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ASCORBIC ACID-LIKE EFFECT OF THE SOLUBLE FRACTION OF RAT BRAIN ON ADENOSINE TRIPHOSPHATASES AND ITS RELATION TO CATECHOLAMINES AND CHELATING AGENTS

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Abstract—The inhibitory effect of the soluble fraction of rat brain on the Na^+, K^+-ATPase, Mg^{2+}-ATPase and K^+-*p*-nitrophenylphosphatase activities of rat brain subcellular particles and its antagonization by catecholamines have been studied. The soluble fraction could be replaced by L-ascorbic acid in the process, but dehydroascorbic acid or cytoplasmic reducing agents such as L-cysteine and L-glutathione did not show the same effect. Catecholamines could be replaced by chelating agents; EDTA, EGTA, *o*-phenanthroline and α,α′-dipyridyl prevented the inhibition by the soluble fraction and by L-ascorbic acid. The inhibition of the enzyme activities was not prevented by catecholamines or chelators when they were added after preincubation of the enzyme preparation in the presence of soluble fraction or of L-ascorbic acid. The effect of EGTA was eliminated by an excess of Ca^{2+} and the effect of EDTA by an excess of Ni^{2+}. Excesses of Ca^{2+}, Mn^{2+} and Co^{2+} did not influence the effect of EDTA. It is supposed that a reoxidizable heavy metal bound to the membrane structures is responsible for the inhibitory effect of the soluble fraction and of L-ascorbic acid. Catecholamines may act by chelating this metal. The possible role of iron in the phenomenon has been investigated and discussed.

THE PARTICULATE fractions of the brain are very rich in both Mg^{2+}-dependent, Na^+,K^+-stimulated ATPase[1] activity and ATPase activity stimulated by the divalent cations, Mg^{2+} or Ca^{2+}. Considering the role of Na^+,K^+-ATPase activity in active cation transport through the cell membrane,[1–4] this enzyme may be very important in the function of neuromembranes. The synaptosomal uptake of certain neurotransmitters related to Na^+,K^+-ATPase function,[5–8] and the activity of this enzyme constitute an important regulatory system of the energy metabolism of the brain.[9–10]

The significance of ATPase activity stimulated by divalent cations in the nervous tissue has not been revealed. According to certain concepts, the Mg^{2+} or Ca^{2+} stimulated ATPase activity in synaptic vesicles and the synaptosomal actomyosin-like ATPase activity may play a role in the storage or release of transmitters.[11–13]

The sum of Na^+,K^+-ATPase activities measured in the individual brain fractions is much higher than that measured in the original homogenate;[14] furthermore after removal of the soluble fraction the specific activities of ATPases in certain particulate fractions increase to a degree greater than can be explained by the removal of the soluble proteins.[11] It was supposed that the specific activities measured were influenced by endogenous inhibitors[15] or structural configurations.[16]

We have demonstrated recently[17] that the soluble fraction of the rat brain contains a heat-stable, dialyzable substance inhibiting both Na^+,K^+- and Mg^{2+}-ATPase activity, the effect of which can be antagonized by catecholamines. This suggested that the ATPase activities of certain membrane structures are regulated by endogenous substances in the brain. Further experiments revealed that the effect of the endogenous inhibitor and of catecholamines was influenced by certain tranquilizers.[18,19] It was of interest therefore to investigate the mechanism of the phenomenon in detail, and to characterize the inhibitory compound in the soluble fraction.

MATERIALS AND METHODS

CFE rats of both sexes weighing 130–180 g were used in these experiments. Microsomes and nerve ending membranes were isolated from whole brain and a partially purified Na^+,K^+-ATPase preparation was obtained.

Microsomes were prepared according to the cell fractionation method of De Robertis *et al.*[20,21] with the modification that the homogenizing solution did not contain any Ca^{2+} and the second centrifugation was carried out at 10,000 *g* instead of 11,500 *g*. The 10,000 *g* supernatant was centrifuged at 100,000 *g* for 1 hr. The pellet, containing the microsomes was washed once with 0·32 M sucrose and was then suspended in 0·32 M sucrose, the protein content of the final suspension being 0·6–0·9 mg/ml. Nerve ending membranes were isolated by sucrose density gradient centrifugation, as described by De Robertis *et al.*,[22,23] from the M_1 fraction isolated by centrifugation at 20,000 *g* after osmotic shock of the crude mitochondrial fraction. Fractions M_1 (0·9), M_1 (1·0) and M_1 (1·2) were collected; the solution was diluted to a final sucrose concentration lower than 0·32 M and the particles were isolated by centrifugation at 30,000 *g* for 30 min; the particles were then suspended in 0·32 M sucrose so that the suspension contained 0·30–0·50 mg of protein per ml.

Na^+,K^+-ATPase preparations were obtained by cell fractionation in the presence of desoxycholate, histidine and EDTA, as described by Skou.[24] After the desoxycholate-histidine-EDTA treatment of the pellet obtained by the second centrifugation at 20,000 *g* for one hour the particles were sedimented by centrifugation at 100,000 *g* for 50 min and were then suspended in 0·32 M sucrose. The suspension contained 0·20–0·30 mg of protein per ml. Preparations were stored at −4°.

The soluble fractions used in the experiments were the primary and the submitochondrial soluble fractions obtained by the cell fractionation method of De Robertis *et al.*[20,21] In the experiments on the microsomal fraction and Na^+,K^+-ATPase preparation the primary soluble fraction was used, while in those on the membrane preparation isolated from the submitochondrial fractions, the submitochondrial soluble fraction was used.

ATPase activities were measured as described previously.[19] In the present experiments Na_2ATP was used, so that Mg^{2+}-ATPase activities were measured in the presence of 1 mM ouabain.

K^+-*p*-Nitrophenylphosphatase activity was measured according to Albers *et al.*[14]

Unless otherwise stated, the system for enzyme activity measurements contained all the test compounds tested during preincubation without the substrate.

