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A SODIUM- AND POTASSIUM-STIMULATED ADENOSINE TRIPHOSPHATASE FROM CARDIAC TISSUES—II

THE EFFECTS OF OUABAIN AND OTHER AGENTS THAT MODIFY ENZYME ACTIVITY*

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Abstract—An ATP-hydrolyzing enzyme system (ATPase) has been found in the 'microsomal' fraction of heart muscle homogenates, which appears to consist of at least two components, one, Mg^{2+}-dependent and the other, $Mg^{2+} + Na^+ + K^+$-dependent.

Various inhibitory agents were employed in order to differentiate these two components or sites of activity. Amytal and ethyl alcohol are representative of compounds that inhibit the $Mg^{2+} + Na^+ + K^+$-dependent activity to a greater extent than the Mg^{2+}-dependent activity. Ouabain and *p*-hydroxymercuribenzoate (POMB) *specifically* inhibit the $Mg^{2+} + Na^+ + K^+$-ATPase activity while having little or no effect on the basic Mg^{2+}-activity. The POMB-sensitive portion of the enzyme system appears to be sensitive to ouabain.

Sodium azide, guanidine, and trinitrobenzenesulfonic acid represent compounds which preferentially inhibit the basic Mg^{2+} activity of the heart muscle, while being almost unreactive on a similar enzyme system from brain. These substances markedly increase the sensitivity of the enzyme system to both ouabain and to sulfhydryl group inhibitors.

The possibility that certain charged groups are involved in the effect of ouabain on the membrane ATPase system from heart muscle is discussed.

IN a previous communication[1] the preparation and 'ageing' properties of an adenosine triphosphatase (ATPase)† from heart muscle were described. This enzyme, associated with the microsomal fraction of the cell, was implicated in the active transport of sodium and potassium ions across membranes. Since the enzyme complex appeared to consist of either two enzymes, or two sites of activity on one enzyme, it was of interest to examine in greater detail some of the characteristics that might be useful in differentiating the two enzymes or sites and possibly in elucidating some aspects of structure. For this purpose several types of compounds were used which have been shown to affect a number of enzyme systems from other tissues. Among these substances, the cardiac glycoside, ouabain, which specifically inhibits active transport[2]

* Preliminary reports of this study have appeared in *Fed. Proc.* **22**, 212 (1963) and in *Life Sci.* **6**, 363 (1963).

† Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; AMPD, 2-amino-2-methyl-1,3-propanediol; EDTA, ethylenediamine tetraacetic acid; SM, suspending medium; POMB, *p*-hydroxymercuribenzoic acid; Tris, tris(hydroxymethyl)amino methane; TBS, 2,4,6,-trinitrobenzene sulfonic acid; DOC, deoxycholic acid; DNP, 2,4-dinitrophenol; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NAD^+, nicotinamide-adenine dinucleotide; NADH, reduced NAD^+; NEM, N-ethylmaleimide.

and membrane ATPases derived from various sources[3–5] was found to affect the cardiac ATPase in a selective manner.[1, 6, 7] This inhibition appeared to be dependent upon certain groupings in the enzyme-containing microsomal preparation.

Since it has been suggested recently that $Na^+ + K^+$-activated ATPases isolated from different tissues may possess specific characteristics which possibly may be related to function,[5, 8] the cardiac ATPase was compared to a microsomal enzyme system isolated from brain. It was of interest to determine whether similarly isolated ATPase preparations from different tissues would exhibit similar sensitivities toward various inhibitory agents.

Electron micrographic studies of freshly prepared and of 'aged' ATPase preparations were carried out in attempts to disclose some possible morphological changes that may occur concomitantly with alterations in enzyme activity.

EXPERIMENTAL

Enzyme preparation and assay

The procedures used for preparation and assay of the enzyme systems were the same as those reported previously[1] with the exception of the "quick-ageing" effect. In order to hasten the loss of the Mg^{2+} activity and hence increase the 'activity ratio', in some experiments the pellet obtained after the centrifugation (80,000 *g*) was suspended in the standard suspending medium (SM) (0·25 M sucrose, 1 mM EDTA, 30 mM histidine, 30 mM AMPD, pH 7·00) and stored for one day at −5°. The suspension was then recentrifuged at 150,929 *g* (average *g*) for 30 min. The particulate material obtained was kept at 5° for two days, after which it was resuspended in a Tris-sucrose medium (the same as above but with Tris replacing the histidine) and kept at room temperature for 1 hr prior to use or incubated for 15 min at 37°. A somewhat similar resuspension technique was used by Skou for a brain and kidney ATPase preparation.[9]

Protein determinations were made by the biuret[10] or Lowry method.[11] It should be mentioned that the AMPD buffer used in the homogenizing and suspending media reacts with both the biuret and Folin–Ciocalteu reagents. Since appropriate blanks were not used in obtaining the results reported previously,[1, 12] the ATPase values in those studies should be increased by a factor of 8.

