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Studies on Phosphorylation by Phosphoroguanidinates. The Mechanism of Action of Creatine: ATP Transphosphorylase (Creatine Kinase)

(solvolyses/creatinine/dimethylphosphoroguanidinate/methylbenzylphosphoroguanidinate/reaction kinetics)

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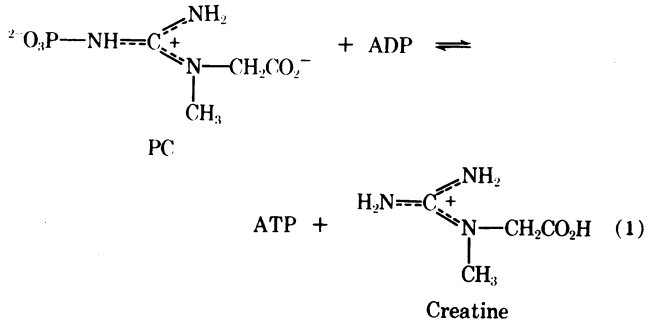
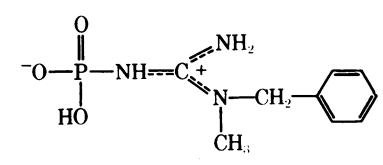
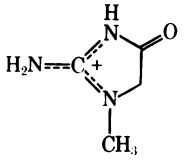
ABSTRACT **The solvolyses of phosphorocreatine (creatine phosphate) and models for phosphorocreatine have been investigated and the results are applied to the mechanism of action of creatine kinase (EC 2.7.3.2). A metaphosphate intermediate appears to be involved.**

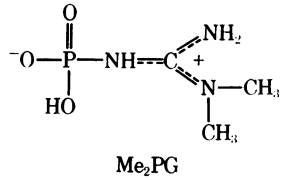
Phosphorocreatine (PC) functions in muscle to buffer the concentration of ATP by phosphorylating ADP (Eq. 1) (1).



Eq. 1 is catalyzed by creatine: ATP transphosphorylase (1) (creatine kinase EC 2.7.3.2), which has been studied extensively by Cohn and coworkers (2, 3). We have completed (4) a detailed study of the phosphorylation of water and alcohols (solvolysis) by PC and the simpler phosphoroguanidinates, Me_2PG and BzMePG, and we communicate in this publication a summary (4) of our results most relevant to the mechanism of action of creatine kinase (5).







RESULTS AND DISCUSSION

Hydrolysis of Me_2PG and BzMePG

The dependence of rate on pH for the hydrolysis of *N,N*-dimethyl-*N'*-phosphoroguanidinate (Me_2PG) reveals a rate maximum at pH 2 (Fig. 1). The same behavior was observed for *N*-benzyl-*N*-methyl-*N'*-phosphoroguanidinate (BzMePG). Such dependence on pH is characteristic of a substrate with two dissociable hydrogens. There are a total of three hydrogens (two P—OH and one N—H) that might be dissociable in reasonable ranges of acidity and basicity. Two of the acidity constants have been determined potentiometrically and are listed in Table 1, together with the first pK, which has been determined from the data in Fig. 1. The smooth curve in Fig. 1 is theoretical and is based upon the values of k_0 (Eq. 2), pK_a^1, and pK_a^2 in Table 1. Table 1 also lists other characteristics of the hydrolysis of Me_2PG. Comparison of the pK values for Me_2PG with other compounds makes it quite clear that the three acidity constants apply to the dissociations in

