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Biological Reactions at Phosphorus

VII. The Nature of Metaphosphate Ion, PO_3^-, as a Reaction Intermediate (*1*)

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In order to improve our understanding of biological phosphorylations by "high-energy" compounds such as ATP, the hypothesis of metaphosphate ion as an intermediate in certain phosphorylation reactions has been critically examined. We have studied the rates and product composition for the solvolysis of the neutral form of *N*,*N*-dimethylphosphoroguanidinate (DMPG) at 30.5°C in various water–alcohol mixtures. The rates of solvolysis were found to decrease as the mole percent of the alcohol increased, but no systematic relationship with dielectric constant or Grunwald–Winstein *y* values was evident. A 1 : 1 correspondence between the percentage alkyl phosphate produced and the mole percent alcohol present was found with methanol, ethanol, and low concentrations of 2-propanol. At higher concentrations of 2-propanol, the product ratio favors water as nucleophile probably due to selective solvation of the metaphosphate precursor by water. These results indicate that metaphosphate mechanisms have a variable amount of nucleophilic participation. Although the reaction of phosphoroguanidines appears to involve metaphosphate ion as a free intermediate, analysis of results in the literature indicate that less reactive metaphosphate precursors react with nucleophilic participation. Extrapolation of these results to biological phosphorylations leads to the conclusion that nucleophilic participation may be an important feature of enzymic transition states due to the favorable orientation of nucleophile and incipient metaphosphate at enzymic active sites.

INTRODUCTION

The original suggestions of metaphosphate ion, PO_3^-, as a possible intermediate in displacement at phosphorus have been fulfilled in a number of chemical studies and in suggestions for metaphosphate ion as an intermediate in biological phosphorylations of the type exemplified by the transfer of a phosphate from ATP (*1d*, *2*). Reactions of "high energy phosphates" are exemplified by phosphoroguanidinates, such as phosphorocreatine, for which our studies of phosphate transfer support a unimolecular mechanism (Eq. [1]) involving a zwitterionic precursor (**1**) for production of the protonated guanidine and the metaphosphate ion which reacts rapidly with nucleophiles (*1*). Metaphosphate ion has not been isolated in stable form, unlike the homologous nitrate ion, and appears to be highly reactive toward water. Presumably the π bond energy of **2** is quite low compared to the energy (3) of the new P–O σ bond in **3**, and this provides a large $-\Delta H$ for **2** → **3**.

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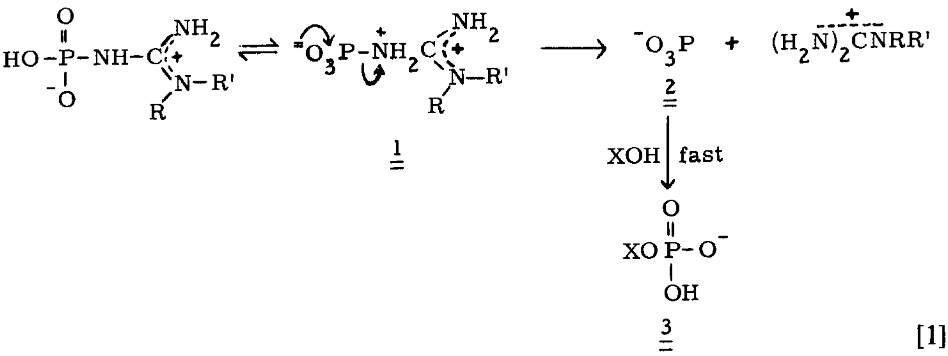


[1]

There are important questions concerning the possible intermediacy of PO_3^- in biological phosphorylations. Is PO_3^- a discrete, free intermediate? Does the transition state have considerable bonding to the attacking and leaving groups? Is a fully pentacoordinate species an intermediate in these phosphorylations? What effects are there on the rate of metaphosphate formation by functional groups at the active sites of enzymes which catalyze these phosphorylations? Since metal ions are required for phosphorylations by ATP and there is evidence from magnetic resonance studies for β, γ coordination of ATP to metal ions, will a positively charged ligand affect the rate of PO_3^- generation? In this paper we present data bearing on these questions.

PO_3^- appears to be highly electrophilic, so that it may have partial bonding to attacking and/or leaving groups at all times. In studies of phosphoramidates (*2b*), evidence was found for participation of nucleophile in the transition state. Valuable experimental evidence concerning the chemical dynamics of a highly reactive, electrophilic species can be obtained by generating it in the presence of competing nucleophiles. In a mixed solvent system consisting of two nucleophilic components of sufficiently similar solvation properties, such an electrophile would be expected to react nonselectively with the two components. The molar ratio of the products of such a reaction should be equal to that of the solvent mixture. Of mixed aqueous solvents, methanol–water meets the criterion of similar solvation closely (*4*). For example, when t-butyl carbonium ion is generated in methanol–water mixtures at 25°C, the molar ratio of t-butyl methyl ether to t-butanol is close to the molar ratio of methanol to water in the solvent (*5*). Similarly, the ratio of methyl phosphate to inorganic phosphate formed on solvolysis of a metaphosphate precursor relative to the molar ratio of methanol to water in the solvent mixture (*2*, *6*) gives evidence about the PO_3^- intermediate. A mechanism of hydrolysis involving free PO_3^- should give indiscriminate reaction with solvent mixture, but nucleophilic participation should give increased amounts of the product from the more reactive nucleophile.

We have shown (*1e*) that neutral DMPG (**1**, R = R = CH_3) is a very reactive phosphorylating species of the type $H\bar{O}_3PX$, and that phosphorylation appears to proceed through a metaphosphate ion (Eq. [1]). In Table 1, a variety of precursors for metaphosphate are summarized. The table demonstrates that phosphoroguanidines are among the most reactive precursors of metaphosphate ion.

