Effects of Phlorizin on Membrane Cation-Dependent Adenosine Triphosphatase and *p*-Nitrophenyl Phosphatase Activities

JOSEPH D. ROBINSON

Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, New York 13210

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SUMMARY

Phlorizin stimulated the K^+-dependent phosphatase activity (measured with *p*-nitrophenyl phosphate) but inhibited the ($Na^+ + K^+$)-dependent adenosine triphosphatase activity in an enzyme preparation obtained from rat brain. The concentration of phlorizin required for half-maximal stimulation of the phosphatase was 0.05 mM, whereas the K_i for the ATPase was 0.06 mM. Of a series of hydroxylated aromatic compounds, phloretin alone also stimulated the phosphatase.

With the phosphatase, 0.03 mM phlorizin decreased the concentration of KCl required for half-maximal velocity ($K_{0.5}$) from 1.92 to 1.17 mM but had little effect on V_{max}. Na^+ inhibited the phosphatase, and phlorizin increased the K_i for NaCl, from 6 to 12 mM. With the ATPase, 0.1 mM phlorizin similarly decreased the $K_{0.5}$ for KCl, from 0.74 to 0.48 mM, and increased the $K_{0.5}$ for NaCl, from 5.0 to 10.5 mM. The positive cooperative allosteric response to Na^+, as indicated by the Hill plot with $n > 1$, was also converted to a negative cooperative response. In addition, phlorizin inhibited the Na^+-dependent phosphorylation of the enzyme.

These data indicate that phlorizin reacts similarly with the cation-dependent ATPase and phosphatase, serving as a heterotropic allosteric modifier to increase the apparent affinity toward K^+ but to decrease it toward Na^+. These findings further imply that the phosphatase represents an aspect of the over-all ATPase reaction. Finally, the data raise the possibility that the inhibition of sugar transport by phorizin and phloretin may be mediated in part through an effect on the Na^+-dependent aspects of that transport system.

INTRODUCTION

Phlorizin, a potent inhibitor of Na^+-dependent glucose transport (1), was shown by Wheeler and Whittam (2) to inhibit ($Na^+ + K^+$)-dependent ATPase activity in kidney cortex. Subsequently Fujita *et al.* (3) reported that although phlorizin inhibited the ($Na^+ + K^+$)-dependent ATPase activity in a brain preparation it also stimulated the K^+-dependent *p*-nitrophenyl phosphatase activity. These disparate effects of phlorizin might appear paradoxical, for considerable evidence has accumulated indicating that the K^+-dependent phosphatase represents the terminal hydrolytic step of the ATPase, following a Na^+-dependent phosphorylation of the enzyme (for reviews and summaries, see refs. 4–6).

To explore this apparent inconsistency, the effects of phlorizin and some related compounds on both the ATPase and the phosphatase were studied: the experiments show that, for both activities, phlorizin increased the apparent affinity for K^+ while decreasing it for Na^+. These results not only

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are consistent with the observed stimulation and inhibition of the two processes, respectively, but also provide further evidence for their fundamental relationship.

It appears unlikely that the inhibition of glucose transport by phlorizin involves a direct effect on the $(Na^+ + K^+)$-dependent ATPase; however, the inhibition of Na^+-dependent glucose transport by the aglycone of phlorizin, phloretin, suggests that the drugs might influence glucose transport through an alteration in the cation-associated aspects of transport. In this respect the phlorizin-induced decrease in affinity of the ATPase for Na^+ may reflect an analogous process between phlorizin or phloretin and the sugar transport apparatus.

METHODS

The $(Na^+ + K^+)$-dependent transport ATPase was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as described previously (7), except that the preparation was finally suspended in 0.01 M Tris-HCl (pH 7.8).

$(Na^+ + K^+)$-dependent ATPase activity was measured in terms of the production of P_i as previously described (7). The standard medium contained 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, 10 mM KCl, and the enzyme preparation (0.2 mg of protein per milliliter). Incubation was conducted for 5–10 min at 30°; activity was linear with time during this period. Activity in the absence of added Na^+ and K^+ ("Mg-ATPase") was measured concurrently; such activity averaged only a small percentage of the $(Na^+ + K^+)$-dependent activity under optimal conditions (7), and was subtracted from the total activity in the presence of Na^+ and K^+ to give the $(Na^+ + K^+)$-dependent activity.

