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Solvent Effects on the Solvolysis of ADPβS

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Solvolysis rates for trialkylammonium and tetraalkylammonium salts of ADPβS at 25°C and ADP at 80°C in water, *N,N*-dimethylformamide (DMF) and water–DMF mixtures are reported. ADPβS undergoes solvolysis much faster than ADP under all conditions. The solvolysis of *tris*-triethylammonium ADPβS is 40-fold faster in DMF than in water at 25°C, but the solvolysis of *tris*-tetramethylammonium ADPβS is only slightly faster in DMF than in water. The solvolysis rate for *tris*-tetramethylammonium ADPβS increases whereas that for *tris*-triethylammonium ADPβS decreases upon addition of small percentage of H_2O to DMF. The corresponding salts of ADP do not undergo detectable solvolysis at 25°C, but react at 80°C, where the trialkylammonium and tetraalkylammonium salts exhibit analogous but much smaller effects on solvolysis rates. The results indicate the operation of two effects of DMF in enhancing the solvolysis of ADPβS: (a) DMF decreases the acid dissociation constant of ADPβS, which leads to an increased fraction being in the solvolytically labile dianionic form. (b) The decreased polarity of DMF relative to water enhances the rate by a medium effect of destabilizing the ground state relative to the transition state in a dissociative mechanism. Comparisons of the solvolytic rates for ADPβS relative to ADP suggest that the two react by different mechanisms, ADP by a S_N2 mechanism via a dissociative transition state and ADPβS by a purely dissociative mechanism via a discrete thiometaphosphate monoanion as an intermediate. © 1990 Academic Press, Inc.

Experience in chemical synthesis of nucleoside thiophosphoanhydrides has shown that thiophosphoanhydrides with terminal thiophosphoryl groups can present problems in reactions conducted in organic solvents. Molecules such as ADPβS[1] often decompose rapidly in solvents such as *N,N*-dimethylformamide, hexamethylphosphoramide, dimethylsulfoxide, pyridine, and triethylphosphate. The decomposition products are generally lower phosphomonoesters such as AMP; however, other products appear in triethylphosphate. Molecules with sulfur at internal positions of phosphoanhydride linkages, such as ADPαS, are much more stable and do not present problems with synthesis in organic solvents. Ordinary phosphoanhydrides are also much more stable than the sulfur analogs with external thiophosphoryl groups.

Earlier studies showed that the coupling of (R_p)- or (S_p)-[β-^{17}O]ADPβS with phenyldichlorophosphate in hexamethylphosphoramide proceeds with epimeriza-

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[1] Abbreviations used: ADPβS, adenosine 5′-(2-thiodiphosphate); ADPαS, adenosine 5′-(1-thiodiphosphate); (Rp)- and (Sp)-[β-^{17}O]ADPβS, adenosine 5′-(2-thio[2-^{17}O]diphosphate) having the R and S configuration at P^2; DMF, *N,N*-dimethylformamide; HPLC, high performance liquid chromatography.

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tion at phosphorus (*1*). This was explained on the basis that reversible cleavage of the [^{17}O]thiophosphoryl group from *P*-chiral [β-^{17}O]ADPβS competes with coupling and leads to epimerization. Reversible cleavage could generate high energy [^{17}O]thiophosphoryl–solvent species or [^{17}O]thiometaphosphate, either of which would rephosphorylate AMP with epimerization at phosphorus. These experiments were consistent with the known instability of ADPβS in organic solvents.

In this paper we report solvolytic rate constants for ADPβS and ADP under various conditions of solvent polarity, temperature, and acidity. The data show that the rate at which ADPβS undergoes solvolytic cleavage in organic solvents depends upon the countercation that is present. The tri-*n*-ethylammonium salt is much more rapidly cleaved in *N,N*-dimethylformamide than the tetramethylammonium salt, which is relatively stable.

