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ASCORBIC ACID: AN ENDOGENOUS INHIBITOR OF ISOLATED Na^+,K^+-ATPase*

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Abstract—During attempts to isolate and identify an endogenous ligand for the glycoside binding sites on Na^+,K^+-ATPase, bovine adrenal glands were found to contain a potent inhibitor of isolated Na^+,K^+-ATPase. The inhibitory principle was extracted from adrenal cortex, following homogenization in $NaHCO_3$ solution and separation on a Sephadex G-10 column. The active principle was recovered from a fraction which eluted from the column after the ^3H_2O peak. The extract inhibited isolated Na^+,K^+-ATPase and the specific [^{3}H]ouabain binding reaction. Sensitivity of the enzyme to the inhibitory action of the extract was species and tissue dependent; however, the pattern and the magnitude of the sensitivity were different from those of the digitalis glycosides. Moreover, the inhibitory principle failed to inhibit sodium pump activity, estimated from ouabain inhibitable $^{86}Rb^+$ uptake by guinea pig brain slices. The activity of the extract to inhibit isolated Na^+,K^+-ATPase was stable under acidic condition but was lost rapidly at neutral pH, and could be eliminated by EDTA. In an acidic medium, the inhibitory principle had an absorption maximum at 244 nm which shifted to 264 nm and decayed rapidly at neutral pH. By using mass spectrometry, the principle was identified to be ascorbic acid, which has been shown previously to inhibit isolated Na^+,K^+-ATPase under appropriate conditions. Because ascorbic acid was incapable of inhibiting the sodium pump in intact cells, this inhibitor of the isolated enzyme does not appear to be the endogenous ligand which regulates sodium pump activity *in vivo*.

The digitalis glycosides specifically bind to Na^+,K^+-ATPase [1], indicating that this enzyme system has the receptor site which selectively recognizes pharmacologically active derivatives of these plant extracts. Glycoside binding results in enzyme and sodium pump inhibition, which has been proposed to increase intracellular Ca^{2+} transients in the cardiac muscle cells increasing the force of myocardial contraction [2–4].

The discovery of endogeous ligands for the opiate receptors [5] has provoked a search for endogenous ligands of the glycoside binding sites on Na^+,K^+-ATPase. If such a ligand exists, it could conceivably have a wide range of physiological functions, such as modulation of neurotransmitter release, stimulus-secretion coupling, renal salt excretion and vascular resistance, in addition to regulating the force of myocardial contraction. Several investigators reported that tissue or plasma extracts have "digitalis-like" activity [6]. More recently, an increase in the level of an endogenous digitalis-like principle in plasma has been reported in experimental hypertensive animals [7, 8] as well as in hypertensive patients [9]; however, isolation and identification of the endogenous principle have not been reported.

Adrenal gland synthesizes a variety of steroids and peptides and thus is a possible site for the synthesis of endogenous digitalis-like principles. Kölbel *et al.* [10] have reported that lipidic extracts of adrenal cortex have digitalis-like activity. In the present study, extracts from bovine adrenal glands have been found to inhibit isolated Na^+,K^+-ATPase. The active principle has been identified to be ascorbic acid. Although ascorbic acid is a potent inhibitor of isolated Na^+,K^+-ATPase under appropriate conditions, it does not meet the criteria of an endogenous ligand for the glycoside binding sites on Na^+,K^+-ATPase.

MATERIALS AND METHODS

Crude extracts. Fresh bovine adrenal glands obtained at the Meat Laboratory of Michigan State University were homogenized in 1 mM $NaHCO_3$ solution (1 ml/g tissue). The homogenate was kept at room temperature for 30 min. Subsequently, 2 vol. of 1 M HCl was added to the homogenate, and precipitated protein was centrifuged at 10,000 *g* for 30 min at 10°. The supernatant solution was kept frozen until use. This fraction was designated as a 1:4 dilution of the adrenal gland.