Since the microsomal enzyme preparations derived from heart muscle are somewhat labile,[1] most of the results in the present paper were obtained with different enzymes stored for varying periods or treated in different ways as described. Each of these preparations consequently served as its own control. In some cases, however, particularly when various ATPase preparations were isolated the same day, but from different animals, and were treated and stored in exactly the same manner, the results were grouped and statistical analyses were performed. In these cases, standard errors of the mean are indicated and Student's 't' test showed significance at the 1 per cent level.

The enzyme system isolated from cerebral tissues of the guinea pig was prepared in the same manner as that from cardiac muscle. These preparative methods were originally suggested by Skou[9, 13–15] and have yielded much more active and stable enzymes than those employed previously.

Electron microscopic studies. These were performed by Dr. K. Smetana, Department of Pharmacology, Baylor University College of Medicine.

The pellet derived from the 80,000 *g* centrifugation was fixed in 2% osmium tetroxide or in 6% glutaraldehyde, followed by treatment with formalin, and stained with uranylacetate during the dehydration procedure. It was then embedded in Maraglas or Araldite (Ciba, 502), sectioned on a Porter–Blum microtome, and observed with either an RCA 3F electron microscope or a Siemens Elmiscope I electron microscope. Two types of studies were made, one utilizing freshly prepared 'microsomal' pellets and the other, pellets which had been stored at −50° for approximately one month.[1]

Reagents and Chemicals

The POMB and ouabain were purchased from California Biochemical Co. The TBS and the AMPD were purchased from Eastman Organic Chemicals and recrystallized. The Tris (Sigma 121 Primary Acidometric Standard) and the ATP (Tris form) were products of Sigma Chemical Co. The sodium content of the ATP was less than 0·1 mmoles/liter. The other reagents and chemicals were all "Baker Analysed" reagent grade. Glass-distilled water was utilized throughout and assayed routinely for sodium and potassium content, both of which were negligible.

RESULTS

The enzyme suspensions derived from heart muscle and used in the present studies consist mostly of fragments presumably derived from the sarcoplasmic reticulum. The 'vesicles' were of the smooth type possessing a membrane, and were between 0·2 and 0·4 μ in diameter. They appeared to be intact. Adjacent spiral arrays of myofilaments were occasionally seen and depicted in the sections appearing in Figs. 1 and 2. The section in Fig. 1 was derived from a freshly prepared microsomal pellet. The ultrastructure of an 'aged' pellet is shown in Fig. 2.

The vesicles appear somewhat enlarged or swollen as compared to the non-aged preparation, and exhibit a rather thickened, membrane-like structure which encloses the vesicle. In addition many of the aged vesicles appear to contain some type of unidentified dense body, usually centrally located.

Several inhibitors and other agents were incubated with the microsomal ATPases from heart and from brain and, in almost all cases, the $Mg^{2+} + Na^+ + K^+$-ATPase activity was considerably more sensitive to the added substance than was the Mg^{2+}-ATPase activity. Figures 3 and 4 are examples of this. Figure 3 shows the effect of varying concentrations of ethyl alcohol on the enzyme system derived from cardiac muscle. After a slight initial stimulation at low concentrations, a marked depression of the $Mg^{2+} + Na^+ + K^+$ component occurred, whereas only a slight depression of the Mg^{2+}-ATPase activity was seen. Amytal produced a similar effect, as shown in Fig. 4.

P-hydroxymercuribenzoate was also found to affect the $Mg^{2+} + Na^+ + K^+$ component of the heart enzyme while having little or no effect on the Mg^{2+}-ATPase activity (Fig. 5 and Table 1, experiments 1, 3–5). Contrary to the heart muscle ATPase, a similar brain preparation showed marked sensitivity to POMB both in the presence of Mg^{2+} alone and $Mg^{2+} + Na^+ + K^+$ (Fig. 6). Maximal inhibition of the heart ATPase occurred at a concentration of 1×10^{-5} M (0·01 mM, Table 1). At this concentration of POMB, addition of ouabain, even in high concentration, produced no further reduction of enzyme activity (Table 1, e.g. experiment 3). As has been shown previously,[1, 15] ouabain does not affect the basic Mg^{2+}-dependent ATPase

