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Solvent Effects on Hydrolysis of the Phosphoenzyme Intermediate in Sodium- and Potassium-Dependent Adenosine Triphosphatase: Correlations with Stimulation of Potassium-Dependent *p*-Nitrophenyl Phosphatase

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SUMMARY

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Studies were undertaken to examine the effects of the solvents deuterated water (^2H_2O) and dimethyl sulfoxide (DMSO) on the breakdown of the phosphorylated intermediate and on the K^+-dependent *p*-nitrophenyl phosphatase activity of rat brain ($Na^+ + K^+$)-ATPase. At 4° phosphoenzyme hydrolysis in the presence of K^+ (0.02–0.33 mM), NH_4^+ (0.33 mM), or Li^+ (0.33 mM) followed a biphasic time course, indicating the presence of comparatively rapid (sensitive) and slow (insensitive) components. Phosphoenzyme breakdown in the presence of 5 mM ADP exhibited similar components. However, when both ADP and K^+ were added together, essentially all of the phosphoenzyme was rapidly hydrolyzed, suggesting that the K^+-sensitive and ADP-sensitive components predominated. ^2H_2O and DMSO stimulated the rate of breakdown of the slower, insensitive component in the presence of K^+ and NH_4^+, but not Li^+. No effect of these solvents was observed in the presence of ADP. The rate of hydrolysis of the rapid component could not be determined, but the solvents appeared to have no significant effect on the amount of phosphoenzyme hydrolyzed within 1 sec. Both ^2H_2O and DMSO stimulated the *p*-nitrophenyl phosphatase activity at 4° in the presence of K^+ (0.02–0.33 mM) or NH_4^+ (0.33 mM), but not of Li^+ (0.33 mM). The stimulatory effects of ^2H_2O and DMSO on K^+-dependent phosphoenzyme breakdown and K^+-dependent *p*-nitrophenyl phosphatase activity appear to be related and are interpreted in terms of a solvent role of H_2O in the conformation of the phosphoenzyme which modulates its interaction with K^+.

INTRODUCTION

It is generally agreed that ($Na^+ + K^+$)-ATPase is involved in the transport of Na^+ and K^+ across the cell membrane (1). A large body of data suggests that two steps may be involved in the over-all ATPase reaction: the Na^+-dependent formation of a phosphoenzyme, followed by its breakdown in the presence of K^+ (for reviews, see refs. 1-6). A phosphatase activity associated with the ($Na^+ + K^+$)-ATPase system has been demonstrated by utilizing a simple substrate, *p*-nitrophenyl phosphate, whose breakdown is stimulated by K^+ and inhibited by ouabain (7, 8). The consistent presence of K^+-*p*-nitrophenyl phosphatase in ($Na^+ + K^+$)-ATPase preparations and the properties of this enzyme

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have led to the belief that it is a manifestation of the terminal step in the reaction sequence of the $(Na^+ + K^+)$-ATPase system (2–8).

Recent studies on the effects of certain solvents have described disparate effects on the two activities (9–11). For example, both ^2H_2O and DMSO[2] were shown to stimulate the K^+-*p*-nitrophenyl phosphatase while inhibiting the $(Na^+ + K^+)$-ATPase. Stimulation by ^2H_2O was due primarily to an increased affinity of K^+ for the enzyme (9), whereas DMSO, in addition to increasing the affinity of K^+ (10), enhanced the affinity of the substrate *p*-nitrophenyl phosphate (11, 12). In view of the possibility that the phosphoenzyme may be an intermediate in K^+-dependent *p*-nitrophenyl phosphatase activity (13, 14), the question arises whether the stimulation of this activity in the presence of ^2H_2O and DMSO may reflect an increased rate of K^+-mediated phosphoenzyme breakdown in the presence of these solvents. The present experiments demonstrate that the breakdown of K^+-mediated phosphoenzyme involves a rapid, sensitive and a slow, insensitive component; ^2H_2O and DMSO significantly enhance the rate of hydrolysis of the latter. It is suggested that this stimulation may correlate with the increased activity of the K^+-*p*-nitrophenyl phosphatase in the presence of these solvents. A preliminary account of this work has been published (15).

