THE ROLE OF SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATASE IN CARDIAC FUNCTION

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ABSTRACT

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By

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The role of (Na^+-K^+)-ATPase in cardiac function was explored by using a relatively specific inhibitor of this enzyme system, ouabain, and comparing the effect of this drug in several mammalian species.

A method to prepare a highly active (Na^+-K^+)-ATPase preparation with low Mg^{++}-ATPase activity and applicable to cardiac tissue of several species was established. This method utilizes deoxycholic acid and LiBr treatment of microsomal fractions.

Maximal stimulation of (Na^+-K^+)-ATPase activity was obtained, in the presence of 100 mM NaCl and 5 mM $MgCl_2$, with KCl concentrations between 15 and 25 mM in all species.

The apparent dissociation constants (K_s) for the potassium-enzyme complex of the enzymes from dog, pig, sheep, guinea pig, and rat, indicate that the affinity of cardiac (Na^+-K^+)-ATPase for K^+ was similar in all five species.

In vitro sensitivity of (Na^+-K^+)-ATPase to ouabain was compared in these animals, and the order of species sensitivity was found to be: dog>pig>sheep>guinea pig>>>rat.

Ouabain sensitivity of the enzyme from the various species was determined by estimation of the apparent inhibitor constants (Ki). The Ki values indicate that the order of affinity for ouabain is: dog>pig and sheep>guinea pig>>rat. This suggests that the species difference in sensitivity to ouabain is due to a difference in enzyme affinity for ouabain.

The interaction between ouabain and (Na^+-K^+)-ATPase was examined under different KCl concentrations. Hunter-Downs plots constructed for each animal species indicated a competitive nature of the inhibition for ouabain in respect to K^+ at KCl concentrations up to 25 mM and a noncompetitive inhibition above that concentration.

To determine a quantitative relationship between the in vitro effects to ouabain and in vivo potencies of the drug, ouabain was administered to various species by intravenous infusion and blood pressure and ECG monitored. Amounts of ouabain needed to produce blood pressure and ECG changes in the rat, guinea pig, sheep, and dog were in good agreement with the concentration of ouabain necessary to inhibit 25 percent of the enzyme activity in vitro. In rats, females were more sensitive to ouabain than males. Female rats pretreated with phenobarbital were less sensitive to ouabain. It is concluded that there is a significant

correlation between the *in vivo* and *in vitro* potency of ouabain in these species.

A significant correlation was observed between the prolongation of the P-Q interval and the reduction of cardiac (Na^+-K^+)-ATPase activity in ouabain infused dogs. Thus, a dose of ouabain that elicited changes in physiological parameters resulted in an inhibition of cardiac (Na^+-K^+)-ATPase activity.

The effect of ouabain infusion on cardiac contractile force, electrocardiogram, blood pressure, and cardiac adenosine triphosphatase activity was studied in the dog. A constant infusion of ouabain produced an increase in cardiac contractile force and inhibition of atrioventricular conduction. A significant correlation was observed between enhancement of cardiac contractile force and inhibition of cardiac (Na^+-K^+)-ATPase activity. Isoproterenol infusion or sympathetic stimulation increased cardiac contractile force but failed to alter cardiac (Na^+-K^+)-ATPase activity. Addition of KCl during ouabain infusion prevented arrhythmias but failed to influence the inotropic stimulation and the reduction of cardiac (Na^+-K^+)-ATPase activity. (Na^+-K^+)-ATPase isolated from kidney, brain, and skeletal muscle of ouabain infused dogs had activities similar to controls.

It appears from this data that a significant portion of the cardiac (Na^+-K^+)-ATPase activity is inhibited during the positive inotropic action of ouabain.

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INTRODUCTION

The sodium-potassium activated adenosine triphosphatase [(Na^+-K^+)-ATPase]: Activity, distribution, and possible role in ion transport.