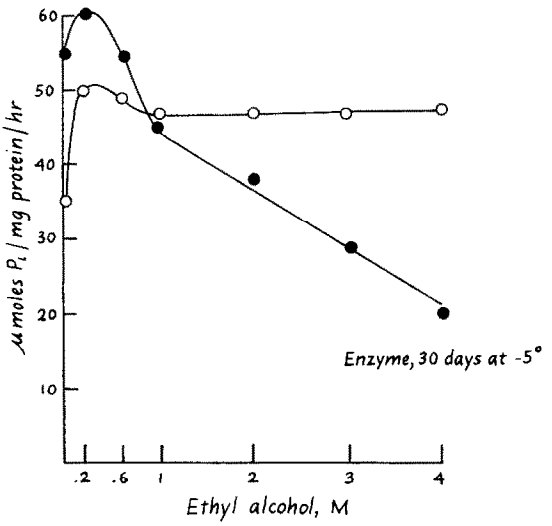

FIG. 3. Effect of ethyl alcohol on a cardiac microsomal ATPase. The conditions of incubation, assay, and the concentrations of ions, buffer, and substrate were the same as described in Table 1. In this and subsequent figures, unless otherwise indicated, the $Na^+ + K^+$ stimulated ATPase includes Mg^{2+}, 3 mM. The curves presented in all figures are representative of at least 3 separate determinations using different enzyme preparations.
○: Mg^{2+}; ●: $Mg^{2+} + Na^+ + K^+$

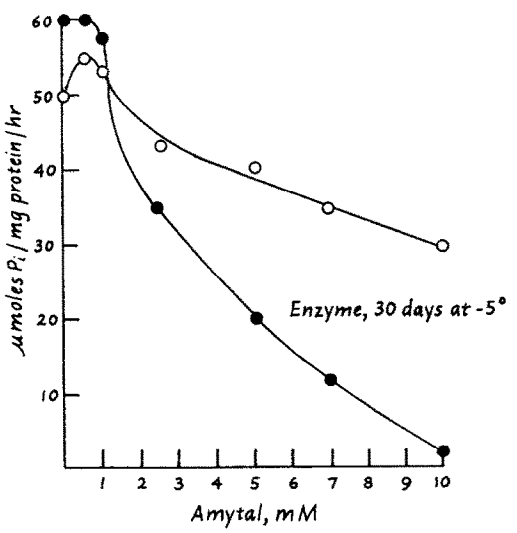

FIG. 4. The effect of amytal on a cardiac microsomal ATPase.
○: Mg^{2+}; ● $Mg^{2+} + Na^+ + K^+$

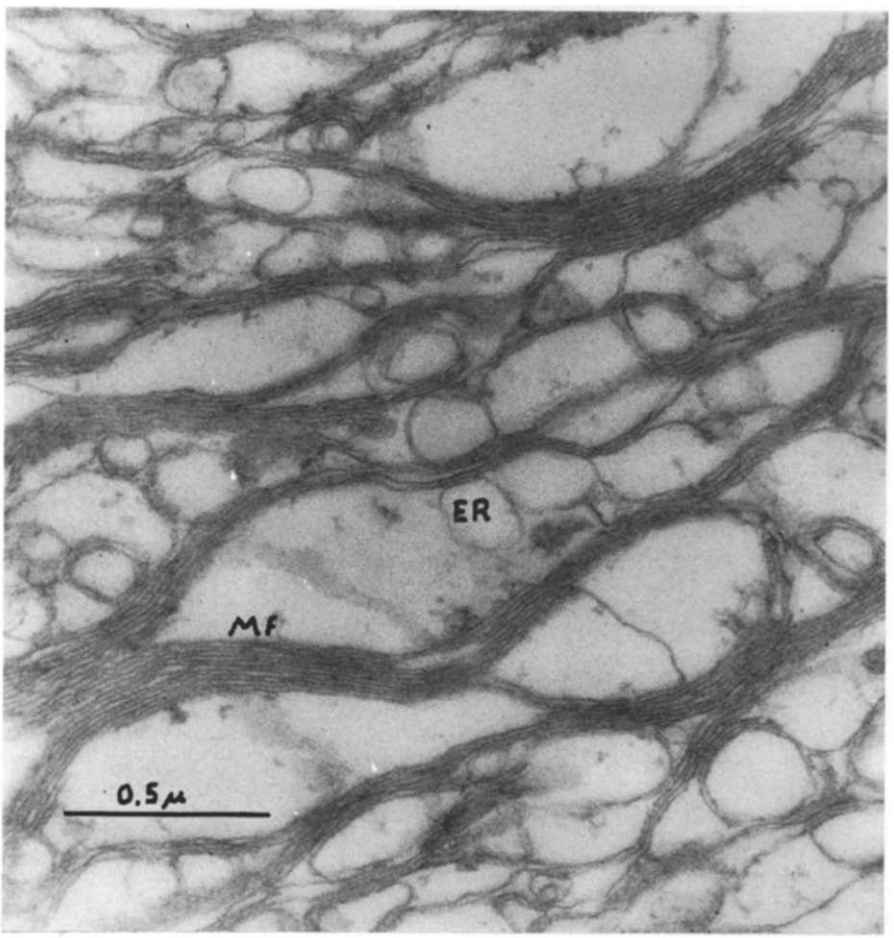

FIG. 1. Electron micrograph of a fresh microsomal pellet from guinea pig cardiac muscle. The microsomes were isolated by differential centrifugation as described in the text and the pellet treated as described in the text; × 77,800. ER = endoplasmic reticulum; MF = myofilaments.

