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VITAMIN C—EFFECTS ON THE Na^+, K^+ ADENOSINE TRIPHOSPHATE PHOSPHOHYDROLASE COMPLEXES OF SEVERAL TISSUES*

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Abstract—The effects of ascorbate and dehydroascorbate on the Na^+, K^+-adenosine triphosphatase (ATPase) preparations of several tissues were studied. Both compounds inhibited the ATPase activities of the preparations obtained from the brains of rat, guinea pig and beef; but they did not affect the enzyme activities of the rat kidney, rat heart, beef heart and human red cells. Both ascorbate and dehydroascorbate produced half-maximal inhibition of the Na^+, K^+-ATPase and the K^+-dependent phosphatase activities of the rat brain enzyme at a concentration of 5×10^{-5} M. Maximum inhibition was obtained at 0·5 mM. Comparison of the inhibitory effects of the vitamin with those of ouabain and oligomycin indicated qualitative differences between the nature of the interactions of these inhibitors with the enzyme complex. The inhibited enzyme, obtained by preincubation with the vitamin, could not be reactivated by washing. Presence of histidine and EDTA did not prevent the inhibitory effects of the vitamin. Of the several simple lactones that were tested, only angelicalactone proved to be an inhibitor of the enzyme. These studies suggest a possible role of vitamin C in the control of active transports of Na^+ and K^+ in certain tissues.

THE DISCOVERY of the membrane-bound Na^+, K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) by Skou,[1] and the subsequent studies of many laboratories[2] have established that this enzyme complex is related to the energy-dependent translocations of Na^+ and K^+ across the plasma membrane. Cardiac glycosides are specific inhibitors of this enzyme activity and the associated active movements of Na^+ and K^+.[2] It has been known for a long time that the presence of an unsaturated lactone on carbon 17 of these glycosides is essential for their inhibitory effects on the Na^+, K^+-pump and the Na^+, K^+-ATPase, and that a variety of simple lactones (saturated and unsaturated) can also inhibit the active transport of Na^+ and K^+.[3] In view of these facts, we were interested to know if vitamin C (which is a lactone in both the oxidized and the reduced forms) inhibits the reactions and the ion translocations that are catalyzed by the Na^+, K^+-ATPase. A search of the literature revealed some previous observations on such effects of the vitamin. Kahn[4] reported that 20 mM ascorbate caused 50 per cent inhibition of active uptake of K^+ in cold-stored human red cells. In the course of studies on the effects of reducing agents on the Na^+, K^+-ATPase of the electric organ of the eel, Glynn[5] found that 10 mM ascorbate inhibited this activity by about 10 per cent. Later, Inagaki[6] reported that 0·1 mM ascorbate was sufficient to produce almost complete inhibition of the Na^+, K^+-ATPase activity of a crude microsomal preparation from rabbit brain. From these data, the need for the re-evaluation of the effects of ascorbate

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on the Na^+, K^+-ATPase became clear to us. In addition, it seemed desirable to determine: (1) whether the effects of ascorbate are due to its reducing properties; and (2) the similarities and differences between the effects of ascorbate and those of other well known inhibitors of the Na^+, K^+-ATPase.

MATERIAL AND METHODS

Na^+, K^+-ATPase complexes from rat kidney and the brains of rat, guinea pig and beef were prepared by the method of Skou.[7] The enzyme complex from beef or rat heart was made according to Matsui and Schwartz.[8] Haemoglobin-free membranes of human red cells were prepared by the method of Hoffman and Parker.[9] The specific activities of all preparations were similar to those described in the original literature. Unless otherwise stated, the mixture for the assay of ATPase activity contained: 50 mM Tris–HCl (pH 7·45), 3 mM $MgCl_2$, 1 mM EDTA, 2 mM ATP and appropriate amounts of enzyme and inhibitor, in a total volume of 2·5 ml. To determine the level of Na^+, K^+-dependent activity, 100 mM NaCl and 30 mM KCl were also included in the mixture. Usually, the reaction mixture without ATP was preincubated at 37° for 10 min, and the reaction was then initiated by the addition of the substrate. The reaction was terminated by the addition of 1·5 ml of 8% $HClO_4$. The deproteinized extract was then assayed for orthophosphate.[10] K^+-dependent phosphatase activity was measured by the previously described method, using umbelliferone phosphate as the substrate.[11]

Dehydroascorbate was prepared by a modification of the method of Roe and Kuether.[12] Activated charcoal (2·5 g) was added to 50 ml of a 1 per cent solution of ascorbic acid in 0·1 N acetic acid. The mixture was shaken vigorously for 20 min and filtered. This time was sufficient for the complete disappearance of ascorbate, as ascertained by the titration of the solution with 2,6-dichlorophenolindophenol.[13] To determine the yield of dehydroascorbate, an aliquot of the filtrate was reduced with H_2S[12] and titrated again with 2,6-dichlorophenolindophenol. Over 96 per cent of the ascorbate was recovered. This indicated that the treatment with charcoal had caused quantitative conversion of ascorbate to dehydroascorbate, and that no appreciable breakdown of dehydroascorbate had occurred.