Column chromatography. The crude extracts were filtered through a Diaflo PM10 ultrafiltration membrane under pressure using an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA). The filtrate was passed through a chromatographic column (Pharmacia, Psicataway, NJ, 2.6 × 100 cm) of

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Sephadex G-10 (Sigma Chemical, St. Louis, MO) eluting with 10 mM HCl. The flow rate was adjusted to approximately 0.5 ml/min and 7.5-ml fractions were collected. [^{3}H]Inulin and ^3H_2O (New England Nuclear, Boston, MA) were used to calibrate the void and exclusion volume of the column respectively. The above procedures were performed at 10°.

Na^+,K^+-ATPase activity. Partially purified Na^+,K^+-ATPase preparations were obtained from the brain as described by Akera *et al.* [11] and from the heart and kidney by the method of Akera *et al.* [12]. EDTA was omitted in the last resuspension solution because EDTA was found to eliminate the inhibitory activity of the adrenal extracts. Protein concentration was assayed by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

Na^+,K^+-ATPase activity was assayed as described by Akera *et al.* [14] at 37° from the amount of inorganic phosphate released from ATP, with the following modification. The total incubation volume was reduced to 0.5 ml, and Mg^{2+}-ATPase activity was estimated using 0.1 mM ouabain to completely inhibit Na^+,K^+-ATPase activity; the Na^+,K^+-ATPase activity was calculated as the difference in activities observed in the absence and presence of 0.1 mM ouabain. The adrenal gland extract was preincubated with the enzyme preparation in the assay medium for 5 min at 37° in the absence of KCl, and was present during the entire incubation period. The Na^+,K^+-ATPase reaction was started by the addition of KCl (final concentration, 15 mM).

[^{3}H]Ouabain binding reaction. The specific binding of [^{3}H]ouabain to Na^+,K^+-ATPase obtained from bovine brain was estimated by incubating the enzyme preparation (0.01 mg protein/ml) in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5) and 5 mM Tris–ATP. After a 5-min preincubation at 37°, the binding reaction was started by the addition of [^{3}H]ouabain (final concentration, 100 nM). Bound and free [^{3}H]-ouabain were separated by filtering an aliquot of the incubation mixture through a nitrocellulose filter (Millipore Corp., Boston, MA; pore size, 0.8 μm). The filter was washed twice with 5 ml each of a 50 mM Tris–HCl buffer solution (pH 7.5) containing 0.1 mM ouabain and 15 mM KCl, and the radioactivity trapped on the filter (bound [^{3}H]ouabain) was estimated as described by Ku *et al.* [15]. Nonspecific binding of [^{3}H]ouabain was assayed concurrently in the presence of 0.1 mM ouabain, and this value was subtracted from the total binding, observed as above, to calculate specific [^{3}H]ouabain binding. The adrenal extract was added to the incubation mixture at the beginning of the preincubation.

$^{86}Rb^+$ uptake studies. Cortical slices were obtained from guinea pig brain by using a McIlwain tissue chopper (Brinkmann, Westbury, NY). The slices (0.5 mm thick) were incubated for 9 min for Na^+ loading in an ice-cold K^+- and Ca^{2+}-free Krebs–Henseleit bicarbonate buffer solution containing 118 mM NaCl, 27.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$ and 11.1 mM glucose [15]. The slices were then transferred to a K^+-free Krebs–Henseleit bicarbonate buffer solution which had the above composition plus 1.25 mM $CaCl_2$, 2 mM RbCl and tracer amounts of ^{86}RbCl (New England Nuclear). This medium was saturated with a 95% O_2, 5% CO_2 gas mixture. The slices were incubated at 37° for an additional 8-min period and subsequently rinsed three times in a cold K^+-free Krebs–Henseleit bicarbonate buffer solution containing 2 mM Rb^+. The slices were blotted dry, weighed, and assayed for radioactivity by using a gamma scintillation spectrometer. Nonspecific $^{86}Rb^+$ uptake was estimated in the presence of 0.1 mM ouabain and subtracted from the above value to calculate the specific (ouabain-inhibitable) [^{3}H]ouabain binding. The adrenal gland extract was added to both solutions used for Na^+ loading and $^{86}Rb^+$ uptake assay.