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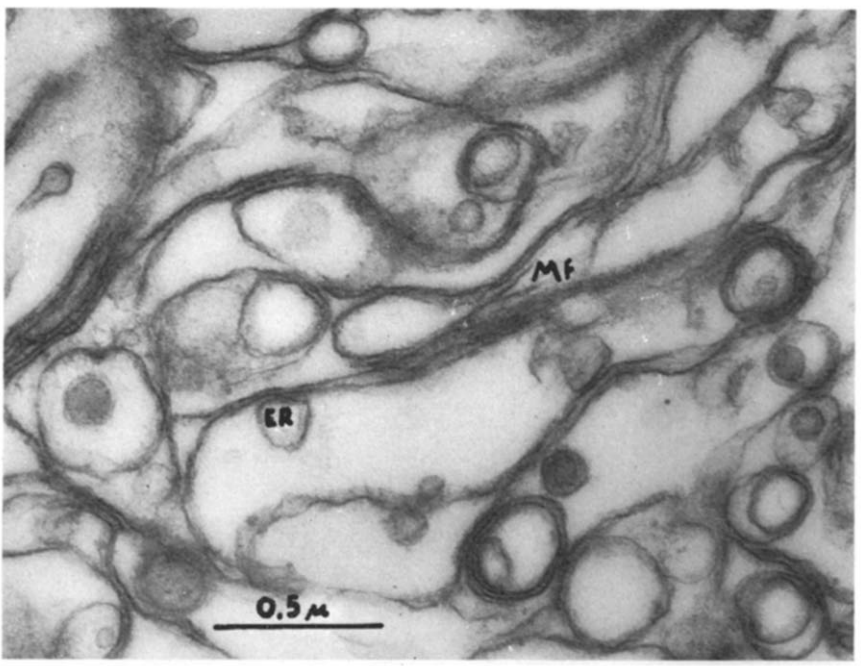

FIG. 2. Electron micrograph of an 'aged' microsomal pellet from guinea pig cardiac muscle. The pellet was obtained as above and stored at −5° for one month; × 65,000.

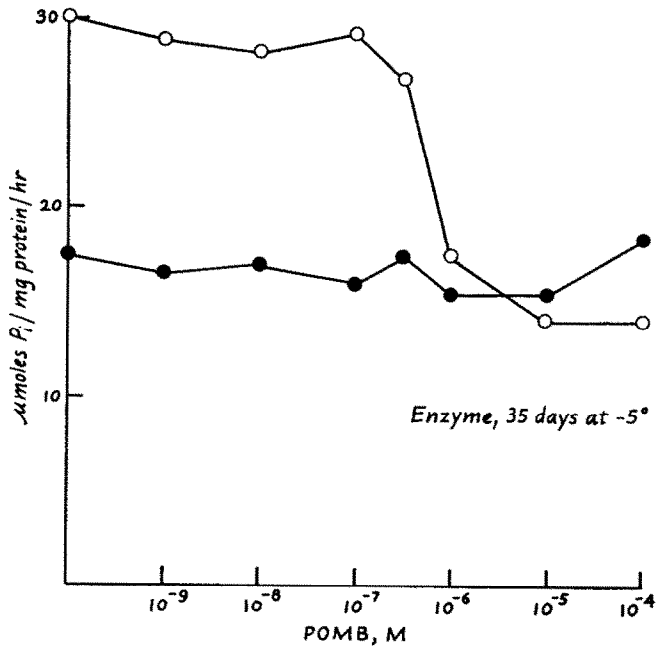

FIG. 5. The effect of various concentrations of POMB on cardiac microsomal ATPase.
○: Mg^{2+} + Na^+ + K^+; ● Mg^{2+}

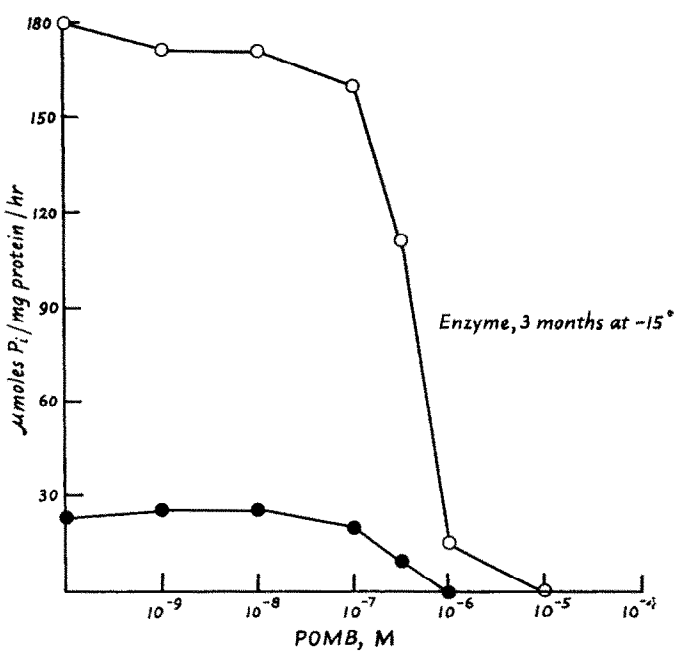

FIG. 6. The effect of various concentrations of POMB on cerebral microsomal ATPase.
○: Mg^{2+} + Na^+ + K^+, ●: Mg^{2+}