METHODS

The triethylammonium salt of ADPβS was synthesized by the method of Ho and Frey (*2*). The tetramethylammonium salt of ADPβS was prepared by addition of tetramethylammonium hydroxide to the triethylammonium salt and evaporation to remove triethylamine. ADP was purchased from Boehringer-Mannheim Biochemicals, and all other chemicals were obtained from Aldrich or Sigma. DMF was stirred with BaO overnight, distilled under vacuum, and stored over Linde 4-Å molecular sieves. Triethylphosphate was stirred with CaH_2 overnight and distilled under vacuum.

All rates were measured using reverse phase HPLC to analyze for product (AMP) formation, using a Waters Model 440 system equipped with an ultraviolet detector, a strip chart recorder, and a Waters Novopak C_{18} reverse phase column (3.9 × 15 cm). The system was operated with a mobile phase consisting of 50 mM K-phosphate buffer at pH 6 at a flow rate of 0.8 ml/min. Authentic samples of ADP, AMP, and ADPβS were used to verify retention times prior to each run. The reaction mixtures were sampled at regular intervals and quenched by dilution into aqueous 50 mM K-phosphate buffer at pH 6.0. The quenched aliquots were analyzed by HPLC for products and unreacted substrates. The integrated areas for reactants and products from the recorder output were measured and used to calculate rate constants.

RESULTS AND DISCUSSION

Solvolysis of ADPβS and ADP in H_2O, DMF and H_2O/DMF mixtures. Rate constants for solvolysis of ADPβS and ADP in various solvents are listed in Table 1. The constants for the triethylammonium salt of ADPβS increase with decreasing polarity of the solvent to as much as 40 times the rate in water when the reaction is carried out in DMF. The rate in DMF is even greater than that in water at pH 2. However, the tetramethylammonium salt of ADPβS does not undergo solvolysis significantly faster in DMF than in water, although both salt forms of ADPβS

TABLE 1

Solvolysis of ADPβS and ADP in Water and DMF[a]

	$K_{OBS} \times 10^{-4}$ (min^{-1})[b]	
Solvent	ADPβS (25°C)	ADP (80°C)
Water (pH 6, 50 mM KP_i)	6.5	13
Water (pH 2, 0.01 M HCl)	40	16
Ethanol	18	—
DMF (Et_3NH^+ salt)	250	60 (Et_3NH^+-salt)
DMF (Me_4N^+ salt)	4.0	41 (Et_4N^+-salt)

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[a] The ADPβS or ADP was 13 mM in the specified solvent; aliquots of 1 or 2 μl were removed at various times and mixed with 75 or 150 μl of 50 mM K-phosphate buffer at pH 6.0 and 0°C. Aliquots of teh quenched samples were analyzed for AMP and ADPβS (or ADP) by HPLC.

[b] Rate constants were calculated from the ratios AMP/ADPβS or AMP/ADP determined by integrating the areas corresponding to the nucleotides on the HPLC chart records. The data were plotted as ln[AMP/(ADPβS + 1)] or ln[AMP/(ADP + 1)] versus time, and the rate constants were calculated from the slopes.

undergo solvolysis at the same rate in water. Therefore, the increased cleavage rate for trimethylammonium-ADPβS in DMF is caused by both the solvent and the counterion. Solvolysis of ADP at 80°C is less sensitive to solvent and countercation effects than ADPβS, although the trends are similar. Cleavage of ADP at 25°C is very slow and not observed under our conditions.