Iron was determined by the spectrophotometric measurement of the dipyridyl-Fe^{2+} complex (530 nm) and the *o*-phenanthroline-Fe^{2+} complex (510 nm) formed in

the reductive medium.[25,26] The system for the determination of the Fe^{3+} content of the ATP preparations contained 10 mM ATP, 1 mM ascorbic acid, 200 mM Tris–HCl (pH 7·4) and 1 mM 2,2′-dipyridyl or *o*-phenanthroline. Determination of Fe^{3+} was carried out by means of a calibration curve which was based on the substitution of ATP for Fe^{3+} in the same system.

Estimation of ascorbic acid was carried out by the dinitrophenylhydrazine method of Roe and Kuether.[27]

Protein was determined by the method of Lowry *et al.*[28]

The materials were obtained from the following sources: ATP disodium, Reanal, Budapest or Sigma, St. Louis; *p*-nitrophenylphosphate disodium, Reanal, Budapest; L-ascorbic acid, Merck, Darmstadt; L-cysteine, Reanal, Budapest; dehydroascorbic acid, Koch-Light, Bucks; L-glutathione, Reanal, Budapest; dopamine hydrochloride, Sigma, St. Louis; EDTA (ethylenediaminetetra-acetic acid), Reanal, Budapest; EGTA (ethyleneglycolbis aminoethylether)-tetra-acetic acid), Calbiochem, Luzern; α,α′-dipyridyl, Reanal, Budapest; *o*-phenanthroline, Reanal, Budapest; ouabain (G-strophanthine), Fluka, Buchs; 2,4-dinitrophenylhydrazine, Reanal, Budapest.

RESULTS

Table 1 shows the total, Na^+,K^+- and Mg^{2+}-ATPase activities of three particulate fractions isolated from rat brain, both in the presence and in the absence of the soluble fraction. The Mg^{2+}-ATPase activity of the microsomal fraction was somewhat higher than the Na^+,K^+-ATPase activity. In the membrane preparation obtained from the crude mitochondrial fraction, Na^+,K^+-ATPase activity was increased compared with Mg^{2+}-ATPase activity, and the specific Na^+,K^+-ATPase activity was also higher than that of the microsomal fraction. A preparation having an even higher Na^+,K^+-ATPase was obtained if desoxycholate, histidine and EDTA were used in the cell fractionation according to the method described by Skou[24] (partially purified Na^+,K^+-ATPase).

In the presence of the soluble fraction, the ATPase activities of each of the three preparations were inhibited. Under the conditions presented in Table 1 and in the case of the above preparations, the extent of inhibition was markedly higher than in our earlier experiments.[17,19]

TABLE 1. THE ATPASE ACTIVITIES OF BRAIN PREPARATIONS IN THE PRESENCE AND ABSENCE OF THE SOLUBLE FRACTION*

Preparation	Protein (mg)†	Soluble fraction‡	Total ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase
Microsomes	0·154 ± 0·012	–	10·88 ± 0·14	6·02 ± 0·11	4·86 ± 0·12
		+	5·96 ± 0·15	4·22 ± 0·10	1·74 ± 0·07
Nerve ending membranes	0·078 ± 0·008	–	27·72 ± 0·38	7·50 ± 0·26	20·29 ± 0·47
		+	16·00 ± 0·35	6·42 ± 0·22	9·58 ± 0·25
Partially purified Na^+,K^+-ATPase	0·055 ± 0·006	–	41·65 ± 0·24	8·63 ± 0·12	33·03 ± 0·37
		+	11·46 ± 0·53	3·89 ± 0·11	7·57 ± 0·57

* ATPase activities are expressed as μmoles P_i/mg protein/15 min. Means of five experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

† Protein content of the preparations in 2 ml reaction mixture. Means ± S.E.

‡ 0·2 ml of the original soluble fraction (see methods) in 2 ml reaction mixture. Inorganic phosphate liberated by the soluble fraction was subtracted.

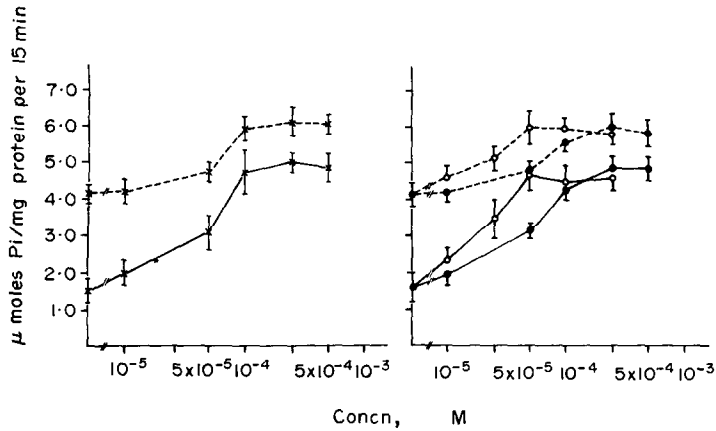

FIG. 1. Effects of dopamine (×), α,α'-dipyridyl (●) and *o*-phenanthroline (○) on the microsomal ATPases in the presence of the soluble fraction. Continuous line, Na^+,K^+-ATPase activity; broken line, Mg^{2+}-ATPase activity. Means of three experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

The Na^+,K^+-ATPase activity of each fraction was inhibited by more than 50 per cent. The sensitivity of Mg^{2+}-ATPase activity to inhibition by the soluble fraction was different for each preparation; the membrane preparation was inhibited by 14 per cent, the microsomal fraction by 30 per cent, while the sensitivity of the Mg^{2+}-ATPase activity of the Na^+,K^+-ATPase preparation was similar to that of Na^+,K^+-ATPase activity: an inhibition of 55 per cent was measured.