The stock solution of dehydroascorbate in acetic acid, obtained by the above procedure, was neutralized with Tris and used in all experiments. This solution was freshly prepared each day.

ATP, ascorbic acid, ouabain, oligomycin, glucoronolactone and ribonolactone were obtained from Sigma Chemical Company (St. Louis, Mo.). All other lactones used in these studies were obtained from K & K Laboratories (Plainview, N.Y.). Umbelliferone phosphate was purchased from Isolabs, Inc. (Elkhart, Ind.). All common chemicals were of reagent grade quality.

RESULTS

Effect of vitamin C on the ATPase activities of various tissues. Enzyme preparations from the brains of rat, beef, and guinea pig were made, and the effects of ascorbate on the Na^+, K^+-ATPase activities were studied. Inhibitory effects of ascorbate similar to those reported by Inagaki[6] for rabbit brain enzyme were observed with all preparations. To determine the possible inhibitory effects of dehydroascorbate, commercial preparations of this compound were purchased and tested. Consistent results could not be obtained. Therefore, fresh preparations of dehydroascorbate were made and

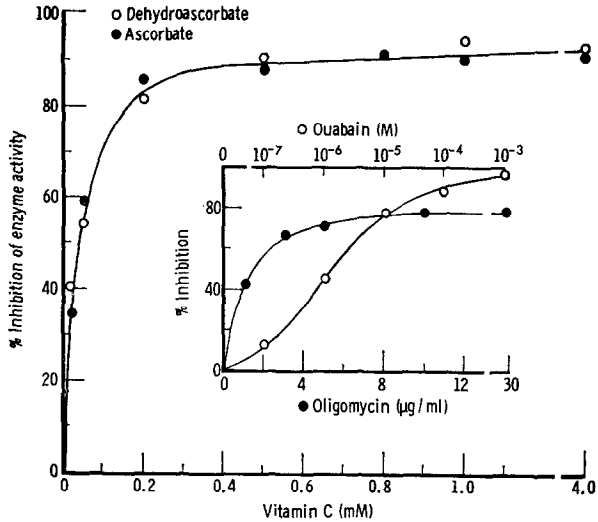

FIG. 1. Effects of varying concentrations of ascorbate, dehydroascorbate, ouabain and oligomycin on the Na^+, K^+-ATPase activity of rat brain. The enzyme was preincubated with appropriate inhibitor and assayed as described in Materials and Methods. The results are expressed as per cent inhibition of the Na^+, K^+-dependent activity. The specific activity of the enzyme under standard conditions was 85 μmoles Pi/mg protein/hr. About 90 per cent of this activity was dependent on the presence of Na^+ and K^+.

used. Figure 1 shows the effects of varying concentrations of ascorbate and dehydroascorbate on the Na^+, K^+-ATPase activity of the rat brain enzyme. For comparison, the effects of varying concentrations of ouabain and oligomycin on the activity of the same preparation are also shown in Fig. 1. The following points are evident from the data: (1) Ascorbate and dehydroascorbate have similar inhibitory potencies on the enzyme activity. (2) The Na^+, K^+-ATPase activity is not inhibited completely by either form of the vitamin, within the tested range of concentrations. (3) In its inability to produce complete inhibition of the activity, the vitamin seems to be acting similarly to oligomycin rather than ouabain.

Although not shown in Fig. 1, the Na^+, K^+-independent ATPase activity was also inhibited slightly by both forms of the vitamin. For example, at 1 mM ascorbate, 20 per cent inhibition of this activity was obtained. Results similar to those presented above were obtained with enzyme preparations from the brains of other species.

The effects of ascorbate and dehydroascorbate on the activities of the enzymes from beef heart, rat heart, rat kidney and human red cells were studied. Neither compound, at concentrations as high as 10 mM, showed any significant inhibitory effects on these preparations.

All of the remaining experiments described in the following sections were performed with the rat brain enzyme.