K^+-dependent phosphatase activity was measured in terms of the production of *p*-nitrophenol after incubation with *p*-nitrophenyl phosphate, as described previously (6). The standard medium contained 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate (as the Tris salt), 10 mM KCl, and the enzyme preparation (0.2 mg of protein per milliliter). Incubation was carried out for 10–20 min at 30°; activity was linear with time during this period. Activity in the absence of added K^+ was measured concurrently; such activity averaged only a small percentage of the K^+-dependent *p*-nitrophenyl phosphatase activity under optimal conditions (6), and was subtracted from the total activity in the presence of K^+ to give the K^+-dependent activity.

Na^+-dependent phosphorylation of the enzyme was measured after incubation with γ-^{32}P-labeled ATP, by a modification of the procedures of Post *et al.* (8) and Gibbs

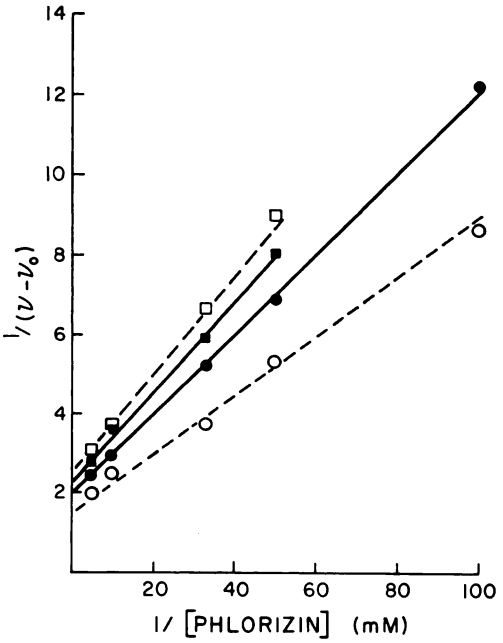

FIG. 1. *Effects of phlorizin on K^+-dependent phosphatase activity*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, and either 10 mM KCl (■——■), 3 mM KCl (○- - -○), 1 mM KCl (●——●), or 10 mM KCl plus 10 mM NaCl (□- - -□), with and without various concentrations of phlorizin. The increase in velocity due to phlorizin ($v - v_0$) was calculated and plotted in the double-reciprocal Lineweaver-Burk form. Velocity is expressed in units relative to the K^+-dependent *p*-nitrophenyl phosphatase activity of controls incubated concurrently in the standard medium, defined as 1.0.

et al. (9). The standard incubation medium contained 50 mM Tris-HCl (pH 7.8), 0.1 mM $MgCl_2$, 0.01 mM ATP containing tracer amounts of ^{32} P-ATP, 30 mM NaCl, and the enzyme preparation (0.4 mg of protein per milliliter). The enzyme was equilibrated at 0° with the incubation medium (in the absence of ATP), and the reaction was initiated by adding ATP; incubation was conducted for 20 sec at 0°. The reaction was stopped by adding trichloracetic acid (to a final concentration of 5%) containing unlabeled ATP, and the mixture was centrifuged. The precipitate was washed with trichloracetic acid and again centrifuged; after the supernatant material had been drained off and the insides of the tubes dried with cotton swabs, the precipitate was dissolved in 2 N KOH. Finally, the clear KOH solution, diluted to 1 N with water, was added to Bray's solution (10), and the radioactivity was measured with a liquid scintillation counter; quenching was monitored by the channels ratio method. Na^+-dependent phosphorylation was considered to be the difference in activity between incubation in the standard medium with NaCl and that of a concurrent incubation without NaCl (values were corrected for the ^{32}P content of zero time incubations).

p-Nitrophenyl phosphate and ATP were purchased from Sigma as the sodium salts, and were converted to the Tris salts. ^{32}P-ATP was synthesized enzymatically (11); $^{32}P_i$ was purchased from New England Nuclear Corporation. Protein was measured by the biuret method, using bovine serum albumin as a standard. All solutions were made in water that had been redistilled in an all-glass still.

Phlorizin was purchased from Calbiochem and ordinarily was used from freshly opened bottles without further purification; however, several series of representative experiments were performed with phlorizin that had been recrystallized after treatment with activated charcoal, and no difference in results was apparent. Phloretin and other compounds were freshly obtained from Nutritional Biochemicals Corporation and were used without further purification.