Carbonium ion chemistry is a useful conceptual model for inquiry into the chemical dynamics of PO_3^-. The more reactive carbonium ion precursors give reactions in which there is less nucleophilic participation. Therefore, phosphoroguanidines should be useful substrates with which to examine closely this criterion of indiscriminate reaction with nucleophiles as a basis for metaphosphate ion as intermediate; we have studied the solvolysis of DMPG in alcohol-water mixtures using methanol, ethanol, and 2-propanol.

RESULTS

Rates of Solvolysis in Alcohol-Water Mixtures

DMPG was solvolyzed in various alcohol-water mixtures at 30.47 ± 0.05°C, ionic strength = 0.20. The rates of solvolysis were measured either spectrophotometrically by observing the decrease in absorbance due to DMPG or by determining the release of inorganic phosphate. The rate constants are tabulated in Table 2 and displayed graphically in Fig. 1.

Product Composition in Alcohol-Water Mixtures

For product determinations the solvolyses of DMPG in various alcohol-water

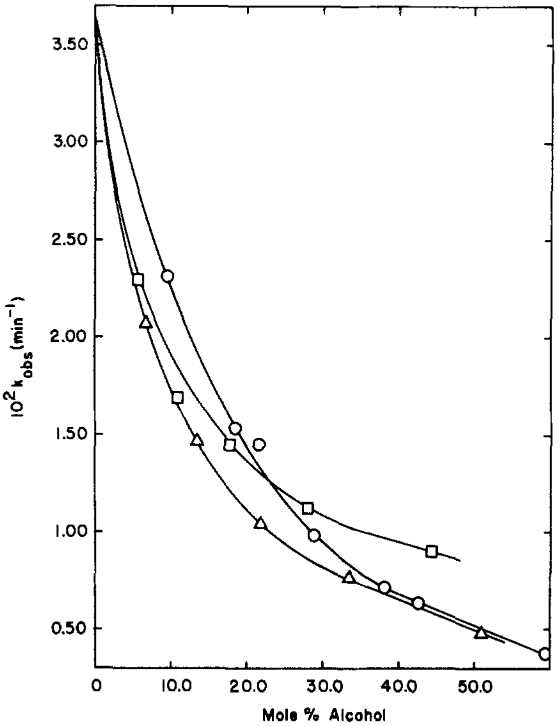
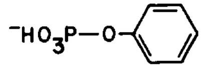
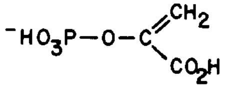
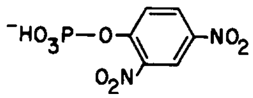
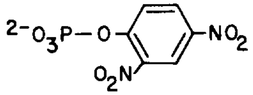
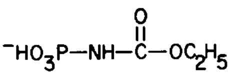
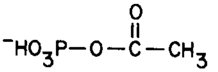
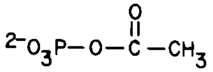
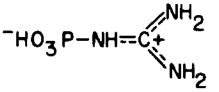
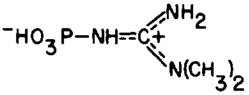


FIG. 1. The first-order rate constants for the solvolysis of DMPG at 30.5°C (μ = 0.20 N) in alcohol-water mixtures (0.01 N in HCl) as a function of the mole percent alcohol present: (○) methanol; (△) ethanol; (□) 2-propanol.

TABLE 1

RATES OF HYDROLYSIS OF VARIOUS METAPHOSPHATE PRECURSORS

Compound	k_{hyd} (min^{-1})	T (°C)	ΔS^* (e.u.)	k^H/k^D	Reference
$^{-}HO_3P-OCH_3$	4.94×10^{-4}	100	−2.2	0.87	[a]
	1.67×10^{-3}	100	−0.6	—	[b]
	8.80×10^{-3}	75	−1.1	1.10	[c]
	3.04×10^{-4}	39	−6.0	1.45	[d]
	1.05×10^{-2}	39	+6.6	1.01	[d]
$^{-}HO_3P-NH_3^+$	0.70×10^{-2}	37	−18.2	—	[b]
$^{2-}O_3P-NH_3^+$	0.42×10^{-2}	37	−1.6	1.20	[b]
 $^{-}HO_3P-NH-C(=O)-OC_2H_5$	3.53×10^{-2}	37	+14.4	0.95	[e]
 $^{-}HO_3P-O-C(=O)-CH_3$	1.3×10^{-2}	37	−3.6	1.03	[f]
 $^{2-}O_3P-O-C(=O)-CH_3$	0.43×10^{-2}	37	+3.7	1.00	[f]
	6.32×10^{-2}	50	—	—	[g]
	33.3×10^{-2} 8.58×10^{-2}	50 37	+0.4	0.90	This work

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[a] C. A. BUNTON, D. R. LLEWELLYN, K. G. OLDMAN, AND C. A. VERNON, *J. Amer. Chem. Soc.* 3574 (1958).

[b] J. D. CHANLEY AND E. FEAGESON, *J. Amer. Chem. Soc.* **77**, 4002 (1955).

[c] S. J. BENKOVIC AND K. J. SCHRAY, *Biochemistry* **7**, 4090 (1968).

[d] A. J. KIRBY AND A. G. VARVOGLIS, *J. Amer. Chem. Soc.* **89**, 415 (1967).

[e] M. HALMANN AND A. LAPIDOT, *J. Chem. Soc.* 419 (1960).

[f] G. DISABATO AND W. P. JENCKS, *J. Amer. Chem. Soc.* **83**, 4400 (1961).

[g] V. M. CLARK AND G. MACRAE, private communication to P. Haake.