TABLE 1. THE EFFECT OF OUABAIN ON A CARDIAC MICROSOMAL ATPASE IN THE PRESENCE OF POMB AND AZIDE*

Experiment	Addition	Mg (A)	Mg + Na + K (B)	(B)/(A)
1	None (fresh)	115	135	1·2
	Ob	118	88	0·8
	P	111	85	0·8
	A	46	100	2·2
	A + P	45	55	1·2
	A + Ob	45	54	1·2
	A + Ob + P	44	53	1·2
2	None (21 days)	28	93	3·3
	Ob	25	38	1·5
	A	8	72	9·0
	A + Ob	5	8	1·6
3	None (25 days)	22	71	3·2
	Ob	23	23	1·0
	P	22	23	1·1
	P + Ob	23	23	1·0
	A	12	72	6·0
	A + P	12	12	1·0
	A + P + Ob	12	12	1·0
4	None (5 mo)	6	11	1·8
	Ob	6	6	1·0
	P	6	6	1·0
	A	2	10	5·0
	A + P	2	2	1·0
	A + Ob	2	2	1·0
5	None (quick-aged)	29	86	3·0
	Ob	29	36	1·2
	A	13	83	6·4
	A + Ob	12	23	2·0
	P	29	24	0·8
	P + Ob	29	24	0·8
	A + P	12	16	1·4

* Different enzyme preparations were used for each of the experiments and were "aged" for varying periods as indicated in parentheses. Unless otherwise stated, the values reported in this and subsequent tables and figures were obtained from guinea pig heart by a method previously described.[1] Incubation was carried out in a medium which included: Tris, 30 mM, pH 7·0; Tris-ATP, 3 mM; $MgCl_2$, 3 mM; NaCl, 100 mM; and KCl, 20 mM. Unless specified, the inhibitors were added to the reaction mixture prior to the addition of the enzyme and the ATP. Concentrations are in mmoles per liter (mM) and represent the amount in the total reaction medium (1 ml). Incubation was usually carried out for 30 min at 37° and the reaction stopped by the addition of 0·1 ml of ice-cold 50% TCA. The ATPase values are expressed as μmoles inorganic phosphate/mg protein/hour. Ob = ouabain, 0·1 mM; P = *p*-hydroxymercuribenzoate (POMB), 0·01 mM; A = azide, 5·0 mM.

activity. POMB in no case produced a stimulation of ATPase, as is the case with myosin ATPase preparations.[16, 17]

The inhibition produced by POMB and other sulfhydryl inhibitors may be completely or partially reversed, depending upon the method of incubation of the reagent with the enzyme by a number of sulfhydryl-containing compounds. The inhibition may be completely reversed if the sulfhydryl compound is added to the reaction mixture *before* incubation (Table 2, experiments 4 and 5). However, if the POMB is allowed to react with the enzyme at 37° for a few minutes, the binding between the inhibitor and the enzyme apparently is enhanced, since the sulfhydryl-containing

agents now only partially reverse the inhibitory effect. The latter is illustrated in Fig. 7, which demonstrates the effects of two such sulfhydryl compounds, β-mercaptoethanol and cysteine, on a POMB-inhibited system.

An example of an inhibitor that preferentially affects the Mg^{2+} component of the

TABLE 2. THE EFFECT OF VARIOUS SULFHYDRYL INHIBITORS ON A CARDIAC MICROSOMAL ATPASE*

Experiment	Addition (mM)	Mg (A)		Mg + Na + K (B)	
		1	2	1	2
1	None	7	6	15	14
	IAA, 10	6	6	13	13
	IAA, 100	5	6	6	7
2	None	47	48	62	62
	S, 0·1	46	47	35	36
3	None	29	29	40	40
	Hg, 0·01	27	28	17	17
4	None	40	20	114	43
	NEM, 0·1	40	20	88	35
	NEM + CYST, 1	40	20	110	45
5	None	13	28	28	112
	P, 0·01	11	28	10	30
	P + CYST, 1	12	29	27	109
6	None	24·6 ± 3·5 (5)		77·7 ± 6·8 (5)	
	A, 5	12·4 ± 0·75 (4)		74·4 ± 5·8 (4)	
	NEM, 1	24·3 ± 2·8 (5)		60·6 ± 3·0 (5)	
	NEM, 10	23·2 ± 1·8 (5)		41·6 ± 3·5 (5)	
	A, 5 + NEM, 10	11·9 ± 0·5 (4)		32·7 ± 2·1 (4)	