TABLE 1

Summary of the effects of phorizin on the kinetic parameters for K^+ and Na^+

This table summarizes the values of the kinetic parameters for K^+ and Na^+ in Figs. 2, 3, 6, and 7. V_{max} for the variable under consideration was estimated graphically from Lineweaver-Burk plots, and K_i from Dixon plots, and $K_{0.5}$ and n were calculated from Hill plots by the method of least squares. The statistical significance of variations in $K_{0.5}$ and n was calculated as described previously (7); the significance of variations in V_{max} and K_i was not estimated.

		K^+-dependent phosphatase		($Na^+ + K^+$)-dependent ATPase	
Cation	Kinetic parameter	Control	Phlorizin (0.03 mM)	Control	Phlorizin (0.1 mM)
K^+	$K_{0.5}$ (mM)	1.92	1.17[a]	0.74	0.48[a]
	V_{max}[b]	1.10	1.20	1.03	0.73
	n	1.44	1.74[a]	1.40	1.54[a]
Na^+	$K_{0.5}$ (mM)			5.0	10.5[a]
	V_{max}[b]			1.02	1.00
	n			1.28	0.46[a]
	K_i (mM)	6	12		
Na^+ + 10 mM KCl	$K_{-0.5}$ (mM)	32	32		
	$\|n\|$	1.46	1.07[a]		
Na^+ + 3 mM KCl	$K_{-0.5}$ (mM)	16	26[a]		
	$\|n\|$	0.96	1.01		

[a] Significantly different from control ($p < 0.05$).

[b] Relative units (see the text).

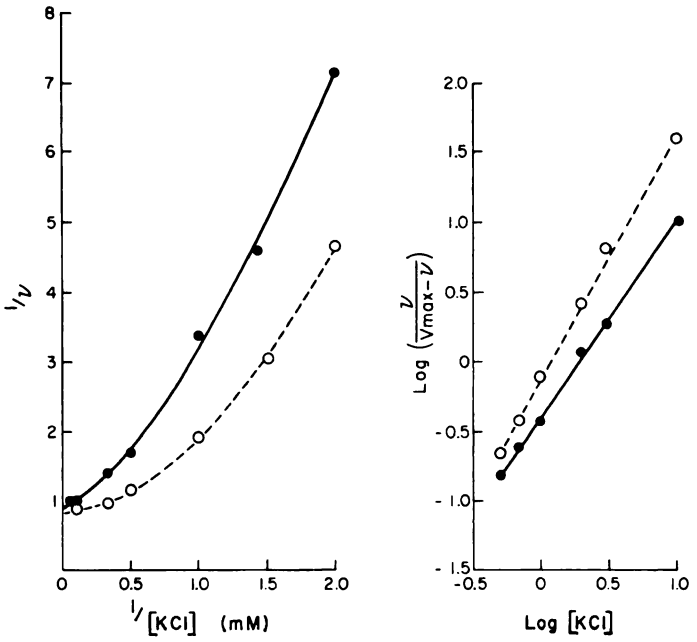

FIG. 2. *Effects of phlorizin on the response of the phosphatase to KCl*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, the concentration of KCl indicated, and either no phlorizin (●——●) or 0.03 mM phlorizin (O- - -O). Velocities are expressed in relative units, and are presented in the left-hand panel in the form of a Lineweaver-Burk plot, and in the right-hand panel as a Hill plot with straight lines drawn by the method of least squares.

