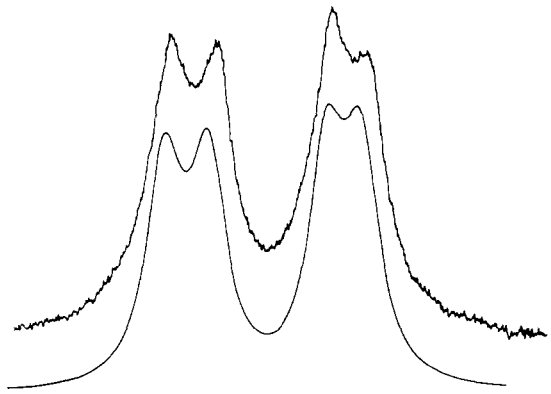
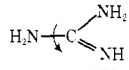
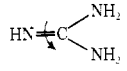
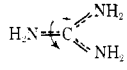
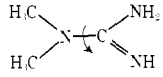
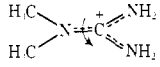
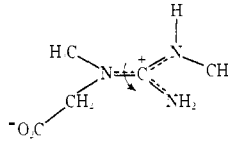


Figure 2. Typical match of an experimental proton NMR spectrum (top, T = −74.72 °C) and a simulated spectrum (botton, k = 2.4) generated by a DNMR3 computer program for the restricted rotation of *N*-methylcreatine. Only N-CH_3 absorptions are represented.

Table II. Calculated and Experimental Values for Barriers to Rotation in Some Guanidines and Guanidinium Ions

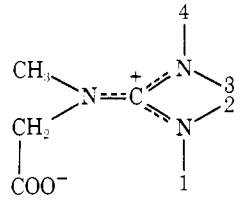
Structure	Calcd barrier to rotation using: CNDO/2, kcal/mol	Calcd barrier to rotation using: *Ab initio*, kcal/mol	Exptl. $\Delta H^{\ddagger}$, kcal/mol
	14.4		
	30.4		
	21.9	14.1	
	11.6		
	19.7		
	22.6		13.6 ± 1.9

[a] The calculated ΔE between the planar and perpendicular conformations; this should correspond to the energy of activation and differ by ~0.6 kcal/mol from the enthalpy of activation. [b] See ref 21.

Even very highly hindered guanidinium ions, such as 1,1,2,2,4,4,5,5-octamethylbiguanidinium perchlorate and 2-methoxy-1,1,2,3,3-pentamethylguanidinium perchlorate, show NMR spectra at room temperature characteristic of relatively free rotation.[11] In these cases, extreme steric hindrance does not seem to inhibit relatively free rotation about the bonds, and the single NMR peak observed for each ion at room temperature indicates barriers of less than 20 kcal/mol.

It is also interesting to note that the calculated barrier to rotation about the double bond in guanidine (30.4 kcal/mol, Table II) is much larger than the barrier to rotation around the partial double bond in the guanidinium ion (21.9 kcal/mol). In contrast, the calculated barrier to rotation in the methylenimmonium ion ($CH_2{=}NH_2^+$) is larger than that in methylenimine ($CH_2{=}NH$).[24] This qualitative difference can be understood by recognizing that in the guanidinium ion rotation of one of the NH_2 groups to a perpendicular position still leaves two lone pairs to stabilize the positive charge; in the methylenimmonium there is no such available lone pair.

Table III. CNDO/2 Calculations of Relative Stabilities of Various Conformations of *N*-Methylamidino-*N*-methylglycine and of Phosphocreatine



Position of CH_3 group	$-COO^-$ not H-bonded, kcal/mol	$-COO^-$ H-bonded, kcal/mol
N-Methylamidino-*N*-methylglycine (*N*-Methylcreatine)		
1	62.7	—
2	26.0	(0)
3	26.1	0.8
4	61.7	36.1
Phosphocreatine		
Position of O_3P^{2-} group		
2	2.7	52.5
3	(0)	21.7

In view of the planarity of the guanidinium group, the only remaining conformational degree of freedom to consider in creatine itself is that of the CH_2-COO^- group. In the *N*-substituted creatines, cis-trans isomerism must also be considered. We have used the CNDO/2 method to examine the relative energies of the conformations of the CH_2-CO_2^- group in creatine using a 30° grid for θ_1 (the C-N-C-C dihedral angle; $\theta_1 = 0$ has the C-C bond eclipsing the C=N) and a 90° grid for θ_2 (theN-C-C-O dihedral angle: $\theta_2 = 0$ has the C=$O^{\delta-}$ bond eclipsing the N-C bond). The minimum energy occurred at $\theta_1, \theta_2 = 330, 90$, where the C=$O^{\delta-}$ group formed a strong hydrogen bond with the N-H. The structure for creatine is in accord with that found by x-ray studies,[22,23] except that in the solid state intermolecular H bonding occurs between the C=$O^{\delta-}$ and N-H.