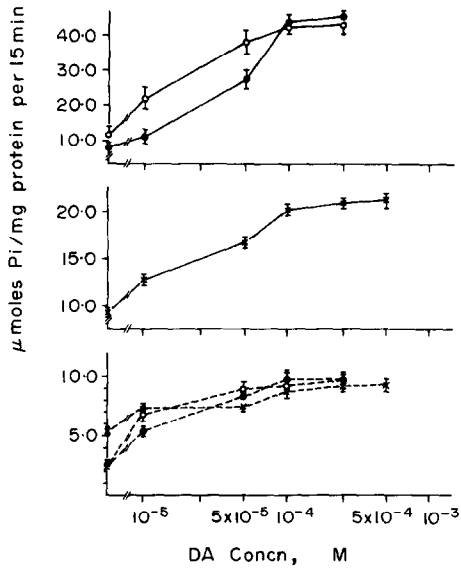

FIG. 2. Effect of dopamine on the ATPases of the nerve ending membrane preparation and on the Na^+, K^+-ATPase preparation in the presence of the soluble fraction. (×) Nerve ending membrane preparation, Reanal ATP was used. (●) Na^+,K^+-ATPase preparation, Reanal ATP was used. (○) Na^+,K^+-ATPase preparation, Sigma ATP was used. Continuous line, Na^+,K^+-ATPase activity; broken line Mg^{2+}-ATPase activity. Means of three experiments in duplicate ± S.E.

Under these experimental conditions, the catecholamine concentration necessary to antagonize the inhibition was also higher than in our earlier experiments. In each preparation 10^{-4} M dopamine (DA) was needed to achieve maximal activity in the presence of the soluble fraction (Figs. 1 and 2). When using Sigma ATP instead of Reanal ATP (Fig. 2), DA was much more effective in lower concentrations and at 5×10^{-5} M the activity measured was nearly maximal. In the case of the membrane and Na^+,K^+-ATPase preparations the maximum values measured in the presence of DA and of the soluble fraction were slightly greater than in the absence of the soluble fraction and in the presence of DA. In the absence of the soluble fraction the ATPase activities of the microsome and membrane preparations were not influenced by DA; the Na^+, K^+-ATPase activity was increased by DA, even in the absence of the soluble fraction (from 39·97 to 46·13). Later this could often be observed with the microsome preparation and also with chelators.

Figure 1 shows the effect of two chelators, in addition to DA, on the microsomal ATPase activities. These compounds, similar to catecholamines, antagonized the inhibitory effect of the soluble fraction. The concentration dependence of the effect of α,α'-dipyridyl was very similar to that of DA; *o*-phenanthroline, however, was effective at a lower concentration. It is noteworthy that the changes in the Na^+,K^+- and Mg^{2+}-ATPase activities due to any of the three compounds were proportional. A similar effect was obtained also by EDTA and EGTA. The effect of EGTA was eliminated by an excess of Ca^{2+} (Fig. 3).

Thus, in these circumstances catecholamines can be substituted for chelators. The following experiments show that an inhibition similar to that produced by the soluble fraction, antagonized by catecholamines or chelators, can be brought about by L-ascorbic acid and under certain conditions, by L-cysteine.

Ascorbic acid inhibits ATPase activity very effectively (Fig. 4); at a concentration of 10^{-5} M, the inhibition of Na^+,K^+- and Mg^{2+}-ATPase activities was similar to

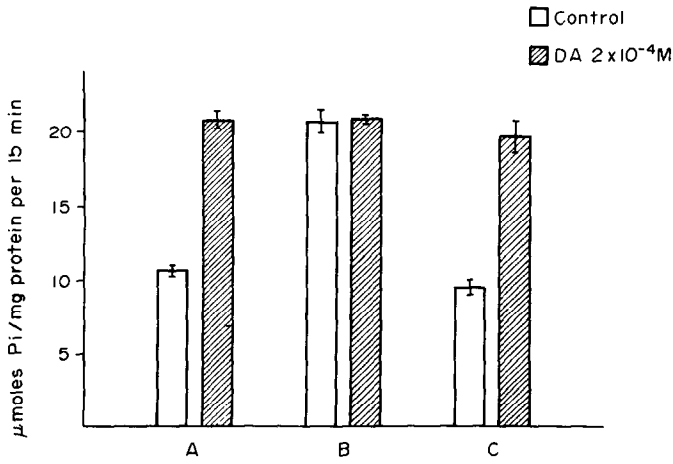

FIG. 3. Effects of EGTA and dopamine on the Na^+,K^+-ATPase activity of the nerve ending membrane preparation in the presence of the soluble fraction. A, Without EGTA; B, with 2×10^{-4} M EGTA; C, with 2×10^{-4} M EGTA and $2{\cdot}5 \times 10^{-4}$ M Ca^{2+}. The enzyme activity was not affected significantly by 5×10^{-5} M Ca^{2+} in the absence of the soluble fraction. Means of four experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

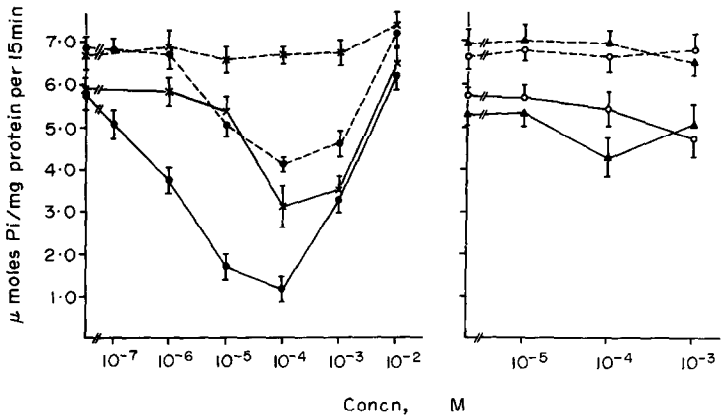

FIG. 4. Effects of L-ascorbic acid (●), dehydroascorbic acid (○), L-cysteine (×) and L-glutathione (▲) on the microsomal ATPases. Continuous line, Na^+,K^+-ATPase activity; broken line, Mg^{2+}-ATPase activity. In the presence of the soluble fraction values of 2·09 ± 0·18 and 4·82 ± 0·32 were measured for Na^+,K^+-ATPase activity and Mg^{2+}-ATPase activity respectively. Means of three experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

that produced by the soluble fraction. The Na^+,K^+-ATPase activity was inhibited also by L-cysteine. The effect was less than that of ascorbic acid and the soluble fraction. Both compounds were ineffective at high concentrations. Cysteine had no effect on Mg^{2+}-ATPase activity. Glutathione or dehydroascorbic acid did not have such a strong effect as cysteine or ascorbic acid, though in the presence of 10^{-4} M glutathione or 10^{-3} M dehydroascorbic acid a slight inhibition of Na^+,K^+-ATPase activity was observed.