Table 2 shows the effect of water content in mixed water–DMF solvents on the solvolysis of the triethylammonium and tetraalkylammonium salts of ADPβS and ADP. Increasing the water content up to 7% decreases the rate for the triethylammonium salt of ADPβS. Increasing the water to 3% increases the rate for the tetramethylammonium salt. The effects of solvent polarity are much smaller for the triethylammonium salt of ADP, again at 80°C, and the solvolysis of the tetrabu-

TABLE 2

Solvolysis Rate Constants for ADPβS and ADP in DMF-H_2O Mixtures[a]

ADPβS (25°C)		ADP (80°C)	
Et_3NH^+ (% H_2O)	Me_4N^+ (% H_2O)	Et_3NH^+ (% H_2O)	Bu_4N^+ (% H_2O)
	$k_{obs} \times 10^2$ (min^{-1})		
2.5(<1)	0.040 (<1)	0.60 (<1)	0.52 (<1)
2.0 (3)	0.36 (3)	0.24 (10)	0.58 (5)
1.5 (5)			0.54 (10)
1.0 (7)			

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[a] The rate constants were measured as described in Table 1.

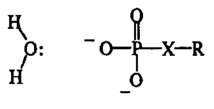
tylammonium salt seems to be insensitive to the water content up to 10% water/DMF.

The major products of solvolysis in water and DMF were AMP and phosphate, which were accompanied by very small amounts of more polar products appearing after very long reaction times. The reaction of ADP in triethylphosphate and mixtures of 5% water in triethylphosphate at 80°C was much more complex, yielding AMP as a minor product, with more than three other products being formed. The other products included several more polar than ADP, one of which was hydrolyzed to AMP by alkaline phosphatase, and one product less polar than AMP that was hydrolyzed to AMP by snake venom phosphodiesterase. These products, which were not identified, were formed only in triethylphosphate; they presumably reflected a participation of the solvent in the cleavage reaction.

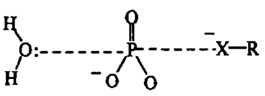
Mechanism rate enhancement by DMF. The effect of DMF in enhancing the solvolysis of ADPβS is qualitatively similar to the effects of other organic solvents such as pyridine, dimethylsulfoxide, and hexamethylphosphoramide, although the rate constants in the latter solvents were not quantitatively measured (*1, 2*). The present results show that the enhancement is counterion dependent, occurring with triethylammonium salts and not with tetramethylammonium salts of ADPβS. Qualitatively similar effects occur in the solvolysis of ADP, although the effects are much smaller, as is the rate.

Apparently analogous effects of organic solvents on the solvolyses of selected phosphomonoesters have been reported in other studies. Dittmer *et al.* (*3*) reported moderate increases in solvolysis rates for *S*-*n*-butylphosphorothioate in mixed dioxane–water and pyridine–water up to 9.8% organic solvent. Kirby and Varvoglis (*4*) reported a marked enhancement in the solvolysis of dianionic 2,4-dinitrophenylphosphate in many mixed organic–aqueous solvents, including DMF–water. Milstien and Fife (*5*) observed an enhancement by methanol of the solvolytic rate for the dianion of *S*-*p*-nitrophenylphosphorothioate.

The two mechanistic rationales for solvolytic rate enhancements in the phosphomonoester field are: (a) The organic solvent tends to stabilize a transition state of decreased polarity relative to the substrate ground state, and this leads to a rate enhancement by the organic solvent. This rationale supports a dissociative mechanism of solvolysis, in which there is little bonding to either the leaving group or the entering nucleophile in the transition state. Such a transition state is characterized by diffuse charge relative to the ground state, as illustrated in structure **2**

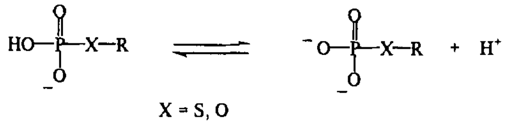


1
Ground State (X = S, O)



2
Transition State (X = S, O)

relative to **1**. An organic solvent, being less polar than water, should destabilize the highly charged ground state relative to the transition state, in which the electrostatic charge is more diffuse (*4, 5*). (b) The second rationale is that the less polar organic solvent decreases the acid dissociation constant of the substrate, so that in the organic solvent an increased fraction of the substrate is in a protonated form, which is more reactive and undergoes solvolysis at its normal, faster rate. In this



$$HO-\overset{\overset{O}{\|}}{\underset{\underset{\_O}{|}}{P}}-X-R \rightleftharpoons {}^{-}O-\overset{\overset{O}{\|}}{\underset{\underset{\_O}{|}}{P}}-X-R + H^{+} \qquad X = S, O \tag{1}$$

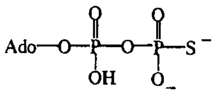
case the organic solvent shifts the equilibrium of Eq. [1] to the left, and the monoanion simply undergoes solvolysis at a faster rate than the dianion (*3*). Both of these effects can be expected in mixtures of DMF and water, since the dielectric constant of DMF is 37 and that of water is 81.