TABLE 2

RATE CONSTANTS FOR THE SOLVOLYSIS OF DMPG IN ALCOHOL–WATER MIXTURES, $T = 30.47 \pm 0.05°C$, 0.01 N HCl, $\mu = 0.20$ IN NaCl

Solvent[a]	mol% ROH[b]	pH[c]	$10^2 k_{obs}$ (min^{-1})
20% CH_3OH	9.6	2.06	2.32
35% CH_3OH	18.5	2.10	1.54
40% CH_3OH	21.7	2.04	1.46[d]
50% CH_3OH	28.8	2.16	0.993
60% CH_3OH	38.3	2.14	0.82[d]
65% CH_3OH	42.6	2.22	0.640
80% CH_3OH	59.5	2.16	0.382
20% C_2H_5OH	6.8	2.09	2.08
35% C_2H_5OH	13.5	2.18	1.47
50% C_2H_5OH	22.0	2.28	1.05
65% C_2H_5OH	33.9	2.36	0.783
80% C_2H_5OH	50.8	2.38	0.485
20% 2-C_3H_7OH	5.4	2.11	2.30
35% 2-C_3H_7OH	10.8	2.20	1.69
50% 2-C_3H_7OH	17.8	2.26	1.45
65% 2-C_3H_7OH	27.9	2.30	1.13
80% 2-C_3H_7OH	44.5	2.30	0.905

[a] Percents given as volume percent.

[b] Calculated on basis of weight.

[c] Measured with glass electrode at 30.5°C.

[d] Determined by measuring inorganic phosphate released with time.

mixtures were run under identical conditions as the kinetic runs, but on a slightly larger scale. The amounts of inorganic phosphate produced at the end of the reactions (after at least 10 half-lives) were determined and the amount of alkyl phosphate was calculated by difference (see Experimental). For a given alcohol–water solvent system, the corresponding alkyl phosphate was shown to be a product of the solvolysis by paper chromatography. The results are collected in Table 3. When the percentage alkyl phosphate produced is plotted against the mole percentage alcohol in the solvent, the slope is 1.0 for methanol and ethanol mixtures, as would be expected from indiscriminate attack of alcohols on a PO_3^- intermediate. In isopropanol–water mixtures, there appears to be indiscriminate attack at low concentrations of isopropanol, but as the concentration of isopropanol becomes large, water becomes the preferential nucleophile (Table 3).

DISCUSSION

Solvent Effect on Rates

The rate of solvolysis of DMPG is depressed upon addition of alcohol, as

TABLE 3

PRODUCT COMPOSITION FOR THE SOLVOLYSES OF DMPG IN ALCOHOL–WATER MIXTURES, 0.01 N HCl, μ = 0.20 IN NaCl, T = 30.47 ± 0.05°C

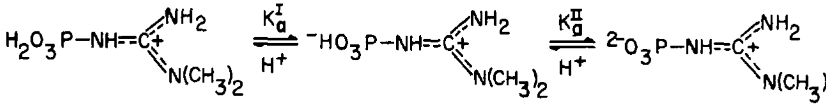
Solvent	mol% ROH[a]	Percentage alkyl phosphate[b]
CH_3OH-H_2O	9.7	11.5
	21.7	22.6
	30.4	33.8
	30.9	28.5
	38.3	38.0
	49.6	52.6
	56.5	59.2
$C_2H_5OH-H_2O$	6.8	8.1
	13.7	15.8
	22.0	22.2
	33.9	32.8
	50.8	44.6
$2-C_3H_7OH-H_2O$	5.4	4.0
	10.8	9.4
	17.8	14.3
	27.9	21.4
	44.5	24.1

[a] Calculated on basis of weight.

[b] Based on theoretical amount of inorganic phosphate. Estimated maximum error, ±2.0. Determined after at least 10 half-lives.

depicted in Fig. 1. No systematic relationship with dielectric constant (*7*) or Grunwald-Winstein *y* values (*8*) could be found. Instead, we look at the mechanism (Eq. [1]) in order to understand the results. The concentration of the reactive species could be decreased or the rates could be decreased.

The metaphosphate reactions were all run between pH 2 and 2.3, where, in aqueous solution, the concentration of the reactive species, neutral DMPG, is at a maximum. The addition of alcohol doubtless changes the dissociation constants of DMPG (Eq. [2]):



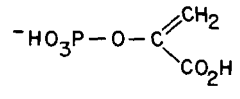
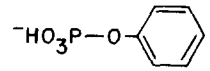
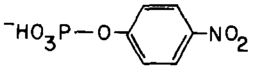
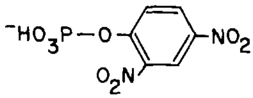
$$H_2O_3P{-}NH{=}\overset{+}{C}(NH_2)N(CH_3)_2 \underset{H^+}{\overset{K_a^{I}}{\rightleftharpoons}} {}^{-}HO_3P{-}NH{=}\overset{+}{C}(NH_2)N(CH_3)_2 \underset{H^+}{\overset{K_a^{II}}{\rightleftharpoons}} {}^{2-}O_3P{-}NH{=}\overset{+}{C}(NH_2)N(CH_3)_2 \quad [2]$$

4 5 6

It would be expected that K_a^2 would decrease upon addition of alcohol since this dissociation involves separating H^+ from an anion, but there should be a smaller solvent effect on K_a^1 since this dissociation involves separating H^+ from a neutral species. In Eq. [3] the pK_a increases from 3.00 in water to 4.05 in 50% (v : v) methanol–water mixtures (*6*). Although no direct results are available concerning

TABLE 4

PRODUCT COMPOSITION FOR THE SOLVOLYSIS OF MONOANIONS OF PHOSPHATE MONOESTERS IN METHANOL–WATER MIXTURES

Compound	*T* (°C)	mol% methanol	Percentage methyl phosphate	Reference
	55	30.9	32.0	(*11*)
 PP	100	15.5	22	(*6*)
	55	15.5	27	
	100	29.2	31	
	55	29.2	42	
	100	48.0	48	
	55	48.0	58	
 PNPP	100	15.5	16	(*6*)
	55	15.5	23	
	37	15.5	22	
	100	29.2	29	
	55	29.2	34	
	37	29.2	38	
	100	48.0	47	
	55	48.0	57	
	37	48.0	58	
 2,4-DNPP	100	15.5	14.3	(*12*)
	39	15.5	32.0	
	39	29.2	38.9	
	39	48.0	52.1	

the effects of added alcohol on pK_a^1, the pK_a's of anilinum ions (*9*) in alcohol-water mixtures indicate that pK_a^1 will decrease on addition of alcohol. Therefore, neutral DMPG (**5**) (*1e*) should be both a weaker acid (pK_a^2 increases) and a weaker base (pK_a^1 decreases) in alcohol–water mixtures