MATERIALS AND METHODS

^2H_2O (99.5% TTS) was purchased from Mallinckrodt Chemical Works and was distilled twice in an all-glass apparatus before use. Analytical-grade DMSO was obtained from Baker Chemical Company. All other details concerning the materials used have been given previously (9, 10, 16). The various salts (KCl, NaCl, $MgCl_2$) used were spectroscopically pure. Twice-distilled water, free of cations, was used to prepare solutions which were stored frozen prior to use. EDTA and ATP were converted to Tris salts by passage through a cation-exchange column. Sucrose and Tris were of the ultrapure grade from Schwarz/Mann.

Preparation of $(Na^+ + K^+)$-ATPase. The details concerning the preparation of the enzyme and its properties have been described previously (16). The enzyme preparation was washed twice in a medium consisting of 0.25 M sucrose, 10 mM imidazole HCl, and 1 mM EDTA, pH 7.4, and was finally suspended in the same medium for storage in small aliquots at −20°. The enzyme was stable for several months, but longer storage was avoided to prevent its contamination by atmospheric NH_4^+. The specific $(Na^+ + K^+)$-ATPase activity of this preparation was generally 120 μmoles of P_i per milligram of protein per hour, of which 95% was ouabain-sensitive. The upper limits of possible cationic contamination in the reaction mixture, containing all components except added cations, were found to be 0.1 mM Na^+, 3 μM K^+, 0.25 μM Mg^{2+}, 5 μM Ca^{2+}, and 20 μM NH_4^+. The contamination by NH_4^+ was accounted for entirely by the presence of 20 μM NH_4^+ in freshly prepared, double-distilled water. The presence of various cations did not contribute additional NH_4^+ to the reaction. The 20 μM level of NH_4^+ is considered equivalent to 6 μM K^+ in terms of its efficacy in substituting for K^+ in the K^+-sensitive reactions of the ATPase.

Procedure for study of phosphoenzyme breakdown. The standard reaction medium for the formation of phosphoenzyme, in a final volume of 2 ml maintained at 4°, consisted of 30 mM Tris-HCl (pH 7.45), 0.3 mM $MgCl_2$, 8 mM NaCl, 0.05 mM [γ-^{32}P]ATP (6×10^4 dpm/nmole of ATP), and 350–450 μg of rat brain microsomal membrane protein. The reaction was initiated by the addition of [γ-^{32}P]ATP and was allowed to proceed for 4 sec, to achieve steady-state formation of phosphoenzyme (16). The reaction was terminated by the addition of 25 ml of ice-cold 5% trichloracetic acid (w/v) containing 15 mM NaH_2PO_4 and 0.6 mM ATP. When phosphoenzyme breakdown was investigated, the chase technique of Post *et al.* (17) was used. To the steady-state phosphoenzyme, obtained as above, 1.0 ml of a solution (chase medium) was added to give a final

[2] The abbreviations used are: DMSO, dimethyl sulfoxide; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; *E*-P, phosphoenzyme.

concentration of 5 mM $MgCl_2$ plus 5 mM ATP with or without KCl at various concentrations. In order to observe ADP-dependent phosphoenzyme breakdown, a chase medium giving a final concentration of 3 mM CDTA (Tris salt) with or without ADP was used. The reaction was terminated at different times (1–4 sec) after the chase. Throughout the experiment, the reaction medium was stirred by means of a magnetic bar rotating at 10 rps. The duration of injection of the chase solution into the reaction was estimated as 0.15–0.20 sec. These procedures are sufficiently rapid to follow the time course used in this study.

After termination the reaction mixtures were placed on ice for 30 min, and the precipitate was then collected on a Millipore filter (0.45 μm pore size) and washed with the above trichloracetic acid medium. The filters were placed in a vial containing 15 ml of Omnifluor liquid scintillation medium (New England Nuclear) for the measurement of radioactivity. The amount of [^{32}P]phosphoprotein formed when NaCl was replaced with KCl in the initial reaction medium was taken as the basal Mg^{2+}-dependent phosphorylation of the membranes and was subtracted from all other values to obtain the Na^+-dependent phosphoenzyme in the ATPase system. Any variations from this procedure are specified in the appropriate figure or table legend.

Measurement of K^+-p-nitrophenyl phosphatase. The procedures for measuring this activity have been detailed in previous papers (8, 9). The standard reaction medium, in a final volume of 2 ml, consisted of 50 mM Tris-HCl (pH 7.45), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate (Tris salt), and varied concentrations of K^+. The reaction temperature was 4°. The amount of enzyme and the time were adjusted to obtain linear rates of K^+-dependent phosphatase activity.