Mass spectrometry. Electron impact mass spectrometry was performed by direct insertion probe at 70 eV on a Finnigan 3200 mass spectrometer (Fin-

Table 1. Inhibition of Na^+,K^+-ATPase and Mg^{2+}-ATPase activities by crude adrenal gland extract

Adrenal gland extract	Dilution	Na^+,K^+-ATPase (μmoles/mg protein/hr)	Mg^{2+}-ATPase (μmoles/mg protein/hr)
None (control)		209.5	4.0
Whole adrenal extract	1:40,000	209.5	3.3
	1:10,000	201.5	5.3
	1:4,000	118.9	3.3
	1:1.000	85.3	9.2
None (control)		234.0	
Whole adrenal extract	1:300	82.8	
Adrenal cortex extract†	1:300	47.2	
Adrenal medulla extract†	1:300	195.3	

* ATPase activities of partially purified enzyme preparations obtained from guinea pig brain were assayed at 37°. Final protein concentration was 0.01 mg/ml. The crude extract was diluted with H_2O and added to the incubation medium, yielding the indicated final dilution. The ATPase reaction was started after a 5-min preincubation of the enzyme preparation in the presence of the extract.

† Adrenal cortex and medulla were visually separated, and crude extracts were prepared.

nigan Instrument, San Jose, CA) with Riber SADR data acquisition and control system (Delsi/Nermag, Houston, TX).

RESULTS

Na^+,K^+-ATPase inhibition by crude extracts. The ability of crude extracts of bovine adrenal gland to inhibit Na^+,K^+-ATPase was examined first. The crude extract obtained from the whole adrenal gland caused a concentration-dependent inhibition of Na^+,K^+-ATPase obtained from guinea pig brain (Table 1). Inhibition was specific to Na^+,K^+-ATPase activity: the crude extract had little effect on ouabain-insensitive Mg^{2+}-ATPase. When adrenal cortex and medulla were visually separated, and crude extracts were obtained separately, the extract obtained from the cortex caused a greater inhibition compared to that obtained from the adrenal medulla at equivalent dilutions.

Purification of the inhibitory principle. To further characterize the inhibitory principle, crude extract obtained from 20 g of bovine adrenal cortex was filtered through a Diaflo PM10 ultrafiltration membrane to remove large molecules and separated for the molecular weight using Sephadex G-10 column eluting with 10 mM HCl. Each 7.5-ml fraction was collected. The marker for the void volume, [^{3}H]-inulin, eluted in fraction 24, whereas the marker for the exclusion volume, ^3H_2O, was found in fraction 50. Each fraction was diluted 10-fold (final dilution) and assayed for its inhibitory effect on Na^+,K^+-ATPase using partially purified enzyme preparations obtained from guinea pig brain. Unexpectedly, inhibitory activity was recovered after the exclusion volume in fractions 54 to 61. Each of fractions 55 to 61 caused approximately 80% inhibition of enzyme activity whereas fraction 54 caused a 50% inhibition. These fractions were pooled and referred to as adrenal gland extract in the following studies. A slight inhibitory activity (approximately 10% inhibition) was found in fractions 48 and 49, i.e. immediately before the ^3H_2O peak. Inhibition by these fractions is probably caused by small ions, such as calcium which has been shown to inhibit Na^+,K^+-ATPase activity at relatively high concentrations [16].