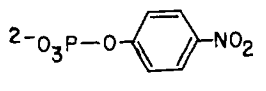
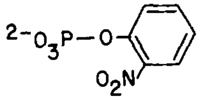
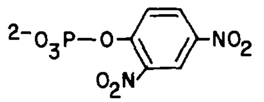
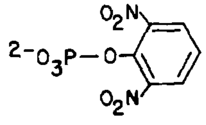
$$^{-}HO_3P{-}\overset{+}{N}H_3 \quad \underset{H^{+}}{\overset{K_a}{\rightleftharpoons}} \quad {}^{-}O_3P{-}\overset{+}{N}H_3 \qquad [3]$$

than in water. Thus, at pH values around 2, addition of alcohol should not significantly alter the concentration of **5**. Therefore, the rates of solvolysis in various alcohol–water mixtures (Table 2) do not reflect a decrease of concentration of **5** but, rather, a decrease in rate of reaction of **5**.

It seems likely that the rate depression is due to suppression of zwitterion (**1**) formation. The zwitterion (**1**), being highly reactive, will be close in structure to the transition state whereas **5** is the ground state. Therefore, the change from ground state to transition state involves a net increase in separation of charge so the transition state is more polar than the ground state. Therefore, addition of alcohol, which lowers the solvent polarity, is expected to depress the rate of

TABLE 5

PRODUCT COMPOSITION FOR THE SOLVOLYSIS OF DIANIONS OF PHOSPHATE MONOESTERS IN METHANOL–WATER MIXTURES

Compound	*T* (°C)	mol% methanol	Percentage methyl phosphate	Reference
	100	15.5	16.9	(*12*)
	100	29.2	29.0	
	100	48.0	46.8	
 2-NPP	100	15.5	23.1	(*12*)
	100	29.2	36.8	
	100	48.0	63.7	
	39	10.2	28.3	(*12*)
	100	15.5	29.5	
	39	15.5	44.8	
	25	15.5	52	(*13*)
	39	29.2	62.7	(*12*)
	25	29.2	67	(*13*)
	25	40.2	88	(*13*)
	39	48.0	79.1	(*12*)
 2,6-DNPP	25	15.5	67	(*13*)
	25	29.2	83	
	25	40.2	88	

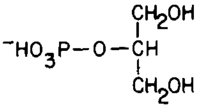
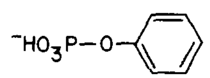
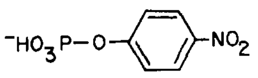
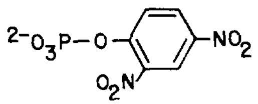
reaction. It is interesting that the rate is depressed more strongly by methanol than 2-propanol at high concentrations of these alcohols (Fig. 1). One might have expected the reverse based on the respective dielectric for these mixtures. It seems likely that this is due to clustering of water around the substrate in 2-propanol mixtures, thereby producing a microscopic environment more favorable to reaction than in methanol (*10*). This is also likely to be the reason for the increased amounts of product due to water as nucleophile in isopropanol–water (Table 3).

Product Composition in Mixed Solvents

The molar ratio of alkyl phosphate produced on the solvolysis of DMPG in alcohol–water mixtures shows a 1 : 1 correlation to the molar ratio of alcohol to water for aqueous methanol, ethanol, and, at low concentrations, 2-propanol (Table 3). It is apparent that DMPG acts as a nonselective phosphorylating agent toward the components of these solvent mixtures. The significance of these results with respect to the postulated metaphosphate mechanism (*2*) has

TABLE 6

PRODUCT COMPOSITION FOR THE SOLVOLYSIS OF PHOSPHATE MONOESTERS IN ETHANOL–WATER MIXTURES

Compound	T (°C)	mol% ethanol	Percentage ethyl phosphate	Reference
	100	31.8	24.2	(*14*)
	100	56.1	44.0	
	100	73.6	64.5	
	100	11.3	10	(*6*)
	55	11.3	11	
	100	22.4	14	
	55	22.4	22	
	100	40.4	25	
	55	40.4	30	
	100	11.3	8	(*6*)
	55	11.3	14	
	37	11.3	9	
	100	22.4	12	
	55	22.4	12	
	37	22.4	15	
	100	40.4	23	
	55	40.4	22	
	37	40.4	24	
	39	11.3	20.5	(*12*)
	39	22.4	35.3	
	39	40.4	58.0	
	39	22.0 (CF_3CH_2OH)	8.3 ($CF_3CH_2OPO_3H_2$)	
	39	36.8 (CF_3CH_2OH)	14.5 ($CF_3CH_2OPO_3H_2$)	

significance for other reactions for which metaphosphate has been postulated to be an intermediate.

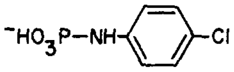
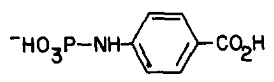
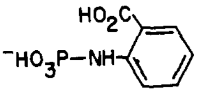
Phosphate Monoesters

The solvolyses of the monoanions of phenyl and *p*-nitrophenyl phosphate in mixtures of methanol–water yield alkyl phosphate and inorganic phosphate in a molar ratio which closely corresponds to the molar ratio of the alcohol–water mixture (*6*). However, such 1 : 1 correlations are not always obtained. A summary of the available data on the product distribution for the solvolysis of monoanions of phosphate monoesters in methanol–water mixtures is presented in Table 4, and for dianions in Table 5. Table 6 summarizes the data on the product distribution for these reactions in ethanol–water and trifluoroethanol–water mixtures. The following important observations emerge from these collections of data.