RESULTS

Effect of ^2H_2O and DMSO on K^+-dependent breakdown of phosphoenzyme. The results given in Fig. 1A show the actual time course of the breakdown of previously formed phosphoenzyme in the absence and presence of K^+ in H_2O or ^2H_2O reaction medium. In the absence of K^+, ^2H_2O did not significantly enhance the rate of breakdown of phosphoenzyme. The plot of phosphoenzyme breakdown, linear on the logarithmic scale, can be extrapolated essentially to 100% of the phosphoenzyme for both H_2O and ^2H_2O. When K^+ was present, the breakdown of phosphoenzyme between 1 and 4 sec after the chase was a linear log function, but it could not be extrapolated back to the original starting level of phosphoenzyme. This suggests that K^+-mediated breakdown of phosphoenzyme does not follow a single linear component. It appears that immediately after the chase with $MgCl_2$ and ATP there is a component which breaks down extremely rapidly (i.e., within 1 sec on our time scale), followed by a relatively slow phase of phosphoenzyme breakdown. In view of the technical limitations, the effect of ^2H_2O on the rate of breakdown of the initial, rapid component cannot be precisely determined at the present time; however, an estimate of the effect of ^2H_2O on the extent of K^+-mediated breakdown of this component was made, and consequently (Table 1) it appears that ^2H_2O has no effect on it. The time course of phosphoenzyme decay between 1 and 4 sec, which is a linear logarithmic process, is amenable to further analysis by means of a "relative plot" (Fig. 1B and C). This was achieved by constructing the graph from Fig. 1A in such a manner that all the lines were arbitrarily made to meet at the 100% value on the ordinate (18). In doing so, the initial, rapid phase of phosphoenzyme hydrolysis was neglected, and the rate constant (k, sec^{-1}) of the breakdown of the slower, linear component was calculated as the inverse of the time at which 1/*e*th of the phosphoenzyme remained. The relative plots in Fig. 1B and C clearly show that ^2H_2O, but not H_2O, significantly stimulates the breakdown of phosphoenzyme in the presence of K^+. The rate constants (k, sec^{-1}) of phosphoenzyme breakdown under various conditions derived from Fig. 1 are given in Table 1, where the differences in rate constants as well as their ratios are tabulated. Also given in Table 1 are the data obtained similarly when the

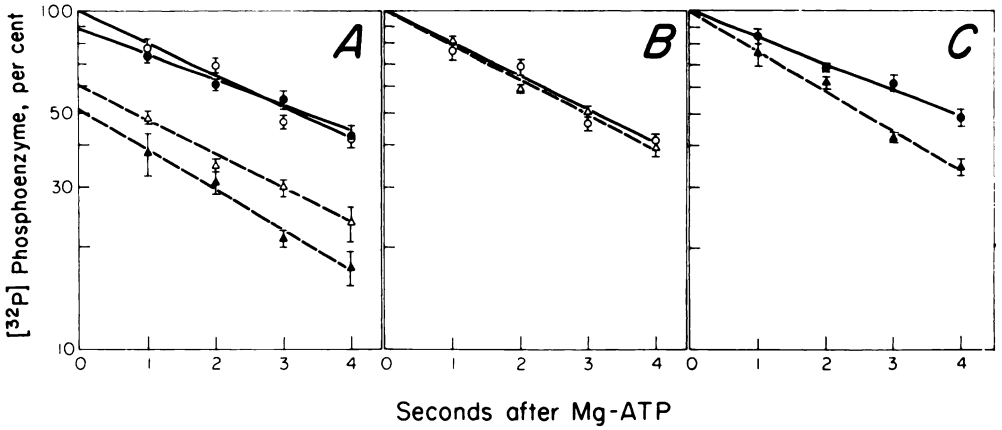

FIG. 1. *Effects of ^2H_2O on K^+-mediated breakdown of phosphoenzyme intermediate of $(Na^+ + K^+)$-ATPase system*