* The values in experiment 6 were obtained from different enzyme preparations isolated the same day and quick-aged as described in the text. The standard error is given along with the number of animals in parentheses. The results in experiments 1–5 were obtained from two different preparations in each case and are indicated in the table under 1 and 2, respectively. NEM = N-ethylmaleimide; A = azide; IAA = iodoacetamide; S = salyrgan (*o*-[(3-hydroxymercuri-2-methoxypropyl)-carbamyl]phenoxyacetic acid); Hg = $HgCl_2$; CYST = cysteine; P = POMB.

cardiac ATPase system is sodium azide, which depressed the activity of the Mg^{2+}-ATPase markedly without very much effect on the $Mg^{2+} + Na^+ + K^+$ activity (Table 1; Table 2, experiment 6; Table 3, experiments 1, 3). In the presence of azide, ouabain inhibited the residual $Na^+ + K^+$-stimulated activity (Table 1). Similarly, POMB in the presence of azide inhibited the remaining $Na^+ + K^+$-stimulated activity. For example, in experiment 3, Table 1, the value of the $Mg^{2+} + Na^+ + K^+$-ATPase in the presence of POMB alone was 23. After azide treatment, POMB depressed the ATPase to a level of 12. Ouabain added to the azide, POMB-inhibited system, caused no further reduction of activity.

The data in Table 2 (experiments 1, 2, 3, 6) illustrate the effects of a number of other sulfhydryl inhibitors on the cardiac ATPase, and all reacted in a manner quite similar to POMB. The main effect was on the $Na^+ + K^+$ stimulation, and azide

pretreatment produced the same effect with at least one of the reagents (N-ethylmaleimide; Table 2, experiment 6) as it did with the POMB or the ouabain (Table 1).

A number of other compounds produced inhibition of the heart ATPase qualitatively the same way as with azide—i.e. a preferential inhibition of the basic Mg^{2+}

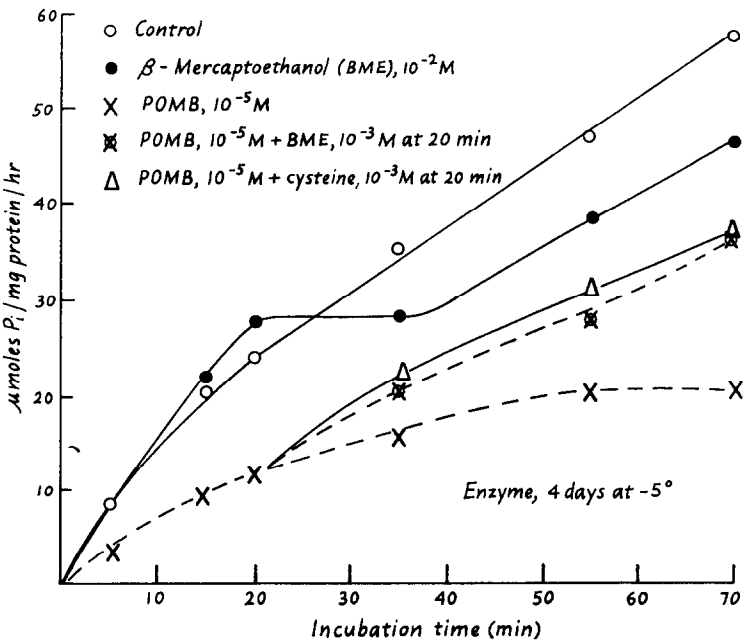

FIG. 7. The effects of sulfhydryl compounds on a POMB-treated cardiac microsomal ATPase in the presence of Mg + Na + K ions. The sulfhydryl compounds were introduced at the 20-min interval, with the controls receiving the same volume of distilled water.

activity (Table 3). Substitution of a methyl group in guanidine markedly reduced the inhibition (Table 3, experiment 2). An acidic substance, TBS, also affected the Mg^{2+} component to a greater degree than the $Mg^{2+} + Na^{+} + K^{+}$ aspect (Table 3, experiment 5).

When the ATPase system from cardiac muscle was compared to one from cerebral tissues with respect to inhibitor reaction, it was observed that azide, e.g., had no effect on the brain ATPase even in concentrations higher than those that markedly inhibited the heart system (Table 4, experiments 1, 2); guanidine reacted similarly (experiments 3, 4).

The cardiac ATPase appears to be quite sensitive to agents that tend to 'solubilize' membranes. For example, deoxycholic acid or Triton X-100 drastically reduced enzyme activity of both the Mg^{2+} and the $Mg^{2+} + Na^{+} + K^{+}$ components (Table 5, experiments 2, 3).