(1) The correlation between the product and solvent ratios is 1 : 1 for the

TABLE 7

PRODUCT COMPOSITION FOR THE SOLVOLYSIS OF PHOSPHORAMIDATES IN ALCOHOL–WATER MIXTURES

Compound	T (°C)	mol% alcohol	Percentage alkyl phosphate	Reference
$^{2-}O_3P-\overset{+}{N}H_3$	37	15.5 (CH_3OH)	54.3	(6)
	37	29.2	72.7	
	37	11.3 (C_2H_5OH)	22.6	
	37	22.4	38.4	
	25	29.2 (CH_3OH)	68.8	(6)
	25	22.4 (C_2H_5OH)	33.4	
	35	2.3 (CH_3OH)	24	(15)
	35	7.7	40	
	35	16.8	54	
	35	31.6	73	
	35	1.6 (C_2H_5OH)	13	
	35	11.6	35	
	35	9.4 ($2\text{-}C_3H_7OH$)	18	
	35	9.8 ($t\text{-}C_4H_9OH$)	19	
	35	2.3 (CH_3OH)	18	(15)
	35	7.7	36	
	35	16.8	55	
	35	31.6	75	
	35	1.6 (C_2H_5OH)	0	
	35	11.6	4	
	35	9.4 ($2\text{-}C_3H_7OH$)	17	
	35	9.8 ($t\text{-}C_4H_9OH$)	0	
$^{-}HO_3P-N(CH_2OH)_2$	39	29.2 (CH_3OH)	50	(16)
	39	48.0	70	
$^{-}HO_3P-NCl_2$	39	29.2 (CH_3OH)	35	(16)
	39	48.0	56	

solvolysis of the dianion, anion, and ethyl ester of phosphoenolpyruvate (PEP) at 55°C, and the aryl phosphates at 100°C (Tables 4, 5).

(2) The correlation between the product and solvent ratios (ROX/HOX) is less than 1 : 1 for the solvolysis of glycerol- 2-phosphate and the aryl phosphates at 100°C (Table 6).

(3) In the solvolysis of aryl phosphates (Tables 4, 5), as the temperature is decreased the product ratio (CH_3OP/HOP) increases relative to the solvent ratio (CH_3OH/HOH). For example in the solvolysis of the dianion of 2,4-DNPP in 15.5 mol% methanol, the yield of methyl phosphate is 29.5% at 100°C, 44.8% at 39°C, and 52% at 25°C (Table 5).

(4) The solvolyses of the monoanions of phenyl phosphate, PNPP, and 2,4-DNPP and the dianions of PNPP and 2,4-DNPP were run in 2-propanol–water mixtures, but in no case was any 2-propyl phosphate detected as a product.

(5) In the solvolysis of PNPP dianion (Table 5) there is a 1:1 correlation between product ratio and solvent ratio.

(6) The percentage methyl phosphate produced is substantially greater than that predicted on the basis of the solvent ratio (CH_3OH/HOH) in the solvolysis of the dianions of 2-NPP, 2,4-DNPP, and 2,6-DNPP (Table 5).

(7) The above phenomenon is observed for the solvolysis of 2,4-DNPP in ethanol–water mixtures, but to a smaller extent, and in trifluoroethanol–water mixtures water is preferentially phosphorylated (Table 6).

(8) The solvolysis of 2,4-DNPP in 70% (v/v) DMSO containing methanol and water in the molar ratio of 29 : 71 (29 mol% methanol relative to water) yields 39% methyl phosphate. This result should be compared to 62.7% methyl phosphate produced with the same molar ratio of methanol to water, but with no DMSO present.

Phosphoramidates

The results of the solvolyses of phosphoramidates in alcohol–water mixtures are summarized in Table 7. The yields of alkyl phosphate are greater than that predicted on the basis of the molar ratios of alcohol to water in the mixtures. It is interesting to note that whereas no 2-propyl or t-butyl phosphate was detected as products on solvolysis of phosphoramidate and *N*-(4-chlorophenyl)-phosphoramidate in 2-propanol–water and t-butanol–water (*6*), good yields of these esters are obtained on solvolysis of *N*-(*p*-carboxyphenyl) phosphoramidate in 2-propanol–water and t-butanol–water mixtures (*15*).

Mechanism of Solvolytic Phosphorylation

There is a paradox in these phosphorylations in that there is, on the one hand, considerable evidence in support of the hypothesis of metaphosphate ions as intermediates as summarized in the preceding paper, yet, on the other hand, there are many discrepancies in Tables 4–7 from the results expected from indiscriminate reaction with PO_3^-.

One possibly important factor is *selective solvation*. It has been proposed (*12*) that the mixed solvolysis data for phosphate esters may be due to nonstatistical distribution of solvent molecules in the solvation shell about the phosphoryl group. Such selective solvation would explain (*12*): (1) 2-Propanol is the least effective ionizing and most sterically hindered solvent and, in consequence, is totally excluded from the solvation shell of the phosphoryl group, and no 2-propyl phosphate is produced. (2) Like other forms of selectivity, it should be reduced at higher temperatures. This picture is unsatisfactory, however, because it might be expected that exclusion from the solvation shell would be greater for the more highly charged and/or more polar substrates. Yet, the percentage methanolysis is greater for the dianion of 2,4-DNPP than for the corresponding monoanion, and it is greater for the dinitrophenyl esters than the mononitrophenyl or phenyl esters. However, in our study, the higher rates of certain 2-propanol mixtures than methanol mixtures could be explained by clustering of water in 2-propanol. This would also explain the product ratios in Table 3.

A likely cause of the above enumerated effects (based on the data in Tables 3–6) is mechanistic situations which are borderline between unimolecular mechanisms involving the production of free metaphosphate ion and bimolecular mechanisms involving nucleophilic attack by the solvent on phosphoryl phosphorus (*15, 16*)—this is analogous to the picture that has emerged for borderline carbonium ion reactions.