Effects of vitamin C on the K^+-dependent phosphatase. In addition to the hydrolysis of ATP, the Na^+, K^+-ATPase complex catalyzes several other reactions.[2] It has been established that all Na^+, K^+-ATPase preparations catalyze the K^+-dependent hydrolysis of some simple organic phosphates such as *p*-nitrophenylphosphate,[2] acetylphosphate[2] and umbelliferone phosphate.[11] Studies on these K^+-dependent phosphatase activities have been of value in demonstrating the different mechanisms of action

of a variety of inhibitors of the Na^+, K^+-ATPase complex. For example, oligomycin and oubain, which are both inhibitors of the Na^+, K^+-ATPase activity (Fig. 1) and the active transport of Na^+ and K^+, have been shown to have drastically different effects on the K^+-dependent phosphatase activity.[14–16] Therefore, it was of interest to compare the effects of vitamin C with those of oligomycin and ouabain on this activity. In Fig. 2, the effects of varying concentrations of ascorbate, oligomycin and ouabain on K^+-dependent umbelliferone phosphatase are shown. From these data and those of Fig. 1, it is evident that: (1) vitamin C, like ouabain and unlike oligomycin, produces

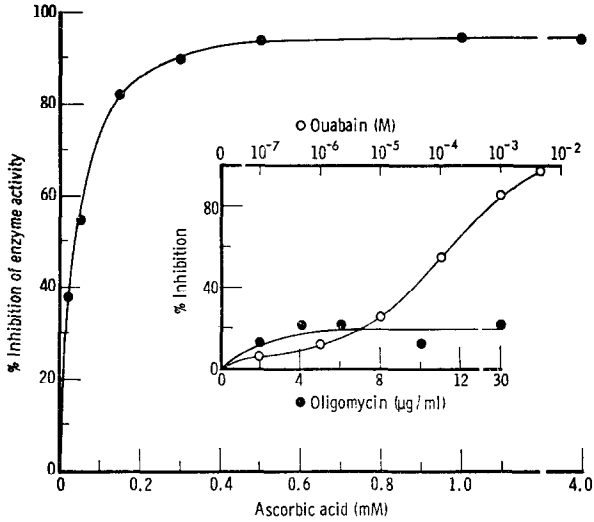

FIG. 2. Effects of varying concentrations of ascorbate, ouabain and oligomycin on the K^+-dependent umbelliferone phosphatase activity of rat brain. Assays were performed using 0·2 mM substrate and 10 mM K^+ by the procedure referred to in Materials and Methods. Specific activity of K^+-dependent phosphatase was 0·55 μmole umbelliferone/mg of protein/hr.

significant inhibition of K^+-dependent phosphatase; (2) the inhibitory potency of vitamin C is the same for both the ATPase and the phosphatase activities; (3) the equal sensitivities of the ATPase and the phosphatase activities to vitamin C are in contrast to the different sensitivities of these activities to ouabain. As originally observed by Fujita *et al.*,[17] and shown again in Figs. 1 and 2, the inhibitory potency of ouabain on the ATPase is greater than its potency on the phosphatase.

Effects of histidine and EDTA on vitamin C inhibition. Inhibitory and activating effects of ascorbate on a variety of enzymes have been reported in the past.[18] In many instances it has been demonstrated that such effects of ascorbate are exerted through the actions of traces of certain heavy metal ions.[18] Therefore, it was of interest to know whether the effect of vitamin C on the enzyme is in any way influenced by metal-complexing agents. The enzyme was preincubated with 0·5 mM ascorbate in the presence and absence of 5 mM histidine and 2 mM EDTA. Control enzyme (without ascorbate) was also preincubated in the same manner. Portions of enzyme samples were then removed at various times and assayed for Na^+, K^+-ATPase activity. The data are presented in Fig. 3. It is evident that both EDTA and histidine protect against the spontaneous inactivation of the control enzyme, but that they offer no protection

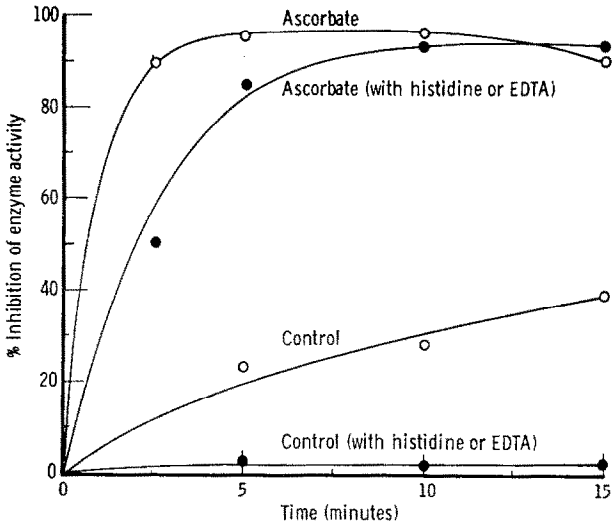

FIG. 3. Time course of ascorbate inhibition in the presence and absence of histidine and EDTA. Experimental conditions are described in text.

against the inhibitory effects of ascorbate. It is also evident that the inhibition of the enzyme by ascorbate is time-dependent. This is similar to the well known time-dependent effects of cardiac glycoside on the enzyme.[2]

Similar results were obtained with several other concentrations of ascorbate and dehydroascorbate.