Inhibition of various Na^+,K^+-ATPase preparations. One specific feature of Na^+,K^+-ATPase inhibition by the digitalis glycosides is that the affinity of the enzyme for a given glycoside is highly variable dependent on the source of enzyme [3]. To determine if the inhibitory principle from the adrenal gland had similar characteristics, its effect on Na^+,K^+-ATPase preparations obtained from several tissues was examined. Enzyme preparations obtained from guinea pig or rat brain had a high sensitivity to inhibition by the adrenal extract and those obtained from dog or guinea pig heart had a low sensitivity, whereas Na^+,K^+-ATPase preparations obtained from rat or bovine heart or guinea pig or bovine kidney had an intermediate sensitivity (Fig. 1). Although the results indicate source-dependent differences in Na^+,K^+-ATPase preparations for inhibitory action of the adrenal extract, this pattern of sensitivity was different from that which has been shown for the digitalis glycosides.

Inhibition of [^{3}H]ouabain binding. To find out if the endogenous principle interacts with Na^+,K^+-ATPase at the glycoside binding sites on the enzyme,

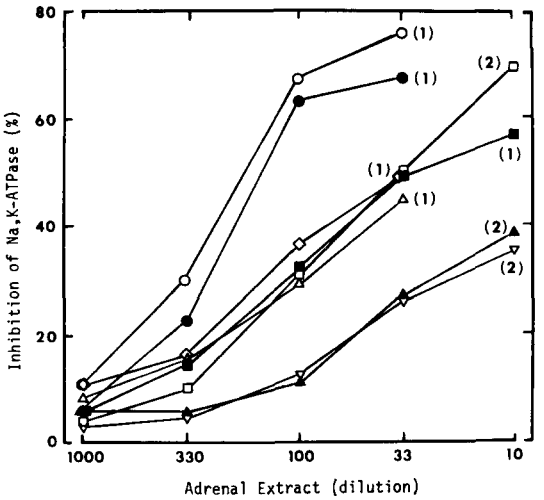

Fig. 1. Sensitivity of Na^+,K^+-ATPase obtained from different sources to inhibition by adrenal extract. Partially purified Na^+,K^+-ATPase preparations were obtained from various sources: (○) guinea pig brain, (●) rat brain, (□) rat heart, (■) bovine heart, (◇) bovine kidney, (△) guinea pig kidney, (▲) dog heart, and (▽) guinea pig heart. Na^+,K^+-ATPase activity was assayed at 37° after a 5-min preincubation with the extract. Enzyme protein concentrations were: brain enzymes, 0.01 mg/ml; heart enzymes, 0.1 mg/ml; and kidney enzymes, 0.04 mg/ml. Adrenal gland extract was diluted in 10 mM HCl. Numbers in parentheses indicate the number of enzyme preparations tested.

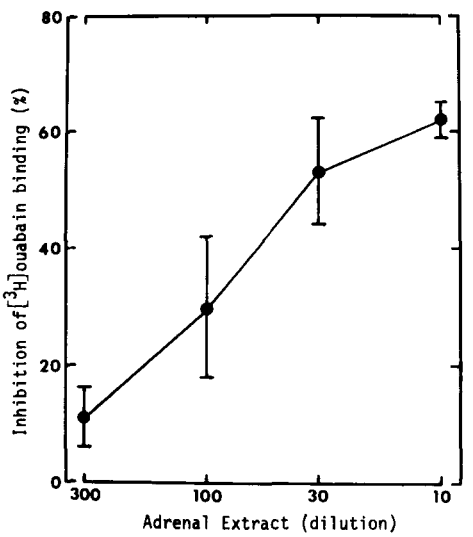

Fig. 2. Inhibition of the specific [^{3}H]ouabain binding by adrenal gland extracts. The specific binding of [^{3}H]ouabain (final concentration, 100 nM) to the Na^+,K^+-ATPase preparation (protein concentration, 0.01 mg/ml) obtained from bovine brain was estimated in the presence of 100 mM NaCl, 5 mM $MgCl_2$ and 5 mM ATP. Four different extracts were used in this study. Control value for the specific [^{3}H]ouabain binding was 31.8 ± 5.1 pmoles/mg protein. Vertical lines indicate S.E.

the ability of the extract to inhibit the specific [^{3}H]-ouabain binding reaction was examined. The specific binding of [^{3}H]ouabain to Na^+,K^+-ATPase preparations obtained from bovine brain was inhibited by the adrenal extract (Fig. 2), although the results do not positively indicate that the inhibitory principle and ouabain are competing at the same binding site.