A. The reaction medium, in a final volume of 2 ml maintained at 4°, consisted of 30 mM Tris-HCl (pH 7.45), 0.3 mM $MgCl_2$, 8 mM NaCl, 0.05 mM [γ-^{32}P]ATP (6×10^3 dpm/nmole of ATP), and 350–450 μg of the rat brain enzyme. The reaction was initiated by the addition of [γ-^{32}P]ATP; 4 sec later, 1.0 ml of a solution (chase medium), in final concentrations of 5 mM $MgCl_2$ and 5 mM ATP without KCl (○) or with 0.33 mM KCl (△), was added. The solid symbols indicate that H_2O was replaced by 80% ^2H_2O throughout: ●, no KCl; ▲, 0.33 mM KCl. At the times indicated, the reaction was terminated by the addition of 25 ml of ice-cold 5% (w/v) trichloracetic acid containing 15 mM NaH_2PO_4 and 0.6 mM ATP. The amount of [^{32}P]phosphoprotein formed when NaCl was replaced by KCl in the initial reaction medium was taken as the basal Mg^{2+}-dependent phosphorylation of the membranes, and was subtracted from the total phosphoenzyme values to determine the Na^+-dependent phosphoenzyme component of the ATPase system. All experimental values were confirmed in a minimum of six experiments.

B and C. Replots of the data in Fig. 1A obtained with H_2O and ^2H_2O, respectively, such that the intercept at zero time was arbitrarily fixed as 100%. The mean values for 100% phosphoenzyme were 171.04 ± 6.29 (SE) pmoles/mg of protein in the H_2O system, and 124.89 ± 3.95 pmoles/mg in 80% ^2H_2O. The control labeling in the presence of 16 mM K^+ was 11.48 ± 1.24 pmoles/mg of protein and was unaffected by ^2H_2O.

experiments were repeated in the presence of other concentrations of K^+. It was found the ^2H_2O increased the rate constant of phosphoenzyme breakdown in the presence of 0.02 mM K^+, 0.05 mM K^+, and 0.33 mM K^+ by 23%, 46%, and 49%, respectively. Similar results were obtained in the presence of DMSO, except that there was a clear reduction in the k values in the absence of K^+, implying a stabilizing effect of DMSO on previously formed phosphoenzyme. However, comparison of the rate constants of phosphoenzyme breakdown in the presence of DMSO alone and in the presence of K^+ clearly indicates a stimulatory effect of K^+ on phosphoenzyme hydrolysis in the presence of DMSO. NH_4^+ at 0.33 mM increased the rate constant in ^2H_2O approximately 15% over that in H_2O, while Li^+ at 0.33 mM showed no stimulation of phosphoenzyme breakdown in the relative plot, in either H_2O or ^2H_2O.

Figure 1B shows that the relative rate of decay of phosphoenzyme in the presence of H_2O and K^+ does not seem to be significantly different from that of the control (i.e., no K^+). However, the observation recorded in Fig. 1C suggests that the presence of ^2H_2O imparts a conformation to at least one component of the phosphoenzyme (i.e., the slow component of its breakdown) that enhances its sensitivity to K^+. This is in accord with the observation that ^2H_2O enhances K^+-*p*-nitrophenyl phosphatase by reducing the apparent K_m for K^+ (9, 10).

In order to ascertain further that these observations were statistically significant, we re-examined the data from Fig. 1A and calculated the intercepts and slopes of the lines and their standard errors without normalizing the data as above in Fig. 1B and C. The various values obtained in H_2O and ^2H_2O reaction media in the absence and presence of 0.33 mM K^+ (along with the fiducial limits of these values) are shown in Table 2. It is obvious that the