At concentrations higher than 10^{-3} M it was necessary to neutralize the ascorbic acid with Tris-buffer in order to keep the pH value of the reaction mixture at 7·4. Previous neutralization did not influence the effect of ascorbic acid at lower concentrations.

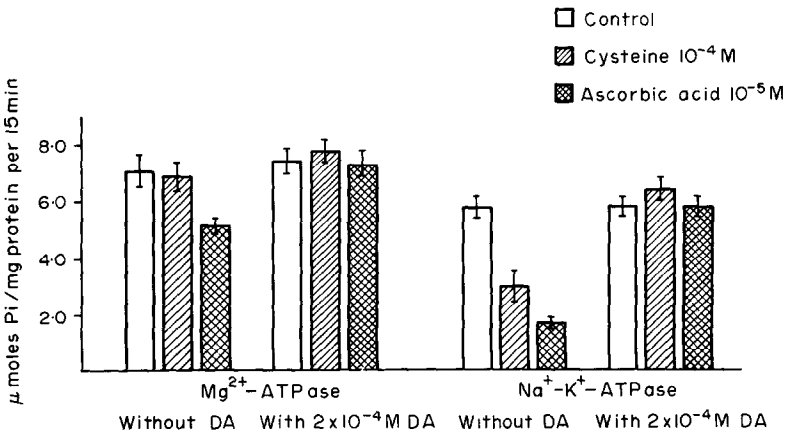

FIG. 5. Effect of dopamine on the microsomal ATPases in the presence of L-ascorbic acid and L-cysteine in inhibitory concentration. Means of four experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

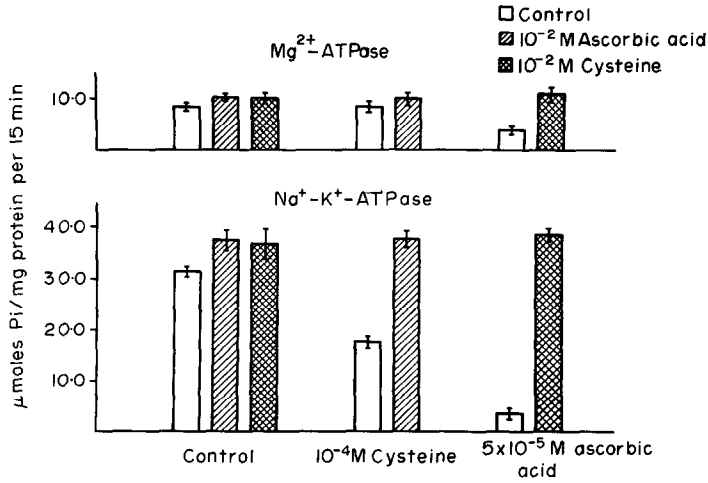

FIG. 6. Antagonization of the inhibitory effect of L-cysteine by 10^{-2} M L-ascorbic acid and the inhibitory effect of L-ascorbic acid by 10^{-2} M L-cysteine on the ATPases of the Na^+,K^+-ATPase preparation. Means of four experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

The inhibition of Na^+,K^+-ATPase and Mg^{2+}-ATPase activities by ascorbic acid and of Na^+,K^+-ATPase activity by cysteine was antagonized by DA (Fig. 5). A similar effect was brought about by the chelators tested. High concentrations of ascorbic acid and cysteine mutually abolish the effect of each other (Fig. 6). Ascorbic acid and cysteine at such concentrations stimulated slightly the ATPase activities of the Na^+,K^+-ATPase preparation. When using Sigma ATP instead of Reanal ATP in the experiments, the inhibitory effect of cysteine could hardly be measured, while the same circumstance did not influence inhibition by ascorbic acid (Fig. 7).

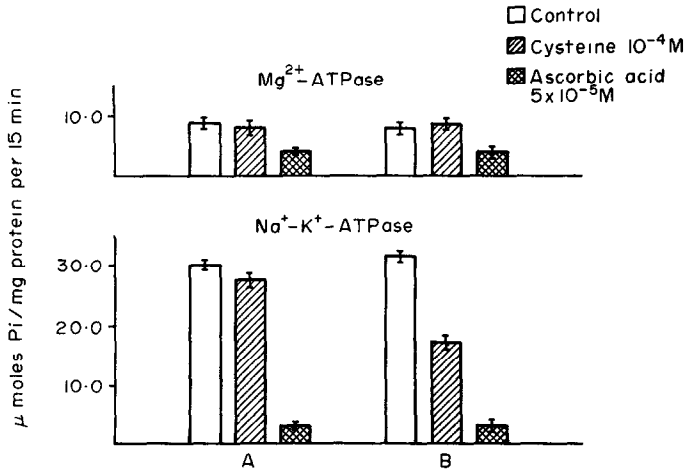

FIG. 7. Effects of L-ascorbic acid and L-cysteine on the ATPases of the Na^+,K^+-ATPase preparation using Sigma (A) or Reanal (B) ATP. Means of four experiments in duplicate ± S.E.