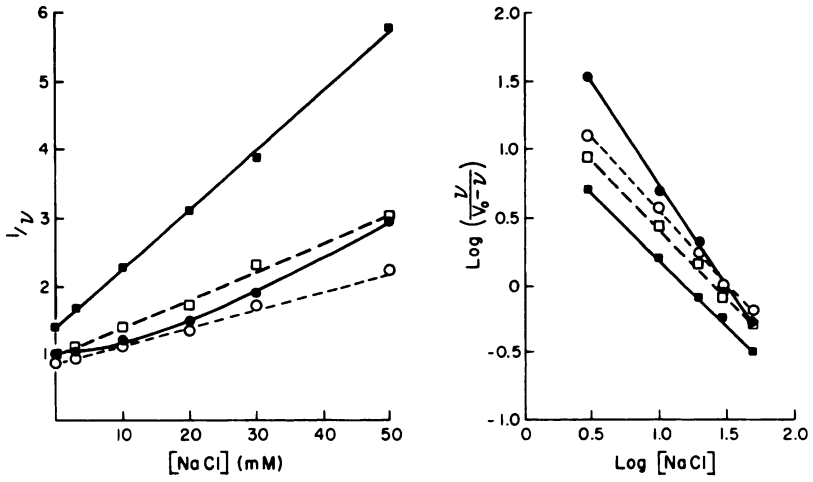

FIG. 3. *Effects of phlorizin on the response of the phosphatase to NaCl*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, the concentration of NaCl indicated, and either 10 mM KCl (●——●), 10 mM KCl plus 0.03 mM phlorizin (O- - -O), 3 mM KCl (■——■), or 3 mM KCl plus 0.03 mM phlorizin (□- - -□). In the left-hand panel relative velocities are presented as a Dixon plot, and in the right-hand panel as a Hill plot in terms of v_0, the velocity in the absence of NaCl.

Experimental points are the averages of five or more experiments performed in duplicate.

RESULTS

Effects of phlorizin on phosphatase activity. K^+-dependent *p*-nitrophenyl phosphatase activity averaged 0.048 μmole of *p*-nitrophenyl phosphate hydrolyzed per milligram of protein per minute in the presence of 10 mM KCl. Because of variations in the absolute activities of different enzyme preparations and a slow decline in activity during storage, enzyme velocities are expressed hereafter relative to the K^+-dependent activity of concurrent control incubations in the standard medium, defined as 1.0.

Phlorizin, over the range 0.003–0.2 mM, had no effect on the *p*-nitrophenyl phosphatase activity in the absence of K^+, but stimulated K^+-dependent activity. The relative increment in activity depended on the concentration of KCl in the medium; 0.03 mM phlorizin in the presence of 1 mM KCl stimulated activity by 64% while with 10 mM KCl it stimulated by only 17%. The concentration of the half-maximal increment in velocity at all concentrations of KCl was 0.05 mM (Fig. 1).

Phlorizin, 0.03 mM, increased the apparent affinity of the enzyme for K^+, changing the concentration of KCl required to produce half-maximal velocity ($K_{0.5}$) from 1.92 mM to 1.17 mM, whereas the V_{max} increased only from 1.1 to 1.2 relative units (Fig. 2 and Table 1). The slope of the Hill plot, n, which is a measure of the cooperative allosteric response, increased with phlorizin from 1.44 to 1.74 (Fig. 2 and Table 1).

NaCl, in the presence of optimal concentrations of KCl, inhibited the K^+-dependent phosphatase, and the response to Na^+ depended on the concentration of K^+ (6). With 0.03 mM phlorizin the K_i for NaCl, estimated graphically from Dixon plots (12), increased from 6 mM to 12 mM (Fig. 3 and Table 1). Furthermore, the cooperative response to Na^+ measured in the presence of 10 mM KCl was reduced by this concentration of phlorizin, with $|n|$ decreasing from 1.46 to 1.07 (Fig. 3 and Table 1), whereas the concentration of NaCl required to decrease the uninhibited velocity by one-

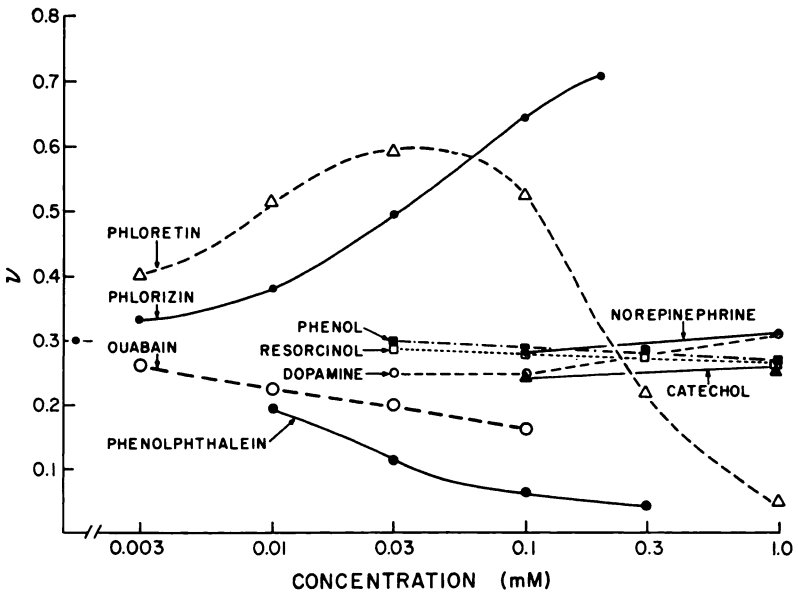

FIG. 4. *Effects of various compounds on K^+-dependent phosphatase activity*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, and 1 mM KCl, with and without the compounds indicated. Velocities are expressed in relative units.

half ($K_{0.5}$) was increased in the presence of 3 mM KCl from 16 to 26 mM.