TABLE 1

Effect of ^2H_2O and DMSO on K^+-mediated breakdown of phosphoenzyme

The conditions in experiment 1 were the same as described for Fig. 1, except that the reaction medium was scaled down to 0.2 ml and 50 μg of enzyme were used. At 4 sec a chase medium (0.8 ml) was added to give final concentrations of 0.1 mM ATP, 0.3 mM $MgCl_2$, 8 mM NaCl, and 30 mM Tris-HCl, pH 7.45, with or without 0.02 mM KCl in either H_2O, 80% ^2H_2O, or 30% DMSO. (To achieve the final 20 μM K^+ concentration in this experiment, the stock chase solution added contained 25 μM K^+.) The reaction was terminated by the addition of 20 ml of the trichloracetic acid medium described in the legend to Fig. 1. The results are the means of duplicate determinations. The data were normalized as in Fig. 1B and C in order to calculate the k value (sec^{-1}), which is the inverse of the time at which 1/eth of the phosphoenzyme remains. The reduced volume and amount of enzyme did not significantly alter the amount of phosphoenzyme per milligram of protein. In experiment 2 the conditions were the same as for Fig. 1, except that the chase medium contained 3 mM CDTA (Tris salt) with or without 0.05 mM KCl (final concentrations). The data for experiment 3 are those depicted in Fig. 1B and C. The rate constants are larger than in experiment 1 because of the different experimental conditions, i.e., chase medium, used in the two experiments; however, each set of experiments was internally controlled to determine the action of ^2H_2O in each case. The values for the rapid component and rate constants were obtained from linear least-squares fits to the data points.

Expt.	Conditions	Loss in 1 sec[a]	Increase due to K^+	k	k (K^+)/k (control) (A)	A (^2H_2O or DMSO)/A (H_2O)
		%	%	sec^{-1}		
1	H_2O, control	0.72		0.126		
	H_2O, 0.02 mM K^+	28.28	27.56	0.120	0.95	
	^2H_2O, control	14.35		0.147		
	^2H_2O, 0.02 mM K^+	41.91	27.56	0.170	1.16	1.23
	DMSO, control	0.49		0.004		
	DMSO, 0.02 mM K^+	23.21	22.72	0.080	20.08	21.24
2	H_2O, control	0		0.149		
	H_2O, 0.05 mM K^+	49.00	49.00	0.159	1.07	
	^2H_2O, control	0.01		0.119		
	^2H_2O, 0.05 mM K^+	36.17	36.16	0.184	1.55	1.46
3	H_2O, control	0.20		0.227		
	H_2O, 0.33 mM K^+	41.28	41.08	0.232	1.02	
	^2H_2O, control	10.94		0.178		
	^2H_2O, 0.33 mM K^+	49.48	38.54	0.272	1.53	1.49

[a] Values were obtained by extrapolation to the intercept at zero time.

observed difference due to ^2H_2O is close to that shown in the last column of Table 1 (experiment 3) and that the ratios (slope, K^+/slope, control) in H_2O and ^2H_2O are statistically different even at ± 2 standard deviations and at a probability level of 99% by Student's t-test.

Effect of ^2H_2O on p-nitrophenyl phosphatase at 4°. The previous observations (9, 10) on the effect of ^2H_2O on K^+-dependent p-nitrophenyl phosphatase were made under standard experimental conditions, employing an incubation temperature of 37°. On the other hand, the present work, dealing with the effect of K^+ on phosphoenzyme breakdown in the presence of ^2H_2O, was performed at 4°. Therefore it was of interest to determine whether ^2H_2O could also stimulate the K^+-dependent activity at 4°. Figure 2 shows that the stimulatory effect of ^2H_2O is apparent even at the reduced reaction temperature. The stimulatory effect of ^2H_2O was also observed in the presence of 0.33 mM NH_4^+, but not 0.33 mM Li^+ (data not shown).

Effect of ^2H_2O and DMSO on ADP-mediated breakdown of phosphoenzyme. It was previously shown that ^2H_2O inhibited the rate of Na^+-dependent ADP-ATP exchange and reduced the steady-state level of Na^+-dependent phosphoenzyme in the (Na^+ + K^+)-ATPase system (10, 15). It was

TABLE 2

Statistical analysis of intercepts and slopes of Fig. 1A

The standard deviations of the intercepts and slopes were derived by procedures given in ref. 19. The fiducial limits of the means were obtained by the t-test at a probability level of $p = 0.01$, and are shown in parentheses.

System	Intercept	Slope[a]	Slope with K^+/slope of control
H_2O	100.21 ± 1.08	−0.098 ± 0.012	
	(102.70, 97.72)	(−0.126, −0.070)	
H_2O + K^+	58.72 ± 1.10	−0.101 ± 0.008	1.022 ± 0.121
	(61.25, 56.19)	(−0.119, −0.083)	(1.30, 0.74)
^2H_2O	89.06 ± 1.03	−0.077 ± 0.005	
	(91.45, 86.67)	(−0.089, −0.066)	
^2H_2O + K^+	50.51 ± 1.05	−0.118 ± 0.008	1.525 ± 0.067
	(52.95, 48.07)	(−0.136, −0.100)	(1.68, 1.37)

[a] These values should be multiplied with 2.303 to compare with values of k in Table 1, expt. 3.