TABLE 2. EFFECTS OF EGTA AND EDTA BOUND BY AN EXCESS OF DIVALENT METALS ON THE MICROSOMAL TOTAL ATPase ACTIVITY IN THE PRESENCE OF ASCORBIC ACID

Substances*	Total ATPase activity†
Control	12·43 ± 0·29
EGTA	14·52 ± 0·43
EDTA	14·43 ± 0·42
Ca^{2+} (chloride)	12·54 ± 0·29
Mn^{2+} (sulphate)	12·99 ± 0·53
Co^{2+} (nitrate)	12·38 ± 0·46
Ni^{2+} (sulphate)	11·74 ± 0·89
Ascorbic acid	6·46 ± 0·43
Ascorbic acid + EGTA	15·23 ± 0·76
Ascorbic acid + EDTA	15·02 ± 0·77
Ascorbic acid + EGTA + Ca^{2+}	4·90 ± 0·70
Ascorbic acid + EDTA + Ca^{2+}	13·07 ± 0·85
Ascorbic acid + EDTA + Mn^{2+}	12·74 ± 0·48
Ascorbic acid + EDTA + Co^{2+}	11·72 ± 0·36
Ascorbic acid + EDTA + Ni^{2+}	7·09 ± 0·43

* The concentration of the chelators was 2×10^{-4} M; ascorbic acid 5×10^{-5} M. The concentration of the metals in the absence of the chelators and the concentration of the metal excess in the presence of the chelators was 5×10^{-5} M.

† Values are expressed as μmoles P_i/mg protein/15 min. Means of four experiments in duplicate ± S.E. Sigma ATP was used in the experiments.

The fact that inhibition by both the soluble fraction and ascorbic acid was antagonized by chelators suggests that a metal ion may play a role in bringing about inhibition. In further experiments we aimed to characterizing the metal ion in question. As shown in (Fig. 3), the antagonism by EGTA of the inhibitory effect of the soluble fraction was eliminated by an excess of Ca^{2+}. It can be seen in Table 2 that, unlike EGTA, the effect of EDTA on inhibition by ascorbic acid was not eliminated by an excess of Ca^{2+}; Mn^{2+} and Co^{2+} were similarly ineffective, while Ni^{2+}, like Ca^{2+} in the case of EGTA, replaced the metal in question in its complex with the chelator. This indicates that the heavy metal involved forms a complex with EDTA having

TABLE 3. EFFECTS OF INHIBITORY METALS ON THE MICROSOMAL ATPase ACTIVITY IN THE PRESENCE AND ABSENCE OF DA*

Metals	Concn (M)	Total ATPase activity without DA	Total ATPase activity with 2×10^{-4} M DA
None	—	10·79 ± 0·18	10·66 ± 0·21
Fe^{2+} (sulphate)	$7{\cdot}5 \times 10^{-6}$	7·41 ± 0·40	10·39 ± 0·24
Zn^{2+} (chloride)	$7{\cdot}5 \times 10^{-6}$	7·79 ± 0·17	8·10 ± 0·13
Cd^{2+} (acetate)	$7{\cdot}5 \times 10^{-6}$	7·68 ± 0·32	7·89 ± 0·26
Cu^{2+} (sulphate)	$1{\cdot}5 \times 10^{-6}$	7·40 ± 0·46	5·49 ± 0·34
Ca^{2+} (chloride)	10^{-4}	8·19 ± 0·19	7·66 ± 0·52
Fe^{3+} (chloride)	10^{-4}	8·30 ± 0·26	8·03 ± 0·38

* ATPase activity is expressed as μmoles P_i/mg protein/15 min. Means of four experiments in duplicate ± S.E. Reanal ATP was used in the experiments.

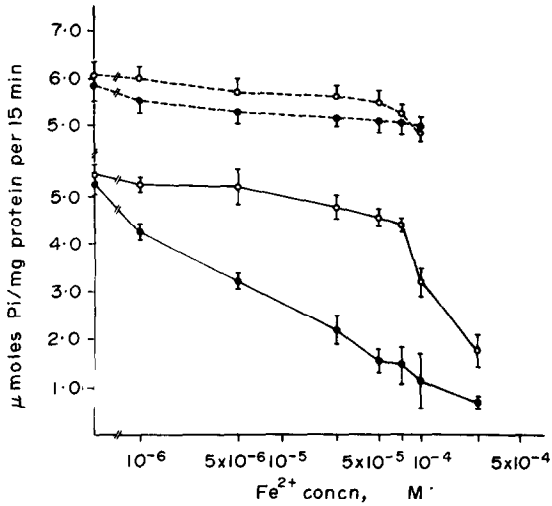

FIG. 8. Effect of Fe^{2+} on the microsomal ATPases in the absence (●) and in the presence (○) of 2×10^{-4} M dopamine in the incubation mixture. Continuous line, Na^+,K^+-ATPase activity; broken line, Mg^{2+}-ATPase activity. Means of three experiments in duplicate ± S.E. Reanal ATP was used.

a stability similar to or higher than those of the Mn^{2+}-EDTA and Co^{2+}-EDTA complexes but lower than that of the Ni^{2+}-EDTA complex.

It seemed promising to investigate the metals inhibiting ATPase activity, whose effect could be eliminated by incubation in the presence of DA. It is known that several divalent cations act as effective inhibitors on Na^+,K^+-ATPase activity.[8,29–31] In the experiment shown in Table 3 some of these metals were introduced to the system in concentrations sufficient to obtain a slight inhibition of the total microsomal ATPase activity. Incubation in the presence of DA suspended only the inhibition by Fe^{2+} ions. Cu^{2+} inhibition of ATPase activity increased in the presence of DA. As shown on Fig. 8, DA antagonized the inhibition of Na^+,K^+-ATPase activity by Fe^{2+} ions very effectively. However Mg^{2+}-ATPase activity was hardly influenced by Fe^{2+} ions; in this case, too, DA prevented the slight decrease in activity observed.