Phosphoroguanidinates

It is evident that nucleophilic solvent participation is unimportant in the solvolysis of DMPG in alcohol–water mixtures. The yield of alkyl phosphate in these reactions is that predicted on the basis of the mole percent alcohol present in the solvent mixture. The solvolysis results for DMPG are consistent with and support the postulated unimolecular mechanism involving the production of free metaphosphate ion as an intermediate, and the metaphosphate ion appears to be very nonselective in its reactions with the nucleophilic solvents. The prime unimolecular reaction pathway for DMPG is predicted on the basis of considerations of nucleophilic susceptibility: (1) bimolecular nucleophilic substitution on DMPG would require expulsion of the neutral guanidine ($pK_a = 13.6$) which is so basic that it is a very poor leaving group; (2) the rate of hydrolysis of DMPG is unaffected (*2*) by the presence of imidazole, an effective nucleophile for the aminolysis of aryl phosphates and phosphoramidates. The only reaction pathway energetically available for the hydrolysis or solvolysis of DMPG, and by analogy all simple phosphoroguanidinates, is by the unimolecular reaction of the neutral zwitterionic form to yield the protonated guanidine and the highly reactive intermediate, metaphosphate ion (Eq. [1]).

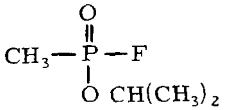
Therefore, it appears that the product ratios in phosphorylations in mixed solvents indicate the extent of nucleophilic participation in the transition state. A 1 : 1 product : solvent correspondence in methanol–water indicates a unimolecular mechanism and a lack of this 1 : 1 correlation indicates merging to a variable extent toward a bimolecular reaction—that is, nucleophilic solvent participation.

The divergence from a 1 : 1 correlation in our work at high concentrations of 2-propanol is probably due to the steric effect in 2-propanol which makes it a poor competitor with water as a solvent and nucleophile. The negatively charged phosphate moiety in the metaphosphate precursor will demand a high degree of protic solvation (*10*). It is interesting to note that even those phosphate monoesters that appear to be nonselective toward methanol–water and ethanol–water mixtures yield no 2-propyl phosphate on solvolysis in 2-propanol–water mixtures.

Nucleophilic attack by amines on both monoanions and dianions of aryl phosphates is known (*17, 18*), but the reaction has a different character from hydrolysis as demonstrated by the activation parameters: hydrolysis of the dianion of 2,4-DNPP, $\Delta H^* = 25.7$ kcal/mol, $\Delta S^* = +7$ e.u.; aminolysis by pyridine of 2,4-DNPP, $\Delta H^* = 16.8$ kcal/mol, $\Delta S^* = -19$ e.u. Hydrolysis, therefore, appears to be fundamentally unimolecular and aminolysis bimolecular.

The Bronsted β in aminolysis of dianions is high ($\beta = -1.23$) for the leaving group and low for the attacking amine ($\beta = 0.13$ for the dianion of PNPP) (*17*).

This lack of sensitivity to the pK_a of the attacking amine is in contrast to that observed with fully substituted phosphate derivatives. For example, the β values for the reactions of Sarin, **9**, are greater than 0.5 and in most cases close to 0.8 (*19–22*). As a class, tertiary amines are more reactive than secondary amines which are more reactive than primary amines. This same order of relative reactivity has also been observed for the aminolysis at phosphorus of acetyl phosphate (*23*). As the pK_a of the leaving group decreases, the sensitivity of the reaction to the pK_a of the attacking amine decreases. The aminolysis of the dianion of 2,4-DNPP (amine pK_a range 0.8–9.2) is characterized by $\beta = 0$. This result can be taken to indicate no bond formation between the amine and phosphorus in the transition state. The reactions, however, are strictly first order in amine, subject to steric hindrance, and characterized by large, negative entropies of activation. These facts appear to be inconsistent with very weak interaction between the substrate and nucleophile in the transition state, but indicate a much more substantial P–N bond. This apparent lack of sensitivity to the pK_a of the attacking amine can be discussed in terms of electrostatic stabilization in the transition state (*24*). The formation of the P–N bond necessarily develops a positive charge on the nucleophile in the vicinity of the negatively charged phosphoryl group, and therefore, the electrostatic attraction between the phosphoryl and amine moieties may make a significant contribution to the stability of the transition state (*24*). This electrostatic effect should be reduced as the charge on the phosphoryl group is reduced. Thus, the rate of aminolysis of aryl phosphate monoanions should depend strongly on the pK_a of the attacking nucleophile and it does (*18–26*). Finally, such electrostatic effects should be relatively unimportant in aminolysis of compounds with neutral phosphoryl moieties. Thus, nucleophilicity of amines toward Sarin (**9**) is strongly dependent on their pK_a's.



$$\mathrm{CH_3-\underset{\displaystyle O\,CH(CH_3)_2}{\underset{|}{\overset{\displaystyle O}{\overset{\|}{P}}}}-F}$$

9

Application of the results on aminolyses to oxygen nucleophiles supports explanation of the solvolysis data (which are divergent from our results with DMPG) in terms of nucleophilic solvent participation. The lack of 1 : 1 correlation between product ratio and solvent ratio then results from preferential nucleophilic attack by one solvent. Factors which should influence the importance of the nucleophilic pathway are:

(1) Nucleophilicity of solvent: The order of reactivity toward bimolecular nucleophilic attack at phosphorus is $CH_3OH > C_2H_5OH > H_2O$ (*6, 15*).

(2) Leaving group: The susceptibility of both mono- and dianions of aryl phosphates to bimolecular nucleophilic attack increases as the pK_a of the leaving group decreases. The monoanions are less susceptible to bimolecular nucleophilic attack than are the dianions of aryl phosphates. Insufficient data are available to extend these generalizations to phosphoramidates.