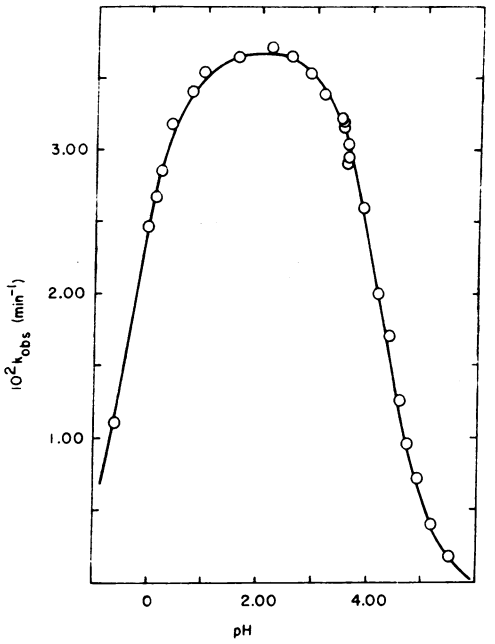
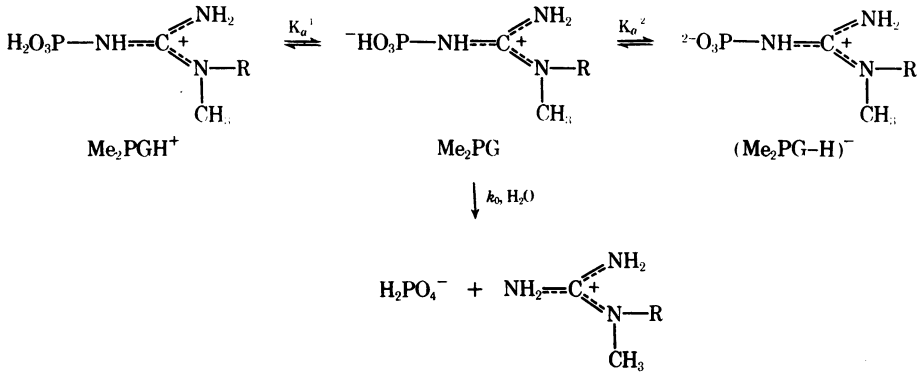


FIG. 1. The dependence on pH for the rate of hydrolysis of Me_2PG at 30.5°C. The *points* are experimental and the *curve* is theoretical, based on the constants in Table 1.

TABLE 1. *Parameters for Me_2PG, 30.5°C*

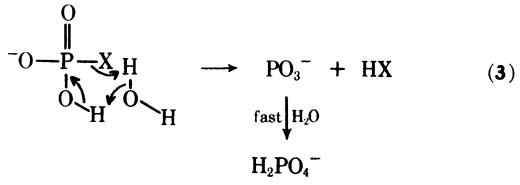
k_0	$= 3.66 \times 10^{-2}$ min^{-1}	ΔH^*	= 22.3 kcal/mol
pK_a^1	= −0.31	ΔS^*	= 0.4 cal/mol°
pK_a^2	= 4.32	k^{H_2O}/k^{D_2O}	= 0.90
pK_a^3	= 11.3		

Eq. 2, and that it is the neutral zwitterion of Me_2PG that is reactive and responsible for the maximal rate at pH 2 (6).



(2)

The pH–rate profile in Fig. 1 and the very fast rate of cleavage of the P—N bond suggest that Me_2PG may react via a metaphosphate intermediate, in the generation of which a proton transfer is important (eq. **3**), as has been suggested for the hydrolysis of monoanions of phosphate monoesters (7). This hypothesis is confirmed by: (*a*) immeasureably slow hydrolysis of Me_2PGH^+, $Me_2PG—H^-$, and especially the zwitterionic benzyl ester of Me_2PG, which has the same charge state and distribution as neutral Me_2PG but no transferrable proton; and (*b*) an entropy of activation, ΔS^*, of 0 entropy units.



(3)

As is generally true for phosphorylation reactions that have been postulated to proceed through metaphosphate intermediates (8), the exact sequence of events is unclear. The deuterium isotope-effect on the reaction rate, k^{H_2O}/k^{D_2O}, is near 1; this indicates that proton transfer must be nearly complete in the transition state. In this case, it seems particularly unlikely that there is a preequilibrium proton transfer because the guanidinium portion of Me_2PG should be very nonbasic. However, although the monoanion should definitely protonate predominantly at phosphoryl oxygen, the presence of two negative charges on the phosphate substituent in ($Me_2PG—H^-$) might make the —PNH— group sufficiently basic so that preequilibrium proton transfer may be possible. Diprotonated guanidines have been observed (9).