The data in Tables 1 and 2 suggest that in DMF the decrease, relative to water, in the acid dissociation constant of ADPβS is an important factor contributing to rate enhancement, effect (b) above. In all the reported experiments substrates were nominally the trianionic forms of ADPβS and ADP. The *tris*-trialkylammonium salts could exist as the equilibrium of Eq. [2],

$$[ADP\beta S]^{2-}(Et_3NH^+)_2 + Et_3N \rightleftharpoons [ADP\beta S]^{3-}(Et_3NH^+)_3 \tag{2}$$

and the dianionic form of ADPβS DMF would be favored to a greater degree in DMF than in water. To the extent that the dianion exists at higher levels in DMF than in water, the solvolysis rate will be enhanced since, as shown in Table 1, the dianion reacts faster than the trianion. The *tris*-tetraalkylammonium salts could not, however, enter into equilibria such as Eq. [2], since there would be no source of protons that could facilitate the equilibrium formation of dianionic ADPβS. Water is a proton source, albeit not as acidic a source as the triethylammonium ion; and in this connection it is significant that 3% added water enhances the solvolysis rate of the tetraethylammonium salt of ADPβS (Table 2). Similar additions of water to the triethylammonium salt of ADPβS slightly decrease the solvolytic rate.

Table 1 also shows that the rate enhancement by DMF is greater than the effect of 10 mM acid in water. At pH 2 ADPβS should be a mixture of dianionic and monoanionic forms, yet the rate is slower at pH 2 than in DMF; therefore, it appears that an equilibrium shift of Eq. [2] does not by itself account for the data. An apolar medium effect would enhance the rate at which dianionic ADPβS reacts. The more reactive form of the dianion is probably the species protonated on the



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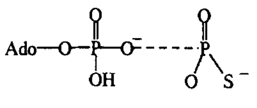

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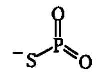
α-phosphoryl group, **3** shown below. In the dissociative transition state **4** the two negative charges are more highly diffused than in the ground state **3**. The reduced polarity of DMF compared with water would tend to enhance the rate owing to greater destabilization of the ground state compared with the transition state. This is the effect advanced by others to account for their observations with monoesters (*4*, *5*).

It seems likely that the *primary* thiophosphoryl group acceptor in DMF is not water, which is present only in adventitious amounts as a contaminant, but rather DMF itself. The question of direct organic solvent participation in the cleavage of ADPβS has been discussed earlier (*1*), and DMF should have the capacity to react as a nucleophile. The primary event, however, is essentially departure of thiometaphosphate, as explained below, and this probably controls the rate.

Relative reactivities of ADPβS and ADP. ADPβS undergoes solvolysis much faster under all conditions than ADP. This is interesting because both reactions involve the expulsion of AMP as the departing nucleophile. The rates we observe for ADP in water are comparable to results presented elsewhere (*6*). The greater reactivity of ADPβS, which much be explained by the solvolysis mechanism and the structures of the two molecules, is consistent with a dissociative mechanism and the known differences in bonding and charge stabilization in these molecules. The relative rates are not consistent with an associative mechanism.