It is well-known that ATP binds iron very strongly, in the form of Fe^{3+} ions.[32] Commercial ATP preparations also contain a considerable amount of chelated Fe^{3+}, released as Fe^{2+} in a reductive medium.[33] In Table 4 the iron contents of the two ATP preparations used in these experiments (Reanal and Sigma) and of two further

TABLE 4. IRON CONTENT OF COMMERCIAL ATP PREPARATIONS DETERMINED SPECTROPHOTOMETRICALLY*

ATP preparation		m-moles Fe^{3+}/mole ATP Determined by dipyridyl	Determined by *o*-phenanthroline
Reanal,	71044850	3·20	3·20
Fluka,	135410 90 K	0·95	1·00
Boehringer,	7222389	1·30	1·25
Sigma,	42C-7290	<0·40	<0·40

* For details see methods.

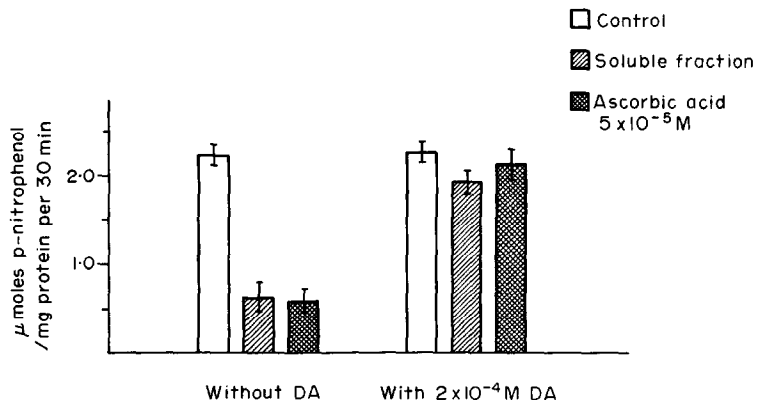

FIG. 9. Effect of the soluble fraction and of L-ascorbic acid on the microsomal K^+-*p*-nitrophenylphosphatase activity in the absence and presence of dopamine. Means of four experiments in duplicate ± S.E.

ATP preparations are listed. The Fe^{2+} content was determined on the basis of the spectrophotometric measurement of Fe^{2+}-*o*-phenanthroline and Fe^{2+}-α,α'-dipyridyl complexes, formed in a reductive medium. The extremely low iron content of Sigma ATP excludes the possibility that iron from ATP plays any role in the phenomenon observed with Sigma ATP. On the other hand, the relatively high iron content of Reanal ATP accounts very well for the differences between the results obtained with the two different ATP preparations (Figs. 2 and 7). It should be noted that values of 5 m-moles Fe^{3+}/mole ATP have been measured in certain commercial ATP preparations.[33] All chemicals present in the incubation medium for enzyme-activity measurements were tested for iron in highly concentrated solutions. Iron contamination was not detected.

Another method of characterizing the phenomenon tested was the measurement of K^+-stimulated phosphatase activity. In the presence of K^+ and Mg^{2+}, Na^+,K^+-ATPase preparations catalyze the hydrolysis of some simple phosphates and this

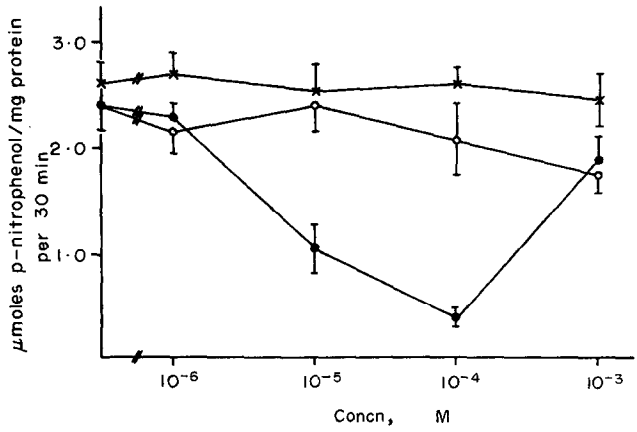

FIG. 10. Effects of L-ascorbic acid (●), dehydroascorbic acid (○) and L-cysteine (×) on the microsomal K^+-*p*-nitrophenylphosphatase activity. Means of four experiments in duplicate ± S.E.

TABLE 5. THE EFFECT OF 5×10^{-5} M ASCORBIC ACID AND OF THE SOLUBLE FRACTION ON THE MICROSOMAL ATPASES IN THE PRESENCE OF 2×10^{-4} M DA ADDED BEFORE OR AFTER PREINCUBATION*

Substances added		ATPase activity†	
Before preincubation	After preincubation	Na^+,K^+-ATPase	Mg^{2+}-ATPase
Ascorbic acid		2·90 ± 0·22	3·51 ± 0·11
Ascorbic acid, dopamine		5·72 ± 0·25	5·73 ± 0·16
Ascorbic acid	dopamine	3·15 ± 0·24	4·27 ± 0·11
Soluble fraction		2·69 ± 0·20	3·94 ± 0·09
Soluble fraction, dopamine		6·11 ± 0·19	5·74 ± 0·08
Soluble fraction	dopamine	2·91 ± 0·11	4·37 ± 0·15

* The microsomes were preincubated for 10 min at 37° without the substrate.

† Values are expressed as μmoles P_i/mg protein/15 min. Means of four experiments in duplicate ± S.E. Sigma ATP was used in the experiments.

reaction is supposed to model the dephosphorylation of the phosphorylated intermediate of Na^+,K^+-ATPase on the effect of K^+.[34] The inhibitory effect of the soluble fraction and of ascorbic acid was demonstrated also on K^+-*p*-nitrophenylphosphatase activity and both inhibitions were antagonized by DA (Fig. 9). The concentration dependence of the inhibition by ascorbic acid was the same as for ATPase (Fig. 10). Ascorbic acid could not be replaced by dehydroascorbic acid. As in the ATPase experiments using Sigma ATP, cysteine proved to be ineffective.