Lack of sodium pump inhibition. When digitalis glycosides bind to Na^+,K^+-ATPase, they invariably inhibit sodium pump activity [3]. Therefore, the effect of the adrenal extract on sodium pump activity was examined in cortical slices obtained from guinea pig brain. Guinea pig brain slices were selected for these studies because guinea pig brain Na^+,K^+-ATPase had the highest affinity for the inhibitory principle among enzyme preparations tested in the present study. The capacity of the sodium pump, estimated from the ouabain-inhibitable $^{86}Rb^+$ uptake observed with Na^+-loaded slices, was markedly inhibited by 10 μM ouabain (Fig. 3). The pooled adrenal extract purified with Sephadex G-10 column, however, failed to inhibit specific $^{86}Rb^+$ uptake in concentrations up to 1:20 dilution (Fig. 3), although this concentration of the adrenal extract caused more than 70% inhibition of Na^+,K^+-ATPase isolated from guinea pig brain (Fig. 1). These results indicate that, unlike ouabain, the adrenal extract does not inhibit sodium pump activity.

Stability of the inhibitory principle. To determine the physical characteristics of the endogenous principle which inhibits isolated Na^+,K^+-ATPase, the stability of inhibitory activity was examined. When the adrenal extract was kept in 10 mM HCl at 37°, the ability of the extract to inhibit Na^+,K^+-ATPase isolated from guinea pig brain was unchanged up to 60 min (data not shown). The ability to inhibit Na^+,K^+-ATPase, however, disappeared rapidly with a half-life of approximately 14 min (mean of four experiments) when the pH value of the extract was adjusted to approximately 7.0 with a 50 mM Tris solution (data not shown). Thus, the endogenous inhibitory principle is stable in an acidic medium but is unstable at neutral pH.

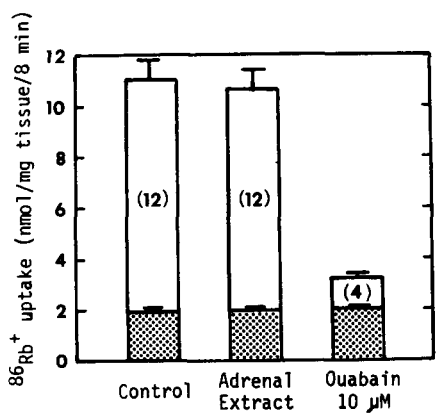

Fig. 3. Lack of the effect of adrenal gland extracts on $^{86}Rb^+$ uptake by cortical slices obtained from guinea pig brain. Cortical slices (approximately 50 mg wet weight) were incubated at 37° in the presence of either vehicle (0.5 mM HCl), adrenal extract (1:20 dilution) or ouabain (10 μM). Shaded bars, non-specific (ouabain-insensitive) uptake; open bars, specific (ouabain inhibitable) uptake. Numbers in parentheses indicate the numbers of experiments. Vertical lines represent S.E.

The ultraviolet absorption spectrum revealed that the adrenal extract purified with Sephadex G-10 column has a single peak at 244 nm when the spectrum was recorded in 10 mM HCl solution (data not shown). When the pH value of the medium was raised to 7.5 using 50 mM Tris solution, the absorption maximum shifted to 264 nm. The absorption at 244 nm observed in an acidic solution was stable whereas that at 264 nm observed in a neutral solution decreased rapidly with a half-life of approximately 15 min. These results strongly suggest that the observed ultraviolet absorption represents that of the principle which is capable of inhibiting isolated Na^+,K^--ATPase.