Irreversibility of vitamin C inhibition. In recent years much work on the mechanism and nature of the binding of cardiac glycosides to the ATPase complex has been done.[2] We have not attempted to measure directly the binding of vitamin C to the enzyme. However, the following simple experiment was done. The enzyme complex was incubated with an inhibitory concentration of the vitamin. The complex was then centrifuged, washed several times, and assayed for Na^+, K^+-ATPase activity after each washing. The data presented in Table 1 show that the activity of the inhibited enzyme cannot be regenerated by this washing procedure.

TABLE 1. IRREVERSIBILITY OF VITAMIN C INHIBITION*

Enzymes	Na^+, K^+-ATPase activity of each pellet (% original activity) No. of washings				
	0	1	2	3	4
Control	100	98	85	80	83
With 0·4 mM ascorbate	10	12	8	6	5

* Two equal portions of the enzyme were mixed with solutions similar to those used for the assay of ATPase activity, in the absence of substrate. Ascorbate (0·4 mM) was added to one. The mixtures were incubated at 37° for 30 min and centrifuged at 10,000 *g* for 15 min. Each pellet was resuspended in the same solution (without ascorbate), a portion was assayed, and the remainder was centrifuged. The same procedure was repeated several times and ATPase activity was determined after each washing. The specific activity of the Na^+, K^+-ATPase prior to incubation and washing was 90 μmoles Pi released/mg of protein/hr.

Stability of vitamin C in the presence of the enzyme. Since both forms of the vitamin seemed to be effective inhibitors, the question arose as to whether either form could be converted to the other by the Na^+, K^+-ATPase complex. Ascorbate and dehydroascorbate were incubated with the enzyme under the same conditions used for the assay of ATPase activity. Possible generation or disappearance of ascorbate was measured by titration with 2,6-dichlorophenolindophenol.[13] No enzymic oxidation of ascorbate or reduction of dehydroascorbate could be detected.

Effects of other lactones. Several other simple lactones were tested for possible inhibitory effects on the Na^+, K^+-ATPase activity. At concentrations as high as 10 mM, the following did not inhibit the activity: β-propriolactone, γ-butyrolactone, γ-valerolactone, glucoronolactone and ribonolactone. Of the tested compounds only β-angelicalactone inhibited the enzyme within the tested range of concentrations. Maximal inhibition (85 per cent) was obtained at 8 mM, and half-maximal inhibition at 1·8 mM angelicalactone.

DISCUSSION

The studies presented here show that both the oxidized and the reduced forms of vitamin C, within the physiological range of concentrations, are inhibitors of the Na^+, K^+-ATPase complex of the brain of several species. The vitamin has no significant effect on the enzyme activities of other tested tissues. The reason for this apparent tissue specificity is not known. It should be pointed out, however, that different sensitivities of various preparations of Na^+, K^+-ATPase to inhibitors other than vitamin C have also been observed. For example, the rat heart enzyme is much less sensitive than the beef heart and the dog heart enzymes to the inhibitory effects of ouabain.[19] Also, in the same species (rat) the brain enzyme is about a hundred times more sensitive to ouabain than are the enzymes prepared from heart or kidney[19] (Fig. 1).

The similar effects of ascorbate and dehydroascorbate, and the inability of the enzyme to either oxidize or reduce the vitamin, clearly show that the redox properties of the vitamin are not related to its inhibitory effects on the transport enzyme system. On the other hand, the effectiveness of at least one other simple lactone and the fact that both cardiac glycosides and oligomycin contain lactone groups point to the possible significance of this structural feature of the vitamin. If the lactone ring is in fact involved in the interaction with the enzyme leading to inhibition, one must assume that other features of these inhibitor molecules influence not only the potency but also the nature of the interaction. For it is evident from the data of this paper and from previous comparisons between oligomycin and ouabain,[14–16] that the effects of vitamin C, cardiac glycosides and oligomycin on two activities (Na^+, K^+-ATPase and K^+-phosphatase) of the same enzyme preparation are qualitatively different.

Perhaps the most important question arising from these studies is: Does vitamin C indeed act as a regulator of the active movements of Na^+ and K^+ under physiological conditions? It should always be considered that agents which affect an enzyme system *in vitro* may not be effective in the intact cell. The possible effects of this vitamin on the activity of the Na^+, K^+-pump in the brain tissue remain to be ascertained.

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