The system used to measure antagonism of the inhibitory effect of the soluble fraction or ascorbic acid by chelators or DA contained both the inhibitor and the compound to antagonize inhibition during the preincubation period. It seemed important to test whether the development of inhibition could be prevented if antagonizing compounds were introduced to the system after a preincubation of the preparation in the presence of ascorbic acid or the soluble fraction. Table 5 shows that after preincubation of the preparation with ascorbic acid or with the soluble fraction a given amount of DA did not prevent the inhibition of ATPase activity. The same results were obtained in the case of K^+-*p*-nitrophenylphosphatase activity, and also if DA was replaced by EDTA.

Finally, the ascorbic acid content of the rat brain was estimated and was found to be 0·324 ± 0·007 (mean ± S.E., n = 4) mg/g wet wt. Ninety one per cent of the total ascorbic acid content found in the brain homogenizate was recovered in the soluble fraction and the ascorbic acid concentration of the soluble fraction was $1{\cdot}69 \pm 0{\cdot}09 \times 10^{-4}$ M.

DISCUSSION

The ability of ascorbic acid to inhibit Na^+,K^+-ATPase was first observed by Glynn on the electric organ of the eel.[35] Later, Inagaki demonstrated a considerably stronger inhibitory effect in experiments carried out on microsomes of rabbit brain.[36] He also observed an inhibition by cysteine similar to that produced by ascorbic acid; cysteine was already known to inhibit liver Na^+,K^+-ATPase activity.[37] Both compounds inhibited also Mg^{2+}-ATPase activity, to a smaller but still considerable extent. Glutathione and methionine were ineffective against both ATPase activities.

Recently, Frey, Pitts and Askari reported a strong inhibitory effect of ascorbic acid, similar to that described by Inagaki on Na^+,K^+-ATPase prepared from the brain of various mammalian species.[38] Both the oxidized and the reduced forms of ascorbic acid were effective; therefore these authors attribute the inhibitory effect of ascorbic acid to its structural features, namely to the lactone structure.

In our experiments oxidized ascorbic acid, dehydroascorbic acid, was ineffective. It must be noted, however, that Frey, Pitts and Askari could not obtain consistent results when using commercial preparations; therefore they used freshly prepared dehydroascorbic acid for experimental purposes. Other aspects of our experiments, however, also indicate that under these conditions an inhibition different from the one described by Frey, Pitts and Askari was observed. The inhibition by ascorbic acid observed by us was antagonized by chelators, while in their experiments the inhibitory effect of ascorbic acid was not affected by the presence of EDTA. At concentrations higher than 0·1 mM the inhibition by ascorbic acid decreased and at high concentrations ascorbic acid was ineffective in our experiments. Regarding the effectiveness of ascorbic acid in low concentrations our results are closer to the results of Inagaki than to those obtained by Frey, Pitts and Askari. Inagaki studied inhibition by ascorbic acid at concentrations up to 0·1 mM. He found inhibition by cysteine, but not by glutathione, similar to that effected by ascorbic acid. In our experiments ascorbic acid could not be replaced either by cysteine or by glutathione. When using Reanal ATP for the experiments, though cysteine inhibits Na^+,K^+-ATPase (Figs. 4–7) and the character of the line of the inhibition resembles the effect of ascorbic acid, the effect was not observed with Sigma ATP (Fig. 7) and cysteine did not act on Mg^{2+}-ATPase activity with either of the ATP preparations. Cysteine was similarly ineffective on K^+-*p*-nitrophenylphosphatase activity, in contrast to ascorbic acid (Fig. 10). In the knowledge of the difference between the iron contents of the two ATP preparations (Table 4) and the fact that Fe^{2+} ions inhibit only Na^+,K^+-ATPase significantly (Fig. 8), it can be established that the inhibition by cysteine of Na^+,K^+-ATPase activity observed with Reanal ATP is an artefact, due to the reduction of Fe^{3+} bound to ATP, thus releasing Fe^{2+}. If the amount of Fe^{3+} measured in the Reanal ATP preparation was added to the Sigma ATP, cysteine produced an inhibition similar to that observed in the case of Reanal ATP. In Inagaki's experiments cysteine also inhibited Mg^{2+}-ATPase activity, similarly to ascorbic acid; furthermore he used Sigma ATP. These facts both preclude the possibility that his result could have been an artefact of the same character.

The type of inhibition caused by ascorbic acid in our experiments could be identified with the inhibition of the particulate ATPase activities by the soluble fraction: the effects of the soluble fraction and of ascorbic acid proved to be identical under all conditions investigated. About 10^{-5} M ascorbic acid inhibited the ATPase activities to the same extent as the soluble fraction (Fig. 4). The ascorbic acid concentration in the original soluble fraction was found to be $1{\cdot}69 \times 10^{-4}$ M and the soluble fraction was ten-fold diluted in the ATPase-assay system. This good agreement indicates that the ascorbic acid-like effect of the soluble fraction was brought about by ascorbic acid itself. Under the conditions used to characterize the endogenous inhibitor in the soluble fraction,[17] ascorbic acid was found to be heat-stable, on the basis of the effect on ATPase activity and the ability to reduce Fe^{3+} ions. Ascorbic acid could not be replaced by dehydroascorbic acid (Figs. 4 and 10), and

if the reducing power of the soluble fraction was abolished by 4×10^{-4} M potassium permanganate the soluble fraction lost its inhibitory effect (unpublished results). Ascorbic acid could not be replaced by other cytoplasmic reducing agents, such as cysteine and glutathione, indicating that the reductivity and the structural features of ascorbic acid may both affect its activity.