Elimination of enzyme inhibition by EDTA. The above results indicate that the endogenous inhibitory principle is unlikely to be a simple cation, such as Ca^{2+} or another multivalent metal ion. Nevertheless, the addition of EDTA to the incubation medium for enzyme activity assay immediately before the addition of adrenal gland extract unexpectedly attenuated the ability of the extract to inhibit isolated Na^+,K^+-ATPase (data not shown). In the absence of EDTA, a 1:200 dilution of the extract caused approximately 57% inhibition of guinea pig brain Na^+,K^+-ATPase. The enzyme inhibition was reduced slightly by 0.02 mM EDTA, reduced significantly by 0.1 mM EDTA and abolished almost completely by 0.2 mM EDTA. It seems therefore that Na^+,K^+-ATPase inhibition by the adrenal extract requires Ca^{2+} or other metal cations as a cofactor.

Identification of the endogenous principle by mass spectrometry. The ultraviolet absorption spectrum of the purified adrenal extract and its instability at neutral pH resembled those of ascorbic acid. That the inhibitory principle is, in fact, ascorbic acid was confirmed by its electron-impact (70 eV) mass spectrum. The spectrum was identical to that of the reference spectrum for authentic ascorbic acid [17], with characteristic ions at $M^+ = 176$, $m/z = 119, 116$ (base peak), 101, 100 and 85.

DISCUSSION

The present results indicate that crude extracts of bovine adrenal cortex contain a principle capable of inhibiting isolated Na^+,K^+-ATPase. A good correlation was observed between the degrees of enzyme inhibition (Fig. 1) and inhibition of the specific [^{3}H]ouabain binding reaction (Fig. 2). Inhibition of Na^+,K^+-ATPase activity was irreversible (data not shown). The inhibitory principle was relatively localized in the adrenal cortex. The inhibitory principle was eluted from a Sephadex G-10 column after the ^3H_2O peak, indicating an interaction of the principle with the column material. This characteristic made it possible to purify the inhibitory principle relatively easily.

The endogenous inhibitory principle was not "digitalis-like" for the following reasons: (1) K^+ inhibits the binding of digitalis glycosides to Na^+,K^+-ATPase. Therefore, digitalis-induced inhibition of

enzyme activity observed in the presence of K^+ is less than that of [^{3}H]ouabain binding which is usually observed in the absence of K^+ [3]. The inhibitory principle, however, inhibited the reactions under the two conditions to the same extent. (2) The inhibitory principle failed to affect sodium pump activity in a concentration which causes a substantial inhibition of isolated Na^+,K^+-ATPase. (3) The pattern of species- and organ-dependent differences in sensitivity of Na^+,K^+-ATPase for the inhibitory principle was distinctly different from that of the enzyme for the digitalis glycosides. Thus, the endogenous inhibitory principle did not meet the criteria for a "digitalis-like" interaction with Na^+,K^+-ATPase.

The present study clearly demonstrates that the inhibitory principle obtained from the adrenal cortex is ascorbic acid. Ascorbic acid was originally isolated from the adrenal cortex of ox [18], and has been shown to have an ultraviolet absorption peak at 245 nm in acidic solution and 265 nm in neutral solution. The amount of "reducing agent" (later identified to be ascorbic acid) found in the adrenal cortex was much higher than that in the adrenal medulla [18], consistent with the distribution of the inhibitory principle. It has also been shown that ascorbic acid is unstable at neutral pH, similar to the inhibitory principle. That the endogenous inhibitory principle obtained from the adrenal extract was, in fact, ascorbic acid was confirmed by mass spectrometry.

That ascorbic acid inhibits isolated Na^+,K^+-ATPase has been reported by several investigators [19–21]. Schaefer *et al.* [22, 23] studied effects of brain extracts on isolated Na^+,K^+-ATPase and concluded from indirect evidence that the inhibitory principle was ascorbic acid. These investigators and others have suggested that ascorbic acid inhibits Na^+,K^+-ATPase by causing peroxidative degradation of unsaturated phospholipids essential for the Na^+,K^+-ATPase activity [23–25]. The lipid peroxidation process requires the presence of membrane-bound metal ions, thus accounting for the antagonistic effect of EDTA on the inhibitory activity of the extract observed in the present study. Enzyme inhibition by the endogenous principle contained in the adrenal extract was irreversible (data not shown), similar to that reported for ascorbic acid [21].