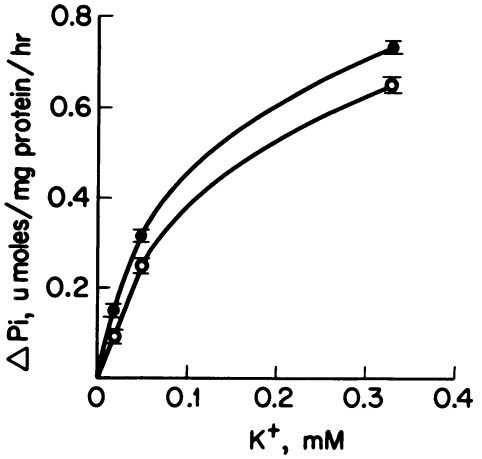

FIG. 2. *Effect of ^2H_2O on K^+-dependent p-nitrophenyl phosphatase activity at 4°*

The assay was performed as described under MATERIALS AND METHODS, except that the temperature was maintained at 4° and the concentrations of K^+ were varied as shown. Conditions for the time of reaction and the amount of enzyme present were established to yield sufficient hydrolysis of the substrate at linear rates of reaction. Appropriate controls to determine the basal Mg^{2+}-stimulated activity were included; this activity was subtracted from that obtained in the presence of Mg^{2+} plus K^+ to obtain the K^+-stimulated phosphatase activity. The standard errors of the means are shown. ○, control; ●, 90% ^2H_2O.

therefore of interest to examine the effect of ^2H_2O on the ADP-mediated breakdown of phosphoenzyme, i.e., the reverse of the phosphorylation step. ADP-stimulated phosphoenzyme breakdown displayed two different kinetic components (one measured within 1 sec, and the remaining, slower component measured between 1 and 4 sec following the chase with CDTA and ADP), which may indicate the presence of a sensitive and a relatively insensitive component in the ADP-mediated breakdown of phosphoenzyme. This observation is in agreement with that of Mårdh (20). Table 3 shows that ^2H_2O does not appear to alter the rate of the slower component of the reaction, but it was not possible to test its effects, if any, on the rate of the rapid component. However, the amount of dephosphorylation within the first measurable time point did not appear to be significantly affected by ^2H_2O.

Phosphoenzyme components sensitive to ADP and K^+. The foregoing results indicate that the phosphoenzyme contains a component which is relatively insensitive to ADP or K^+. The question arises whether the material which is insensitive to K^+ is sensitive to ADP, and vice versa. To examine this point, the previously formed phosphoenzyme (obtained under standard experimental conditions) was treated with a chase solution consisting of final concentrations of 3 mM CDTA, 5 mM ADP, and 0.33 mM K^+. This resulted in a reduction of phosphoenzyme to background levels within 2 sec, suggesting that the Na^+-dependent phosphoenzyme consists entirely of K^+- and ADP-sensitive components.

TABLE 3

Effect of ^2H_2O on ADP-mediated breakdown of phosphoenzyme

The conditions were the same as in experiment 2, Table 1, except that the chase was performed with CDTA (Tris salt) and ADP to give final concentrations of 3 mM CDTA and 5 mM ADP. The results are the means of two separate experiments. The values for the rapid component and the rate constants were obtained from linear least-squares fits to the data points.

Conditions	Loss in 1 sec	Increase due to ADP	k from relative plot	k (ADP)/k (control) (A)	A(^2H_2O)/A(H_2O)
	%	%	sec^{-1}		
H_2O, control	0		0.149		
H_2O, 5 mM ADP	37.53	37.53	0.262	1.76	
^2H_2O, control	0.01		0.119		
^2H_2O, 5 mM ADP	43.56	43.55	0.201	1.69	0.96