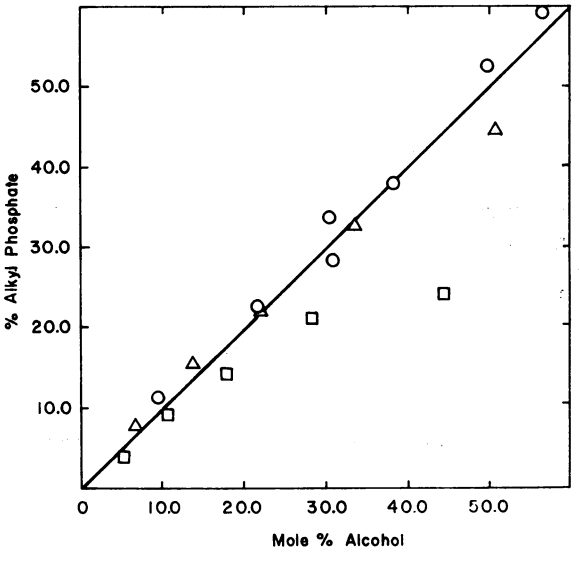


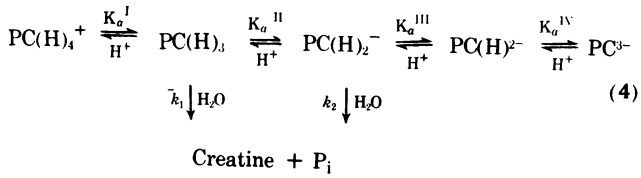
FIG. 2. The product composition for the solvolysis of Me_2PG at 30.5°C (μ = 0.20 N) in alcohol–water mixtures (0.01 N in HCl) as a function of the mole % of alcohol present: ○, methanol; △, ethanol; □, 2-propanol. The line has a slope of 1.0.

Of all the phosphates postulated to hydrolyze through metaphosphates (8) Me_2PG is among the most rapid to hydrolyze. Consistent with this, it appears to be the most indiscriminate phosphorylating agent when phosphorylation is studied in alcohol–water solvents (Fig. 2). Whereas in previous attempts to demonstrate metaphosphate as a highly reactive, indiscriminate intermediate there have been deviations of product ratios from those expected on the basis of the mole fractions of the solvents present, Me_2PG phosphorylates completely indiscriminately in methanol–water and ethanol–water and shows much more reaction with isopropanol in isopropanol–water, than any other phosphorylation that has been examined. These results indicate that a pure metaphosphate mechanism would be expected to be completely indiscriminate, and that the metaphosphate intermediate is a very highly reactive electrophile.

The deviations in Fig. 2 in isopropanol–water and the deviations observed in other studies appear best explained by borderline unimolecular mechanisms with variable amounts of nucleophilic participation by solvents in transition states (4).

Phosphorocreatine (PC)

The hydrolysis of PC is significantly different from Me_2PG, in that there is reaction in acidic solution (Fig. 3). The rate in the region of pH 2 is a broad shoulder, due to the hydrolysis of two different species (Eq. **4**). Analysis for products from PC has revealed that creatinine is produced in the following amounts: tending to 100% in acidic solutions, 10% at pH 1, and 5% at pH 5. These results support the claim of nonenzymatic cleavage of PC to creatinine at physiological pH, and also indicate that urinary creatinine is produced by first-order breakdown of PC (10).



(4)

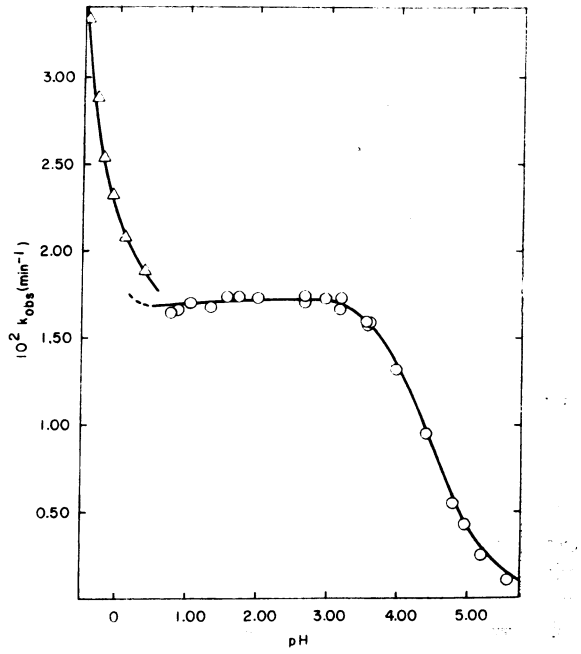


FIG. 3. The pH–rate profile for the hydrolysis of PC at 30.5°C O, μ = 0.20 N; Δ, μ = 4.00 N.