The concentration of phlorizin giving a half-maximal increment in velocity in the presence of both 10 mM NaCl and 10 mM KCl was also 0.05 mM (Fig. 1).

Effects of related drugs on phosphatase activity. Phloretin also stimulated the K^+-dependent *p*-nitrophenyl phosphatase activity; the concentration that produced a half-maximal increment in activity was 0.01 mM. At concentrations above 0.1 mM, however, phloretin inhibited the phosphatase (Fig. 4). A number of related hydroxylated aromatic compounds had little effect in this concentration range, whereas phenolphthalein and ouabain were inhibitory (Fig. 4). The data presented are for experiments with 1 mM KCl; similar results were obtained with 10 mM KCl and with 10 mM KCl plus 10 mM NaCl.

Effects of phlorizin on ATPase activity. $(Na^+ + K^+)$-dependent ATPase activity averaged 0.60 μmole of P_i liberated per milligram of protein per minute in the presence of 90 mM NaCl plus 10 mM KCl (as with the *p*-nitrophenyl phosphatase, enzymatic velocities are hereafter expressed relative to the $(Na^+ + K^+)$-dependent activity of concurrent control incubations in the standard medium, defined as 1.0).

Phlorizin inhibited the ATPase, the inhibition being greater at low Na^+:K^+ ratios (Table 2). The K_i for phlorizin inhibition, estimated graphically from Dixon plots, was about 0.06 mM (Fig. 5), in close agreement with the concentration giving a half-maximal increment in velocity with the *p*-nitrophenyl phosphatase (Fig. 1). Phloretin also inhibited the $(Na^+ + K^+)$-dependent ATPase, with a K_i near 0.01 mM. Neither phlorizin nor phloretin measurably affected the $(Na^+ + K^+)$-independent ATPase.

As with the *p*-nitrophenyl phosphatase, phlorizin increased the apparent affinity for K^+: with 0.1 mM phlorizin the $K_{0.5}$ for K^+ decreased from 0.74 to 0.48 mM, although V_{max} also decreased from 1.03 to 0.73 relative units (Fig. 6 and Table 1). The slope of

TABLE 2

Effect of Na^+:K^+ ratio on inhibition of ATPase by phlorizin

$(Na^+ + K^+)$-dependent ATPase activity was measured during incubations in the standard medium (see METHODS) and in media containing the concentrations of NaCl and KCl shown. The percentage inhibition caused by 0.1 mM phlorizin is listed for the various Na^+:K^+ ratios.

NaCl concentration	KCl concentration	Na^+:K^+ ratio	Inhibition by phlorizin
mM	mM		%
90	1	90	8
20	1	20	16
10	1	10	26
90	10	9	28
10	10	1	39

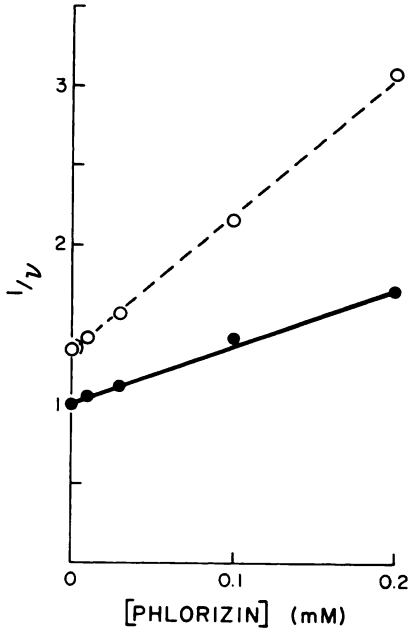

FIG. 5. *Effects of phlorizin on $(Na^+ + K^+)$-dependent ATPase activity*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM ATP, 3 mM $MgCl_2$, and either 90 mM NaCl plus 10 mM KCl (●——●) or 10 mM NaCl plus 10 mM KCl (○- - -○), with the concentration of phlorizin indicated. Velocities are expressed in units relative to the $(Na^+ + K^+)$-dependent ATPase activity of controls incubated concurrently in the standard medium, defined as 1.0, and the data are presented in the form of a Dixon plot.