DISCUSSION

These results indicate that the phosphoenzyme hydrolysis initiated by the addition of K^+ follows a rapid, K^+-sensitive phase and a slower, K^+-insensitive phase. This biphasic nature of phosphoenzyme breakdown is unlikely to be an artifact due to contact of the phosphoenzyme molecules with higher concentrations of K^+ at the time of addition of the K^+-containing chase solutions. This view is supported by the finding that both 0.33 mM K^+ and 20 μM K^+ produced curves of similar shape; furthermore, in experiment 1 of Table 1, 25 μM KCl was added to achieve a 20 μM final concentration of K^+ during the chase. Mårdh (20) has also observed the presence of K^+-sensitive and K^+-insensitive components during dephosphorylation of the phosphoenzyme of bovine brain (Na^+ + K^+)-ATPase. Similarly, biphasic curves were obtained when the breakdown of phosphoenzyme was followed after chasing the reaction with ADP, suggesting the presence of relatively sensitive and insensitive components. On the other hand, when both ADP and K^+ were added together in the chase, the phosphoenzyme was completely hydrolyzed within the earliest time examined. Thus a possible explanation of the biphasic curves obtained in the presence of K^+ (or ADP) is that the Na^+-dependent phosphoenzyme formed in the absence of K^+ consists of a mixture of E_1-P (or K^+-insensitive, ADP-sensitive) and E_2-P (K^+-sensitive, ADP-insensitive) forms (21). As observed by Tobin *et al.* (22), rat brain (Na^+ + K^+)-ATPase appears to yield phosphoenzyme which consists of considerable amounts of both the E_1-P and E_2-P forms; it also appears that the proportions of these two types of phosphoenzyme in a given (Na^+ + K^+)-ATPase preparation may vary with the tissue and species (22).

The above experiments illustrate that in the presence of ^2H_2O the K^+-insensitive phase of phosphoenzyme breakdown after the addition of K^+, measured between 1 and 4 sec, becomes sensitive to K^+, as evidenced by a significant increase in the rate constant of hydrolysis of the phosphoenzyme in the presence of K^+ and ^2H_2O compared with K^+ and H_2O. The effect of ^2H_2O was the same during both the phosphorylation and dephosphorylation reactions, or when added only at the start of dephosphorylation during the chase experiment. Under similar experimental conditions, the K^+-dependent *p*-nitrophenyl phosphatase reaction was stimulated by ^2H_2O. Experiments with DMSO gave essentially the same results, although in this case, in agreement with others (23), stabilization of the phosphoenzyme was noted as well.

Our results indicate that the extent of the rapid component of phosphoenzyme breakdown (i.e., between 0 and 1 sec) observed in the presence of K^+, NH_4^+, or Li^+ was essentially unchanged in the presence of ^2H_2O or DMSO. For technical reasons, we could not determine the effect of these solvents on the rate of the rapid phase of phosphoenzyme hydrolysis following the addition of K^+ or ADP. Nonetheless, our

data clearly demonstrate that ^2H_2O and DMSO enhanced the K^+ sensitivity of the phosphoenzyme species which was insensitive to K^+ in H_2O. Previous studies have shown that ^2H_2O (9, 10), and DMSO (11, 12) increase the apparent affinity of K^+ for the K^+-dependent *p*-nitrophenyl phosphatase. To our knowledge, the present work is the first demonstration that ^2H_2O and DMSO enhance the sensitivity to K^+ of the phosphoenzyme intermediate; this may be related to their stimulation of the K^+-*p*-nitrophenyl phosphatase activity in the (Na^+ + K^+)-ATPase system. We have previously suggested that H_2O plays a role in putative conformational changes associated with Na^+ and K^+ interactions with the ATPase (10, 16), so that H_2O promoted the E_2 form whereas loss of H_2O promoted the E_1 form. This would imply that Na^+ interaction may be favored by the removal of H_2O from the active center, whereas K^+ interaction may be enhanced by the presence of H_2O. ^2H_2O and DMSO may act by increasing hydrophilicity in the region of the active phosphoenzyme intermediate. The effects of ^2H_2O described here are unlikely to be due to an isotopic substrate effect on the hydrolysis of the phosphoenzyme in which H_2O is involved; in that case ^2H_2O would be expected to retard the hydrolytic reaction. Rather, the solvent effects of ^2H_2O may cause conformational changes in the phosphoenzyme and/or the hydration of K^+ which result in modulation of interaction of K^+ with the phosphoenzyme. The arguments which favor conformational effects as the mode of action of these solvents on the (Na^+ + K^+)-ATPase, rather than simple effects on cation hydration alone, have been detailed previously (16).

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