Therefore, the amount of PC in the body should determine the amount of urinary creatinine, and the use of the concentration of urinary creatinine in medical diagnosis has its basis in the fundamental chemistry of PC. The available data on the rate of cleavage of PC in acidic solutions indicate that C—N bond formation and P—N bond cleavage occur in one step (4).

Mechanism of action of creatine kinase

The fundamental chemistry summarized above indicates that the most likely mechanism for phosphorylation of ADP by PC (Eq. 1) involves a highly reactive metaphosphate intermediate that is generated in the rate-determining step by proton transfer and cleavage of the P—N bond of PC. Direct nucleophilic attack (2) by a β-phosphoryl oxygen to ADP on the phosphorus atom of PC is much less likely.

The most important role that creatine kinase must play in catalysis of this reaction involves protonation of the P–N nitrogen of PC. If PC is bound in the $-PO_3^{2-}$ state, the enzyme must function as a general acid catalyst. Alternatively if PC is bound in the $-PO_3H^-$ state, the enzyme must serve as an effective proton transfer agent. The latter mechanism is shown in Fig. 4. There is evidence that a sulfhydryl group (cysteine residue) and an imidazole (histidine residue) are involved in catalysis at the active site of creatine kinase (1). Either the —SH group or the imidazolium group could function as a general acid catalyst for protonation of the P–N nitrogen. Molecular orbital calculations on guanidinium ion, $(H_2N)_3C^+$, demonstrate that two of the three filled π-molecular orbitals are nonbonding. It might be possible therefore, to have some hydrogen bonding of the P–N nitrogen with an acid catalyst before protonation. Creatine phosphate may be bound in a geometry that polarizes the guanidinium moiety. This would increase the basicity of the P–N nitrogen atom and make protonation more facile, an effect that we have

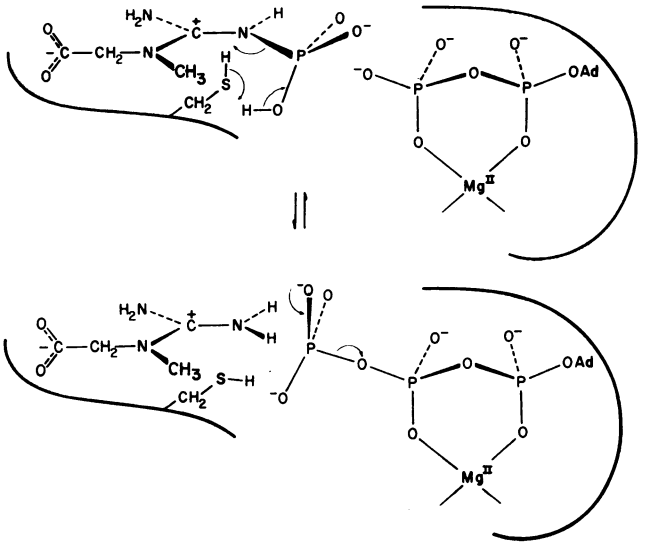


FIG. 4. Proposed mechanism for creatine kinase.

shown to increase the rate of hydrolysis of phosphorylguanidinium ions (4).

These results also afford some insight into the reverse reaction—phosphorylation of creatine by ATP. By the tenets of microscopic reversibility, this reaction must also have metaphosphate character. Since generation of metaphosphate would be inhibited by coordination of metal ion to the γ-phosphate group, the metal should be coordinated only to the α and β phosphates of ATP. Direct displacement is implied in any mechanism that involves coordination of metal ion to the γ-phosphate, since the metal ion will enhance nucleophilic attack, but it should slow the rate of generation of metaphosphate ion, which appears to be stabilized by increased electron density. The function of enzymic catalysis in the phosphorylation of creatine by ATP must also involve proton abstraction from creatine by a base as creatine reacts with metaphosphate (Fig. 4).

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