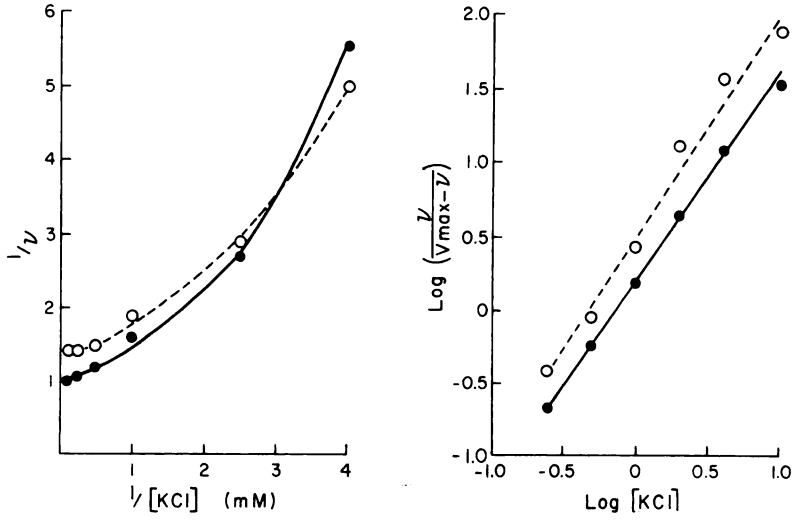

FIG. 6. *Effects of phlorizin on the response of the ATPase to KCl*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, the concentration of KCl indicated, and either no phlorizin (●——●) or 0.1 mM phlorizin (O- - -O). Velocities are expressed in relative units, and are presented in the form of a Lineweaver-Burk plot (left) and a Hill plot (right).

the Hill plot, n, was also slightly increased, from 1.40 to 1.54.

The $K_{0.5}$ for Na^+ was markedly increased from 5.0 to 10.5 mM, by the addition of 0.1 mM phlorizin, although V_{max} was scarcely affected (Fig. 7 and Table 1). The slope of the Hill plot was drastically reduced, n decreasing from 1.28 to 0.46.

Effect of phlorizin on Na^+-dependent phosphorylation. Na^+-dependent phosphorylation of the enzyme was decreased by 0.1 mM phlorizin to 79 ± 8% of control values, in accordance with the degree of inhibition of the ATPase by this concentration of phlorizin.

Reversibility of the effects of phlorizin.

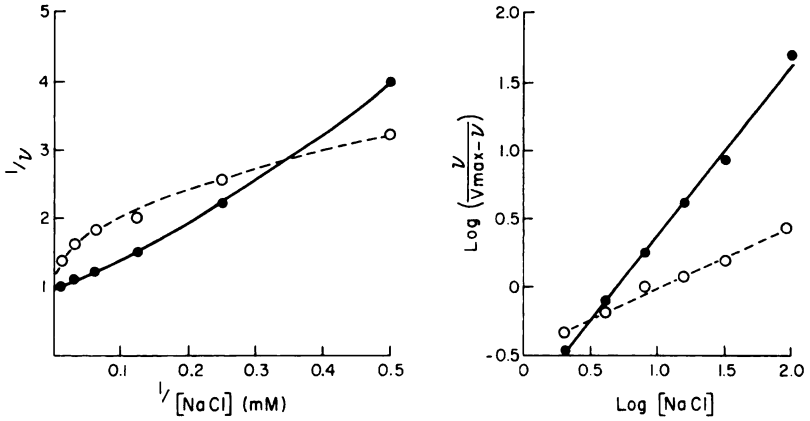

FIG. 7. *Effects of phlorizin on the response of the ATPase to NaCl*

The enzyme preparation was incubated in a medium containing 50 mM Tris-HCl (pH 7.8), 3 mM ATP, 3 mM $MgCl_2$, 10 mM KCl, the concentration of NaCl indicated, and either no phlorizin (●——●) or 0.1 mM phlorizin (O- - -O). Velocities are expressed in relative units, and are presented in the form of a Linew eaver-Burk plot (left) and a Hill plot (right).