Dinitrophenol, an agent that increases the activity of mitochondrial ATPases, had little or no such action on the cardiac microsomal ATPase (Table 5, experiment 1).

TABLE 3. EFFECTS OF GUANIDINE, METHYLGUANIDINE, UREA, AND TRINITROBENZENESULFONIC ACID ON A CARDIAC MICROSOMAL ATPASE*

Experiment	Addition (mM)	Mg (A)	Mg + Na + K (B)	(B)/(A)
1	None (fresh)	34	62	1·8
	G, 20	18	46	2·6
	A, 10	18	46	2·6
2	None (2 mo)	16	34	2·1
	G, 5	16	34	2·1
	G, 10	11	33	3·0
	G, 20	9	31	3·5
	G, 40	6	27	4·5
	MG, 5	16	34	2·1
	MG, 10	16	34	2·1
	MG, 20	12	33	2·7
	MG, 40	10	29	2·9
	U, 1 M	16	34	2·1
	U, 5 M	13	32	2·5
	U, 10 M	8	27	3·4
	U, 20 M	2	17	8·5
3	None (3 mo)	6	11	1·8
	G, 40	4	10	2·5
	A, 10	4	10	2·8
4	None (quick-aged)	26	80	3·1
	Ob, 0·1	23	22	0·9
	G, 20	17	74	4·4
	G, 20 + Ob, 0·1	17	17	1·0
5	None	31 ± 4·3 (4)	91 ± 13·3 (4)	2·9 ± 0·3
	TBS, 1	12 ± 4·0 (4)	47 ± 7·0 (4)	3·9 ± 0·9

* The values in experiments 1–4 were obtained from different enzyme preparations stored for varying periods as indicated. In experiment 5, four different preparations were used, all isolated the same day and quick-aged as described in the text. G = guanidine; MG = methylguanidine; U = urea; Ob = ouabain; A = azide; TBS = 2,4,6-trinitrobenzenesulfonic acid.

TABLE 4. EFFECTS OF AZIDE AND OTHER COMPOUNDS ON A BRAIN MICROSOMAL ATPASE*

Experiment	Addition (mM)	Mg (A)	Mg + Na + K (B)
1	None	43	179
	A, 0·25	42	181
	A, 0·5	43	182
	A, 1	44	184
	A, 5	46	188
	A, 10	46	188
2	None	29 ± 9 (4)	150 ± 40 (4)
	A, 15	30 ± 10 (4)	153 ± 43 (4)
3	None	38	205
	G, 20	37	202
	G, 40	35	199
4	None	45	196
	Ob, 0·1	41	32
	G, 20	40	201
	G + Ob	40	32

* The values in experiments 1, 3, and 4 were obtained from enzyme preparations isolated from different guinea pig brains and each was stored at −15° for 3 months prior to use. The values in experiment 2 represent a composite of 4 different preparations with standard error. In experiment 2 the azide was preincubated with the enzyme in the absence of ATP for 15 min at room temperature. The control was treated in the same way. A = azide; G = guanidine; Ob = ouabain.

TABLE 5. THE EFFECT OF 2,4-DINITROPHENOL, DEOXYCHOLATE, AND TRITON ON A CARDIAC MICROSOMAL ATPASE*

Experiment	Addition	Mg (A)		Mg + Na + K (B)	
		1	2	1	2
1	None	24	33	99	54
	DNP. 0·1	24	33	104	33
2	None	52	3	74	13
	DOC, 0·001%	49	3	73	13
	DOC, 0·01%	42	2	60	11
	DOC, 0·1%	21	1	23	1
	DOC, 0·2%	12	6	8	0·4
	DOC, 0·4%	7		7	
3	None	17	8	75	11
	Trit, 0·1%	11	1	13	1

* This table represents part of a more complete study (in preparation). The conditions of incubation and assay were the same as in the previous tables. DNP = 2,4-dinitrophenol; DOC = deoxycholate; Trit = Triton X-100.

DISCUSSION

The results reported in this paper lend further support to the presence of an ATPase system concerned with cation transport which is associated with the sarcoplasmic reticulum of heart muscle. The activity of this cardiac microsomal ATPase is markedly altered by ageing. While the specific activity is depressed, the activity ratio is greatly enhanced.[1] The results of the electron micrographic studies suggest the interesting possibility that a swelling and/or membrane alteration of the 'microsomal vesicles' may somehow be responsible for this ageing effect.