Enzyme preparations which hydrolyze adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and inorganic phosphate (Pi), and thus release the energy from the high energy terminal phosphate bond of ATP, have been known for some time. Since ATP is almost exclusively utilized as the energy source in living organisms for chemical, electrical, and mechanical processes, it is reasonable to assume that any enzyme that is associated with a mechanism which requires energy can hydrolyze ATP and may be recognized as an ATP phosphohydrolase (ATPase)[1] under appropriate conditions. Furthermore, a system which synthetizes ATP may also have ATPase activity if the synthetic reaction can proceed in the reverse direction. Therefore, ATPases should be abundant in living organisms.

The first report of the presence of such an enzyme was made by Engelhardt (1930) who observed that tissue preparations have the ability to hydrolyze labile phosphate esters.

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[1]An enzyme which hydrolyzes ATP to ADP and Pi is called an ATPase and that which hydrolyzes ATP to AMP and pyrophosphate is called an apyrase.

The same author (1939) also reported the ATPase activity of myofibrils, later related to myofibril contraction by Szent-Gyorgi (1945).

Aged or 2,4-dinitrophenol-treated liver mitochondrial fractions were also shown to possess ATPase activity (Kielly and Kielly, 1951; Hunter, 1951; Potter et al., 1953).

The presence of ATPase activity in liver microsomal fractions (Jones and Ernster, 1960) from nerve tissue (Abood and Gerard, 1954), and in the sarcotubular system of heart muscle (Kielly and Meyerhof, 1948; Kielly and Meyerhof, 1950; Muschatello et al., 1961) was also reported.

In 1950, Utter reported a Na^+-dependent ATPase activity of brain homogenate. Since the stimulation obtained with sodium was relatively small, the author did not recognize the possible significance of this stimulation.

In 1957, Skou reported the presence of an enzyme which was isolated from the peripheral nerve of sea shore crab which hydrolyzed ATP into ADP and Pi and was activated by Mg^{++}. It could be further stimulated several fold by the simultaneous addition of Na^+ and K^+.

Skou postulated that this enzyme system could be responsible for the transport of Na^+ and K^+ across cell membranes for the following reasons: (1) the enzyme system was found in the "microsomal fraction" (which is considered to be an organelle originating from the cell membrane and the endoplasmic reticulum); (2) was stimulated by Na^+; (3) had cation requirements which included K^+; and (4) hydrolyzed ATP.

If this were the case, this enzyme system would be responsible for maintaining the transmembrane cation gradients, which in turn regulate the excitability of cells. Since transmembrane cation gradients are observed in all cellular tissues, this enzyme system should then be found in all such animal tissues. One would expect highest specific activity of this enzyme in excitable tissues where replenishment of cation gradients is frequently required.

The most comprehensive study on the distribution of this enzyme system was that of Bonting et al. (1961). They found significant (Na^+-K^+)-ATPase activity in 29 of 36 different tissues studied in the cat. The highest specific activities were found in nervous tissue and in tissues such as the kidney and retina which are concerned with active transport of ions. Seven tissues where (Na^+-K^+)-ATPase activity was absent were the cornea stroma, whole lens, lens capsule, lens fiber, vitreous, adipose tissue and blood serum. They also studied some 21 tissues of 10 different animal species all of which were found to have significant (Na^+-K^+)-ATPase activity (1962). Thus, the enzyme system has been shown to be widely distributed throughout animal tissues where one would expect active cation transport to play a major role in cellular function.

Before a correlation can be made between this (Na^+-K^+)-ATPase system and active cation transport, however, certain requirements for an active cation transport system must be satisfied. Ussing (1960) found that increases in the rate

of tissue oxygen consumption in the giant cells of the alga, Halicystis ovalis, were always proportional to the rate of Na^+ transport, and that Na^+ was transported against an electrochemical gradient, as determined by intracellular and extracellular ion measurements. Also, Post et al. (1966) working with human red blood cells, found an absence of cationic transport with low intracellular Na^+ concentrations or low extracellular K^+ concentrations. The data of Ussing and Post et al. indicate that Na^+ is actively transported from the cytoplasm to the interstitial fluid through cellular membranes and that this transport is dependent upon the intracellular Na^+ and the interstitial K^+ concentrations. It would appear that the enzyme responsible for this transport must be closely related to the cellular membrane and have sites on the inside and outside of the membrane which have specific affinities for Na^+ and K^+, respectively.