To determine whether phlorizin was a reversible or irreversible modifier of the enzyme activities, experiments were performed to see if the effects of phlorizin would be removed by washing. Incubations were performed for 10 and 20 min at 0° and at 30° in the standard medium without subtrate (*p*-nitrophenyl phosphate or ATP) in the presence and absence of phlorizin (0.1 mM for the ATPase and 0.03 mM for the phosphatase). The incubation mixtures were then diluted with 10 volumes of 0.01 M Tris-HCl (pH 7.8), and the mixtures were centrifuged. The sedimented enzyme preparations were again washed with the same volume of buffer, centrifuged, and then resuspended. On incubation of these preparations under the standard conditions, no discernible effect of the prior incubation with phlorizin was apparent on the ATPase or phosphatase activities whether phlorizin was present or absent during the final incubation.

Furthermore, prior incubation of phlorizin for 10 or 20 min at 0° and at 30° with the enzyme in the standard medium (without substrate) did not alter the inhibition of the ATPase or the stimulation of the phosphatase measured after subsequent addition of substrate. Thus, no time dependence for the effects of phlorizin was apparent.

DISCUSSION

Previous kinetic studies (7, 13, 14) of the effects of monovalent cations on the $(Na^+ + K^+)$-dependent ATPase have been interpreted in terms of allosteric processes by which Na^+ and K^+ are both cooperative homotropic allosteric activators (15) of the enzyme. In addition, K^+-dependent phosphatase activity, which appears to represent the terminal K^+-dependent hydrolytic step for the ATPase (5, 6), also exhibits allosteric effects with monovalent cations: K^+ is a cooperative allosteric activator, but Na^+ is a cooperative allosteric inhibitor (6). The kinetic constants for the activators of the ATPase and the phosphatase differ, but the variation probably reflects the heterotropic allosteric effects (15) of the substrate, for incubation of nucleotides with the phosphatase can decrease the $K_{0.5}$ for K^+ markedly (6, 16).

Although phlorizin stimulated the phosphatase but inhibited the ATPase, it appeared to have a similar effect on both enzymatic activities: phlorizin may be interpreted as a heterotropic allosteric modifier, decreasing the $K_{0.5}$ for K^+ but increasing the $K_{0.5}$ (or $K_{-0.5}$ or K_i) for Na^+. Phlorizin also appeared to affect the cooperative response to Na^+ (both as an activator and as an inhibitor) as measured by the slope of the Hill plot, and for the ATPase it converted Na^+ from a positive to a negative (17) cooperative allosteric activator. These analogous effects on the apparent cation affinities of both enzymatic activities, as well as the similar concentrations required for half-maximal efficacy of stimulation or inhibition, also strengthen the argument relating the two activities (the inhibition of the Na^+-dependent phosphorylation by phlorizin is also consistent with this interpretation).

Of a series of related compounds, phloretin alone stimulated the phosphatase, implying a certain specificity for the interaction. Formby and Clausen (18) recently reported that high concentrations (1–10 mM) of dopamine and norepinephrine stimulated the activity of a brain phosphatase preparation under somewhat different experimental conditions. The inhibition by phenolphthalein of an ATPase preparation obtained from intestine was not associated with a change in K_m for Na^+ (19).

Furthermore, the effects of phlorizin on the apparent affinities of the ATPase toward cations may be relevant to its effect on sugar transport. Phlorizin is a competitive inhibitor of glucose transport in certain tissues (20, 21), implying an interaction between the glycoside portion of the molecule and the sugar transport site. However, phloretin, the aglycone of phlorizin, also inhibits sugar transport (although it is far less potent), and is not a competitive inhibitor (20, 21). Since the sugar transport inhibited by phlorizin is Na^+-dependent (1), the latter observations suggest that both phlorizin and phloretin may interfere with sugar transport by affecting cation-associ-

ated aspects of the mechanism (the far greater potency of phorizin would reflect its additional interaction through the glycoside group).

Monod *et al.* (15) suggested that allosteric enzymes may have arisen in evolutionary development through the association of originally distinct entities into polymeric complexes of catalytic subunits and regulatory subunits, the latter containing receptor sites for the allosteric modifiers. This formulation might be extended to the various Na^+-dependent systems involved in translocation of a number of different solutes: thus the Na^+-regulatory sites involved in Na^+-dependent transport at various cellular loci might represent similar subunits having a common ancestor. Then reagents (such as phlorizin) that influence Na^+ affinity at one site (e.g., the regulatory subunit of the sodium pump) might be expected to influence its homologue at another site (e.g., the regulatory subunit of the sugar pump).

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