The enzyme might well have a cationic group on its surface which binds the carboxyl group of creatine and pulls it to a more "extended" (intermolecularly H bonded) conformation than that found for the isolated molecule; we chose $\theta_1, \theta_2 \approx 180, 90$ as a model for this conformation. The calculated difference in energy between this conformation and the intramolecularly H-bonded one was 26.0 kcal/mol for creatine. Although this H-bond energy may be overestimated by our theoretical method, it is not out of line with what one would expect for ion-pair interactions in the gas phase.[25]

Table III summarizes results of the calculated energies of different *N*-methyl and *N*-phospho conformations of both *N*-methylcreatine and phosphocreatine considering both intramolecularly H-bonded and non-H-bonded conformations of the CH_2COO^- group. In the case of *N*-methylcreatine, these calculations indicate a much lower stability for conformers with the methyl group in positions 1 and 4, a result which had been previously inferred from steric considerations. They also support our interpretation of the low-temperature NMR spectrum of *N*-methylcreatine as showing an approximately 50:50 mixture of **3b** and **3c**.

N-Methylcreatine has been found to be neither a substrate nor an inhibitor in the creatine kinase-catalyzed reaction.[7] This suggests that it has at best a very weak affinity for this enzyme. The binding data[7] allow an estimate of the lower limit of the difference in free energy of binding for *N*-methylcreatine vs. that of creatine itself, ~4 kcal/mol. It follows that neither of the two conformers being considered (**3b** or **3c**) are capable of binding well to the enzyme. Lack of activity for the conformer with the methyl group in position 3 (see structure **5**) is easily explained, as the studies with 1-carboxymethyl-2-iminoimidazolidine (**4**) have indicated that phosphorylation can take place at that specific position.[26] Substitution with a methyl group in position 2 of structure **5** should also sterically interfere with phosphorylation since the steric interference between the phospho group and methyl at positions 2 and 3 should be at least as great as the steric repulsion found for *N*-methylcreatine between the methyl on the tertiary nitrogen and the methyl in position 1 (Table III).

It presumably is the hydrophobic nature of the *N*-methyl group which prevents the binding of *N*-methylcreatine to creatine kinase; this lack of observed binding cannot be attributable to bulk since the even bulkier phosphocreatine binds well. One possibility is that enzyme-bound creatine forms a specific hydrogen bond with some H-bond acceptor at the active site of creatine kinase; enzyme-bound phosphocreatine could be stabilized in turn by a favorable electrostatic interaction between the PO_3^{2-} group and the ϵ-ammonium form of the lysyl residue postulated by James and Cohn[27] to be in the active site region. *N*-Methylcreatine would be expected to show neither of these stabilizing interactions.

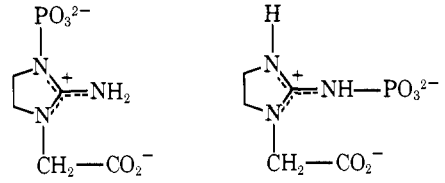
The CNDO/2 calculations (Table III) for the various conformations of phosphocreatine indicate some preference (ca. 2.7 kcal/mol) for the phosphoryl moiety to be in the 3 position of structure **5**, when the carboxyl group is fully extended away from the primary nitrogen; i.e., conformer **2b** predominates. When the carboxyl oxygen hydrogen bonds to the hydrogen in the 1 position, the preference for conformer **2b** is much more pronounced due to the closer proximity of the anionic COO^- and PO_3^{2-} groups. Indeed, Herriott and Love[17] have found just such a conformational preference for the disodium salt of phosphocreatine in the solid state, as revealed by an x-ray crystallographic study.

Summary and Conclusions

The CNDO/2 calculations predict creatine and its derivatives to have planar structures around the guanidinium group and a minimal energy structure with a $COO^-\cdots$H-N intramolecular H bond in vacuo. However, in solutions of water (or other protic solvents), one might expect intermolecular H bonding to predominate, leaving the CH_2-CO_2^- group in an extended conformation. For N-Me substitution, this will leave conformational isomers **3b** and **3c** of roughly equal energy, and this is consistent with our low-temperature NMR results. For N-PO_3^{2-} substitution, the 3 position of structure **5** will be preferred over the 2 position because of electrostatic repulsions, although this preference is predicted to be only 2.7 kcal/mol with the CH_2-COO^- fully extended. Positions 1 and 4 appear to be less favorable on steric grounds, and our calculated energies reflect this. It should be emphasized that we are not relying heavily on the quantitative accuracy of the theoretical calculations, but that the qualitative theoretical findings, together with the relevant NMR, x-ray crystallographic, and biochemical information allow one to put forward an internally consistent picture of the ways in which these creatine derivatives may present themselves to the active site of creatine kinase.

The magnitude of the importance of the electrostatic repulsion between the COO^- and PO_3^{2-} groups in phosphocreatine in solution is still uncertain; nevertheless, it appears that phosphocreatine may prefer conformation **2b** at pH values >6, even at room temperature. Thus creatine kinase may have evolved preferentially to bind the most stable available conformer of phosphocreatine. This in turn could be reflected in a stereospecific phosphorylation of creatine catalyzed by this enzyme. In support of this postulate, we have recently demonstrated that the highly reactive creatine analogue **4** is stereospecifically phosphorylated by creatine kinase on the ni-

trogen furthest removed from the COO− group, generating **6a** rather than **6b**.[26,28]



6a **6b**

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Phosphorus-31 Nuclear Magnetic Resonance Studies on Nucleoside Phosphates in Nonaqueous Media

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Abstract: ^{31}P 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-Gyorgi[2] 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 tripolyphosphate 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 relationship 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 proposed[1-10] included mono- or polyvalent cations, particularly Mg^{2+} and Ca^{2+} 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 intramitochrondrial environ-