The enzyme complex appears to consist of a Mg^{2+}-dependent and a $Mg^{2+} + Na^+ + K^+$-dependent enzyme or site, and is similar in some respects to membrane ATPases isolated from brain[18–20] and other tissues.[4, 8, 21–24]

These conclusions are based in part upon the preferential effects of a number of inhibitory agents on the enzyme systems. Ethyl alcohol and amytal, for example, are representative of a number of compounds that affect the $Mg^{2+} + Na^+ + K^+$ activity to a much greater extent than they do the Mg^{2+}-ATPase activity. The marked sensitivity of the enzyme to alcohol was first reported by Jarnefelt[18] for a brain microsomal ATPase preparation.

This effect appears to be similar to the inhibitory action of detergent-like substances such as DOC on brain microsomal ATPase[15] and, in the present study, DOC and Triton X-100 on the heart preparation, which suggests that a lipid or a lipoprotein structure may be necessary for at least part of the microsomal ATPase activity.

The marked effect of amytal on the $Na^+ + K^+$-stimulated activity points to a possible involvement of flavoprotein in the microsomal ATPase, an observation that has been reported by Skou for a brain preparation.[15] The concentrations of amytal used were in a range which have been shown to inhibit the flavoprotein site of the mitochondrial NADH–cytochrome c–reductase system.[25] The action of amytal, in the present study, probably is not due to mitochondrial contamination, since the electron

micrographs revealed no mitochondria or mitochondrial fragments. In addition DNP, in concentrations that normally produce an increase of mitochondrial ATPase activity, had little or no effect on the microsomal ATPase.

Observations presented in the present investigation clearly indicate the specificity of both POMB and of ouabain on the Mg^{2+}-dependent, $Na^+ + K^+$-stimulated component of the heart microsomal ATPase. Neither POMB nor other –SH inhibitors (e.g. N-ethylmaleimide, $HgCl_2$, iodoacetamide, salyrgan) affected to any significant degree the basic Mg^{2+}-ATPase activity. Some of these agents did, however, markedly depress the Mg^{2+} activity of microsomal ATPases isolated from brain (Ref. 13 and Fig. 6). This may be of basic functional significance, but only after further enzyme purification and characterization studies can this be revealed. It should be emphasized here that the microsomal ATPase of heart muscle varies in specific activity and in 'activity ratio' depending upon the procedure of isolation and the method and length of storage of the preparation. This is readily seen in the tables and figures of the present study. Of importance, however, is that fact that, regardless of these variations in activities, the effects of the various reagents employed are quite consistent. This was true, furthermore, regardless of the method of enzyme-inhibitor reaction—i.e. whether the inhibitor was preincubated with the enzyme or added before the enzyme or substrate in the presence or absence of ions.

Sodium azide, which is representative of a number of agents that preferentially inhibit the Mg^{2+}-ATPase activity, has a rather interesting effect on the residual (i.e. the $Na^+ + K^+$ stimulation) enzyme activity. The sensitivity of the $Mg^{2+} + Na^+ + K^+$ activity to POMB, NEM, or to ouabain is markedly enhanced after azide treatment. This represents another difference between the ATPase from the heart and that isolated from brain, in that azide was shown to have little or no effect on the brain enzyme in concentrations that exerted a considerable inhibitory effect on the heart muscle enzyme. The action of azide and the effects of guanidine, which were similar to azide, and of its methyl substituted derivative, suggest the possible requirements of certain charged groups in the protein for enzyme activity. Habeeb[26] and Stracher and Chan[16] indicate that guanidation of proteins, e.g., modifies $-NH_3^+$ groups without causing drastic alterations in the tertiary structure of the protein. This may account for the markedly diminished inhibitory effect of methylguanidine, as compared to guanidine, on the microsomal ATPase. The possible importance of charged groups for the microsomal ATPase activity is further emphasized by the effects of trinitrobenzenesulfonic acid. By a substitution of a trinitrophenyl group on the ϵ-amino of lysine in the protein, it is thought that TBS eliminates the positive charge of amino groups.[27] If this is true in the present studies, it may account for the inhibition observed in the microsomal ATPase from brain[20] and cardiac tissue after TBS treatment.

Identification of the azide, guanidine, and TBS reactive sites in the microsomal ATPase systems is in progress. It is hoped that these studies may yield more information on the mechanism of inhibition of sulfhydryl reagents and their relationship to the action of the cardiac glycosides on the microsomal ATPase of heart muscle.

A remark of caution concerning the action of ouabain on the microsomal ATPases, on active cation transport in isolated systems,[2] and their possible relationship to therapeutic effects on heart muscle is in order. The results in the present investigation, with respect to the cardiac glycoside effects, are based upon the use of a high concentration of drug (10^{-4} M), a concentration which is probably in the toxic range.[28]

Other investigators have also used high levels of glycosides in their inhibitory studies of membrane ATPases,[3, 4, 6, 7, 18, 22–24] although very much lower levels of digitalis drugs have been employed in a few studies[20, 29] and unpublished data. Any relation between these observations on isolated *in-vitro* systems and possible beneficial clinical effects of the cardiac glycosides, however, is still obscure.

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