(3) Temperature effect: The rate of unimolecular reaction increases faster with

temperature than the rate of bimolecular nucleophilic substitution. This is a result of the different activation parameters for the two processes (*12, 14*).

SUMMARY

This paper provides the generalization that phosphorylation by reactive phosphates proceeds by a mechanism which is borderline unimolecular with variable amounts of nucleophilic solvent participation. DMPG appears to be the clearest example of a unimolecular mechanism with metaphosphate intermediate found to date. Despite the large difference expected in nucleophilicity of methanol and water, the product ratio indicates random attack on the metaphosphate ion. This random result is consistent with phosphoroguanidines having the highest reactivity of metaphosphate precursors. Therefore, there appears to be a blend of metaphosphate mechanisms ranging from those that are predominantly bimolecular with large amounts of nucleophilic participation in the transition state to phosphoroguanidines which, based on our previous results (*1d, 1e*) and this paper, are cleanly unimolecular. This picture is consistent with results on carbonium ions for which transition states have a variable amount of nucleophilic participation depending on a number of factors including the reactivity of the carbonium ion precursor. Our results are consistent with isotope effects (*27*), but raise questions about the interpretation of Rebek's ingenious three-phase experiments on metaphosphates (*2e*).

These results have important implications for the important problem of "high-energy" phosphate transfer catalyzed by enzymes. Although the reactions will definitely be expected to have metaphosphate character, how does the enzyme surface affect the extent of nucleophilic participation in the transition state? We suggest that the high degree of orientation which characterizes enzymic reactions (*28*) will lead to more extensive nucleophilic participation than in chemical reactions. The presence of metal ions at enzymic active sites may also increase the extent of nucleophilic participation.

EXPERIMENTAL

Methanol and 2-propanol were purified by distillation from a molecular sieve. The alcohol–water mixtures were made up in tared 50-ml volumetric flasks by addition of the appropriate amounts of the alcohol, 4.75 ml of a 2.00 *N* NaCl solution, 5.00 ml of a 0.10 *N* HCl solution, and diluted with H_2O to the mark. The mole percent alcohol was calculated on the basis of the weight of the alcohol and the total weight of the solution. All solutions were 0.10 *N* in HCl and the ionic strength was 0.20 *N*.

Kinetics

The rates of solvolysis of DMPG (*1*) at 30.47 ± 0.05°C in various alcohol–water mixtures were obtained as described by observing the disappearence of DMPG at 225.0 nm. The runs were initiated by the addition of 15 μl of DMPG stock solution to 3.00 ml of the kinetic solution, previously equilibrated at 30.47°C, to give an initial DMPG concentration of 5.0×10^{-4} *M*. Excellent first-order kinetics were obeyed for all runs to at least 85% reaction.

Paper Chromatography

All paper chromatography was run by the ascending technique (*29*) on Whatman No. 1 filter paper. The solvent system employed was *n*-butanol: acetic acid: H_2O (5 : 2 : 3). Development time was ca. 1.5 hr, which corresponds to ca. 100 mm advancement of the solvent front. The spots were detected by spraying the chromatogram with the Hanes–Isherwood (H–I) reagent (*30*), and subsequent exposure to uv light. The R_f values and behavior to the H–I reagent are listed below.

Compound	R_f	Behavior to H–I reagent	Detection limit (μg)
Methyl phosphate	0.50	Blue in uv	0.5
Ethyl phosphate	0.54	Blue in uv	0.5
2-Propyl phosphate	0.68	Blue in uv	0.5
Inorganic phosphate	0.42	Yellow, turns blue in uv	0.1

Product Identification

The solvolysis of DMPG was carried out at 30.5 ± 0.1°C in 10.00 ml of 80% (v : v) alcohol–water mixtures (59.5 mol% methanol, 50.8 mol% ethanol, and 44.5 mol% 2-propanol.) with initial [DMPG] = 0.010 *M*. After 10 half-lives, 1-μl aliquots were analyzed by paper chromatography. The only detectable products of the solvolysis of DMPG in alcohol–water mixtures were inorganic phosphate and the corresponding alkyl phosphates.

Product Composition

To 10.00 ml of the appropriate alcohol–water mixture in a volumetric flask, previously equilibrated to 30.5 ± 0.1°C, was added 50 μl of DMPG stock solution to give an initial DMPG concentration of 5.0×10^{-4} *M*. After 10 half-lives, 1.00-ml aliquots were withdrawn and analyzed for inorgnic phosphate (*2*). The alcohol in the 1.00-ml aliquots diluted the i-butanol layer, giving absorbance values slightly less than expected for given concentrations of inorganic phosphates. It was determined that 95% of the alcohol present in the 1.00-ml aliquots was extracted into the i-butanol layer, so where Abs is the observed absorbance at 660 nm, Abs (corr) = Abs $(5.00 + x)/5.00$ which is the corrected absorbance value used to calculate the amount of inorganic phosphate present where x is the volume of alcohol in the 1.00-ml aliquot. The validity of this method was checked with standard solutions of $KHPO_4$. The entire procedure was found to be reproducible to within 5%.

The percentage alkyl phosphate produced was calculated by the equation: percentage (alkyl phosphate) = 100 [millimoles P_i (theoretical) − millimoles P_i (observed)]/millimoles P_i (theoretical) where millimoles P_i (theoretical) and millimoles P_i (observed) are the theoretical amount of inorganic phosphate and the observed amount of inorganic phosphate, respectively. The theoretical amount of inorganic phosphate is that amount of inorganic phosphate produced on the solvolysis of DMPG in the absence of alcohol. The value so obtained was, within experimental error, identical with the calculated initial concentration of DMPG.