The most important characteristic of the effect of the soluble fraction and of ascorbic acid is that it can be antagonized by chelators and catecholamine. Rather high concentrations of chelators or catecholamine were needed to antagonize inhibition by the soluble fraction (Figs. 1 and 2). When using Sigma ATP, however, instead of Reanal ATP, DA was much more effective in a lower concentration (Fig. 2). This may be due to part of the DA forming a complex with Fe^{2+} ions released from Reanal ATP by the reducing effect of the soluble fraction. DA abolished the inhibition of Na^+,K^+-ATPase by cysteine observed with Reanal ATP for the same reason (Fig. 5). It has been known for a long time that catecholamines may form stable binary or, in the presence of ATP, ternary complexes with divalent cations.[39,40] The great affinity of DA to Fe^{2+} in the system could be proved and DA was found to be highly specific for Fe^{2+} (Fig. 8 and Table 3).

In all probability the chelator-like effect of DA was due to the formation of a DA–metal complex. The fact that inhibition by the soluble fraction or ascorbic acid was antagonized by chelators indicates that a metal ion bound to the membrane structures is involved in the development of the inhibition. The above observations regarding DA–Fe^{2+} interaction suggest that the metal may be iron, towards which DA acts like a chelator. The necessity of a Fe^{3+}-Fe^{2+} transformation to bring about the inhibition would explain the role of the reductivity of ascorbic acid. Our experiments show that the metal involved in inhibition by ascorbic acid is a heavy metal which cannot be replaced by Ca^{2+}, Mn^{2+} and Co^{2+} in its complex with EDTA; Ni^{2+}, however, can replace it. The logarithmic stability constants of the complexes of these metals with EDTA are 10·7, 13·6, 16·2 and 18·6 respectively, at 20° in a solution of 0·1 M ionic strength.[41] The logarithmic stability constant of the Fe^{2+}-EDTA complex is 14·3. Though the stability constant of the complex with Co^{2+} is higher than that with Fe^{2+} it is possible that under the conditions of the experiments the difference is insufficient for the replacement of Fe^{2+}.

The assumption of the role of iron is supported by the fact that the artefact inhibition by cysteine through the iron contamination of the Reanal ATP is of the same character, as the inhibition by ascorbic acid (Fig. 4) and at higher concentrations the two compounds can mutually abolish the effect of each other (Fig. 6). It seems worth mentioning that both ascorbic acid and cysteine at a concentration of 10^{-2} M antagonize also the effect of the soluble fraction. It is considered that the protective effect of high concentrations of ascorbic acid and cysteine may be a chelating effect, too.

The iron content of the biological preparations used could not be measured by the photometric method used. However, according to the results obtained by the atomic absorption method a considerable amount of iron is present in the subcellular fractions of the brain, bound to the particulate fractions.[40] It must be supposed, however, that if the metal involved in the inhibition is actually iron, its characteristics when bound to cellular structures are somewhat different from those of Fe^{2+} introduced into the system, inhibiting only Na^+,K^+-ATPase (Fig. 8). It is also possible, that the inhibition of Mg^{2+}-ATPase activity results from some kind of synergism

between Fe^{2+} and ascorbic acid, which cannot be brought about when only Fe^{2+} is present.

The possible role of an intrinsic heavy metal in the regulation of Na^+,K^+-ATPase activity was previously suggested by Bader, Wilkes and Jean on the basis of experiments carried out on kidney cortex Na^+,K^+-ATPase with hydroxylamine.[29] In some preparations hydroxylamine strongly inhibited Na^+,K^+-ATPase activity, and the inhibition could be abolished by incubation in the presence of 10^{-4}–10^{-5} M EGTA. The effect of EGTA could be stopped by the addition of an excess of Ca^{2+}. The authors supposed that an intrinsic heavy metal was bound to a "storage site" of the enzyme. Hydroxylamine decreased the affinity of the heavy metal to this site, thus making it bind to an "inhibitory site", which resulted in the inhibition of the enzyme. It is presumed that this mechanism may also work *in vivo*, with the participation of other compounds, and may act as the control mechanism of Na^+,K^+-ATPase activity.

Our experiments lead to similar conclusions as regards the possible regulatory role of an intrinsic heavy metal. The metal in question is a reoxidizable metal, probably iron, which is reduced by ascorbic acid and brings about such changes in the membrane structure that lead to the inhibition of Na^+,K^+-ATPase and to a different extent of Mg^{2+}-ATPase activity. In the presence of chelators or catecholamines, presumably also forming a complex with the intrinsic metal, inhibition of the ATPase activities does not occur.

In relation to the work of Bader, Wilkes and Jean it seems worth mentioning that, although the effect of hydroxylamine on Na^+,K^+-ATPase activity is discussed in another aspect, hydroxylamine is a reducing agent which may also account for some of its effects. In our preparations hydroxylamine was not inhibitory and ascorbic acid could not be replaced by other cytoplasmic reducing agents. It cannot, however, be excluded that in certain preparations (kidney cortex) and under certain conditions the intrinsic metal is accessible for other reducing agents too, which would explain why hydroxylamine is not always active.

In our experiments the phenomenon discussed could be brought about by preparations isolated from the brain and by compounds present there under physiological conditions; moreover, inhibition was observed also in the presence of the soluble fraction of the brain. The significance of our results *in vivo* still raises several problems, especially whether the inhibition is due or not to an aspecific change in the *in vitro* system. Inhibition by ascorbic acid and by the soluble fraction can only be prevented by DA or chelator, but inhibition, once existing, cannot be abolished (Table 5). If inhibition occurs under physiological conditions, it must be supposed that the role of catecholamines would be to decrease or antagonize inhibition, depending on the catecholamine concentration present; while the re-establishment of the original, non-inhibited state would be the result of some other processes such as oxidation. The fact that the phenomenon can be influenced by tranquilizers like chlorpromazine, tetrabenazine,[18,19] reserpine (in preparation) emphasizes its possible importance. The protection against the inhibitory effect of the soluble fraction and ascorbic acid by these compounds is evidently not a chelating effect, but it may indicate a change in the membrane structures decreasing their sensitivity.

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