The concentration of ascorbic acid in the present extract is estimated to be approximately 28 μM in a solution representing 20-fold dilution of the adrenal cortex based on the absorbance examined in the present study and reported absorbance value of authentic ascorbic acid at 245 nm [26]. It may be seen that the extract produces substantial enzyme inhibition at approximately 100-fold dilution (Fig. 1). Based on the above calculation, the concentration of ascorbic acid in an incubation medium representing a 100-fold dilution of the adrenal cortex is 5.6 μM. This value is substantially lower than the concentration of ascorbic acid (50 μM) reported to cause a 50% inhibition of Na^+,K^+-ATPase activity [18]; however, it is not possible to compare the potency of the inhibitory principle or ascorbic acid estimated under different assay conditions because inhibition of Na^+,K^+-ATPase by ascorbic acid is apparently irreversible, is most likely to be mediated by formation of lipid peroxidates, and is therefore affected by the presence of membrane-bound metal ions or EDTA. Under the present experimental conditions, 1 μM authentic ascorbic acid caused a 60.5% inhibition of Na^+,K^+-ATPase obtained from guinea pig brain (data not shown). Therefore, it seems that ascorbic acid contained in the adrenal extract can account for its ability to inhibit isolated Na^+,K^+-ATPase.

The reason for the apparent source-dependent differences in sensitivity of Na^+,K^+-ATPase to inhibitory action of the adrenal extract or ascorbic acid may be explained from the differences in distribution of unsaturated phospholipids among Na^+,K^+-ATPase of different sources. Frey *et al.* [21] reported that Na^+,K^+-ATPase from rat kidney, rat heart and beef heart was insensitive to ascorbic acid. In the present study, however, enzymes from rat and bovine heart were relatively sensitive to the inhibitory principle. This observation, too, is likely to result from differences in the composition and amount of lipids or trace metals contained in the enzyme preparations.

The inability of the adrenal extract or ascorbic acid to inhibit the sodium pump suggests that the inhibitory site(s) is inaccessible to the compound in intact cells when added to the incubation medium. Consistent with this finding, the adrenal extract failed to elicit a positive inotropic effect in isolated cardiac muscle preparations (data not shown).

Ascorbic acid is widely distributed in various tissues. We have isolated apparently the same inhibitory principle from brain and skeletal muscle of guinea pigs (data not shown). Our findings therefore raise a question whether enzyme inhibitory activity of endogenous substances reported by other investigators as "digitalis-like" principle could be accounted for by ascorbic acid. This possibility has never been examined.

Recently, arachidonic acid or related unsaturated fatty acids have been shown to inhibit Na^+,K^+-ATPase, [^{3}H]ouabain binding and the ouabain-sensitive $^{86}Rb^+$ uptake [27]. Again, whether these compounds are truly "digitalis-like" has not been examined. Inhibition of Na^+,K^+-ATPase activity or [^{3}H]ouabain binding observed with isolated enzyme preparations, relatively specific localization, and differences in sensitivity of the enzyme dependent upon source of the enzyme may not be sufficient to allow a conclusion that an inhibitor is digitalis-like.

In conclusion, ascorbic acid is a potent Na^+,K^+-ATPase inhibitor which can be easily isolated from animal tissues. Due to the ubiquitous nature of this compound, care must be taken to ascertain that ascorbic acid is not the major principle or a contamination of an extract claimed to be endogenous digitalis-like substance. Ascorbic acid does not meet the criteria for digitalis-like activity with respect to its interaction with Na^+,K^+-ATPase.

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