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REFERENCES

1. (a) B. Springs and P. Haake, *Tetrahedron Lett.* 3223 (1977); (b) B. Springs and P. Haake, *Bioorg. Chem.* **6**, 181 (1977); (c) L. Atwood and P. Haake, *Bioorg. Chem.* **5**, 1165 (1976); (d) G. W. Allen and P. Haake, *J. Amer. Chem. Soc.* **98**, 4990 (1976); (e) G. W. Allen and P. Haake, *J. Amer. Chem. Soc.* **95**, 8080 (1973); (f) P. Haake and G. W. Allen, *Proc. Nat. Acad. Sci. USA* **68**, 2691 (1971), this communication summarized some of the material which is presented in the present paper.
2. (a) W. W. Butcher and F. H. Westheimer, *J. Amer. Chem. Soc.* **77**, 2420 (1955); (b) C. A. Vernon, *Spec. Pub. Chem. Soc.* **8**, 17 (1957); (c) J. R. Cox, Jr., and O. B. Ramsay, *Chem. Rev.* **64**, 317 (1964); (d) D. L. Miller and F. H. Westheimer, *J. Amer. Chem. Soc.* **88**, 1507, 1511, 1514 (1966); (e) S. J. Benkovic and K. J. Schray, "The Enzymes" (P. D. Boyer, Ed.), Vol. VIII, p. 201; Academic Press, New York, 1973. (f) J. Rebek and F. Gavina, *J. Amer. Chem. Soc.* **97**, 3221 (1975). (g) L. M. Loew and W. R. MacArthur, *J. Amer. Chem. Soc.* **99**, 1019 (1977).
3. S. B. Hartley, W. S. Holmes, J. K. Jacques, M. F. Mole, and J. C. McCaubrey, *Quart. Rev.* **17**, 204 (1963).
4. H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed. Reinhold, New York, 1958.
5. L. C. Bateman, E. D. Hughes, and C. K. Ingold, *J. Chem. Soc.*, 881 (1938).
6. J. D. Chanley and E. Feageson, *J. Amer. Chem. Soc.* **85**, 1181, 4002 (1963).
7. (a) J. G. Kirkwood, *J. Chem. Phys.* **2**, 351 (1934). (b) G. Scatchard, *Chem. Rev.* **10**, 229 (1932).
8. E. Grunwald and S. Winstein, *J. Amer. Chem. Soc.* **70**, 846 (1948).
9. B. Gutbezahl and E. Grunwald, *J. Amer. Chem. Soc.* **75**, 559 (1953).
10. The negatively charged oxygens in HO_3^- PX and in **1** will have a very strong affinity for protic solvents. Each oxygen should have hydrogen bonds to 2 or 3 protic solvent molecules as discussed by J. W. Henderson and P. Haake, *J. Org. Chem.* **42**, 3989 (1977). Although methanol and water may be able to solvate randomly, 2-propanol's steric effect from the two methyl groups would prevent full protic solvation if it hydrogen bonds to these oxygens. Therefore, water will preferentially form the inner solvation shell about phosphates.
11. S. J. Benkovic and K. J. Schray, *Biochemistry* **7**, 4090 (1968).
12. A. J. Kirby and A. G. Varvoglis, *J. Amer. Chem. Soc.* **89**, 415 (1967).
13. C. A. Bunton, E. J. Fendler, and J. H. Fendler, *J. Amer. Chem.* **89**, 1221 (1967).

14. P. A. T. SWOBODA, *Chem. Soc. Spec. Pub.* **8**, 41 (1957).
15. S. J. BENKOVIC AND P. A. BENKOVIC, *J. Amer. Chem. Soc.* **89**, 4714 (1967).
16. W. P. JENCKS AND M. GILCHRIST, *J. Amer. Chem. Soc.* **86**, 1410 (1964).
17. A. J. KIRBY AND W. P. JENCKS, *J. Amer. Chem. Soc.* **87**, 3209 (1965).
18. A. J. KIRBY AND A. G. VARVOGLIS, *J. Chem. Soc. B.* **135**, (1968).
19. G. F. ENDRES AND J. EPSTEIN, *J. Org. Chem.* **24**, 1497 (1959).
20. R. W. SWIDLER, R. E. PLAPINGER, AND G. M. STEINBERG, *J. Amer. Chem. Soc.* **81**, 3271 (1959).
21. A. L. GREEN, G. L. SAINSBURY, B. SAVILLE, AND M. STANSFIELD, *J. Chem. Soc.*, 1583 (1958).
22. (a) J. EPSTEIN, D. H. ROSENBLATT, AND M. M. DEMEK, *J. Amer. Chem. Soc.* **78**, 341 (1956); (b) J. EPSTEIN, R. E. PLAPINGER, H. O. MICHEL, J. R. CABLE, R. A. STEPHANI, R. J. HESTER, C. BILLINGTON, JR., AND G. R. LIST, *J. Amer. Chem. Soc.* **86**, 3075 (1964). (c) J. EPSTEIN, H. O. MICHEL, D. H. ROSENBLATT, R. E. PLAPINGER, R. A. STEPHANI, AND E. COOK, *J. Amer. Chem. Soc.* **86**, 4959 (1964).
23. G. DISABATO AND W. P. JENCKS, *J. Amer. Chem. Soc.* **83**, 4393, 4400 (1961).
24. G. W. JAMESON AND J. M. LAWLOR, *J. Chem. Soc. B.* **53**, (1970).
25. A. J. KIRBY AND M. YOUNAS, *J. Chem. Soc. B.*, 510 (1970).
26. W. P. JENCKS AND M. GILCHRIST, *J. Amer. Chem. Soc.* **87**, 3199 (1965).
27. D. G. GORENSTEIN, Y.-G. LEE, AND D. KAR, *J. Amer. Chem. Soc.* **99**, 2264 (1977).
28. M. I. PAGE, *Chem. Soc. Rev.* **2**, 295 (1973); W. P. JENCKS, *Advan. Enzymol.* **43**, 219 (1975).
29. R. J. BLOCK, E. L. DURRUM, AND G. ZWEIG, "Paper Chromatography and Paper Electrophoresis." Academic Press, New York, 1958.
30. C. S. HANES AND F. A. ISHERWOOD, *Nature (London)* **164**, 1107 (1949).