In a dissociative mechanism, the faster rate for ADPβS relative to ADP must result from a greater propensity to undergo P–O bond cleavage on the approach to the transition state. The P–O bond energies should not be identical in ADPβS and ADP, except by coincidence, but there is little reason to expect them to differ greatly. On the other hand, the greater the stability of the incipient electrophile, thiometaphosphate or metaphosphate, respectively, the easier and faster the bond will be cleaved in a dissociative mechanism. Thiometaphosphate, **5**, should be more stable than metaphosphate, **6**, since the negative charge will be more stably accommodated by sulfur than by oxygen, even though the charge is more highly delocalized in metaphosphate. The factors favoring charge localization on sulfur in phosphorothioate anions are presented elsewhere (*7*); to the extent that these factors also apply to thiometaphosphate and metaphosphate, thiometaphosphate will be the better incipient electrophilic species in a dissociative transition state.



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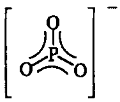

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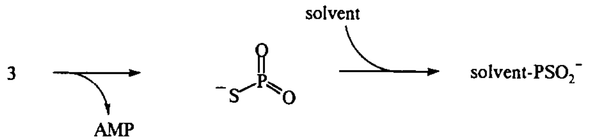
The associative mechanism involves a higher degree of bonding to the entering and leaving groups than does the ground state. Since bond formation to an entering nucleophile is important in the associative transition state, the electrophilic reactivity of phosphorus is important; the more electrophilic the phosphorus the faster the rate, all other things being equal. Reactions proceeding by the associative mechanism should be sensitive to the substitution of sulfur for oxygen, since sulfur is less electronegative than oxygen and will *decrease* the electrophilicity of phosphorus, slowing the reaction with a given nucleophile. The effect of sulfur in ADPβS is the reverse of what is predicted for an associative mechanism.

The mechanism of phosphoryl group transfer is best described as one in which the transition state is dissociative and metaphosphate-like, but with detectable bonding between an incipient metaphosphate and both the entering nucleophile and the leaving group (*8–11*). Structure **2** is intended to represent such a transition state. This view is supported by a compelling body of physical organic chemical information, including extensive structure–function and stereochemical evidence, and ADP can be expected to undergo solvolysis by this mechanism.

The mechanism of thiophosphoryl transfer may differ from phosphoryl transfer owing to the greater stability of thiometaphosphate. Evidence indicating that thiophosphates undergo solvolysis via free thiometaphosphate monoanion is that racemization accompanies the solvolysis of *P*-chiral thiophorphomonoesters (*12, 13*). Therefore, solvolysis of ADPβS may involve fragmentation of the molecule into AMP and thiometaphosphate via the transition state **4**, followed by solvent-capture of thiometaphosphate, as shown in Eq. [3].



[3]

The mechanism of Eq. [3] differs from the probable S_N2 mechanism for solvolysis of ADP, and the difference may account for the difference in solvolysis rates. The requirement for solvent participation in the cleavage of ADP, even via a dissociative transition state such as **2**, is a constraint that is absent in Eq. [3] and can favor the pure dissociative mechanism via the discrete intermediate. The S_N1-dissociative mechanism is presumably possible when the intermediate is not too unstable. Thiometaphosphate may be such an intermediate; if so, the faster solvolysis of ADPβS relative to ADP may be attributed to the difference in reaction mechanism.

ACKNOWLEDGMENT

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Note added in proof. The solvent effects observed in this work are *much* smaller than the 10^6- to 10^7-fold rate enhancements reported earlier for p-nitrophenyl phosphate dianion in 95% dimethyl sulfoxide (14). The leaving groups differ, although they have similar pK_a values in water. The relative stabilities of *p*-nitrophenolate and AMPS in 95% dimethyl sulfoxide probably are not reflected by the pK_a values for their conjugate acids in water, however, since the phenyl group is much less polar than the 5-adenosyl group and the negative charge is much less localized in *p*-nitrophenolate than in AMPS. Thus, the large organic solvent effect on the hydrolysis of *p*-nitrophenyl phosphate may reflect the special properties of the leaving group. This large effect does not appear to extend to AMP or AMPS as leaving groups.

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