The (Na^+-K^+)-ATPase system is found in the cell membrane fraction of liver cells (Emmelot and Bos, 1962), as well as in the microsomal fraction of differentially centrifuged tissue homogenates (Hanson and Toschi, 1959; Glynn, 1962; Hoffman, 1960; Hoffman, 1962; Whittam, 1962; and Bonting and Caravaggio, 1962), and is believed to be an integral part of the cell membrane, since attempts to solubilize the enzyme from cell membranes or microsomal fractions have not been successful.

Baker (1963) has further demonstrated in the intact nerve of the walking legs of the spider crab (Maia squindo) that Na^+ is needed in the cytoplasm and K^+ is needed outside

the cellular membrane for enzyme activity to occur. By measuring the Pi released as an indication of enzyme activity in the incubation mixtures of intact nerves, he observed that high intracellular Na^+ concentrations stimulated enzyme activity whereas low intracellular K^+ concentrations resulted in low enzyme activity. Also, high extracellular K^+ concentrations activated enzyme activity while low extracellular K^+ concentrations resulted in low activity. In addition, a kinetic analysis of the effect of cations on the enzyme system by Skou (1960) indicated that the enzyme had two sites with specific affinities for Na^+ and K^+. When Mg^{++} alone was present in the incubation mixture, the addition of Na^+ caused a slight increase in enzyme activity above the Mg^{++}-activated activity. However, with both Mg^{++} and Na^+ present, the addition of K^+ or other cations such as NH^+_4, Cs^+, Th^+, Rb^+, and Li^+ in concentrations ranging from 5 mM to 120 mM, greatly enhanced enzyme activity. Increasing the concentration of K^+ above 120 mM, however, caused an inhibition of enzyme activity. This is believed to be the result of the K^+ displacing the Na^+ from the Na^+ site. From these data, Skou calculated that at one site on the enzyme the affinity for Na^+ is 6-7 times higher than that for K^+ and that at another site on the enzyme the affinity for K^+ is several times higher than the affinity for Na^+. It is evident, then, that the basic requirements of this enzyme for cations mentioned above have been met.

It has been shown by several authors that the energy necessary for cation transport is supplied by ATP (Caldwell,

1956; Dunham, 1957; and Hoffman, 1960). Inosinetriphosphate (ITP) can support the cation transport less effectively, but other nucleotides are ineffective (Hoffman, 1960). Therefore, if this enzyme is responsible for this transport, its activity must require ATP specifically. ATP is preferentially hydrolyzed by this enzyme (Hoffman and Ryan, 1960) and other substrates such as ITP, guanosinetriphosphate (GTP), and uridinetriphosphate (UTP) cannot replace ATP (Hoffman and Ryan, 1960; Post et al., 1960; and Skou, 1960). ITP can be hydrolyzed at a reduced velocity by this enzyme system, but this is minimal (Hoffman and Ryan, 1960; Post et al., 1960; and Skou, 1960). Thus, the nucleotide specificity for both cation transport and this enzyme system is similar.

Cardiac glycosides and ion movement.

It has been shown by Schatzman (1953) that cation transport in the erythrocyte cell membrane is inhibited by relatively low concentrations of cardiac glycosides. This observation has also been supported by the work of other investigators (Glynn, 1957; Kofoed and Johnsen, 1958; Whittam, 1958). The (Na^+-K^+)-ATPase system is also inhibited by low concentrations of glycosides, as reported by many investigators (Auditore and Murray, 1962; Repke et al., 1965; Schwartz and Laseter, 1964; and others). This inhibition is specific for the (Na^+-K^+)-stimulated activity and ouabain has no effect on the Mg^{++}-stimulated activity. It is important to note also that concentrations of cardiac

glycosides which inhibit cation transport in the intact erythrocyte are similar to the concentrations of cardiac glycosides necessary to inhibit the (Na^+-K^+)-activated enzyme system of erythrocyte cell membranes (Post et al., 1960; and Dunham, 1961). Also, a correlation has been shown between the inhibitory effect of different types of cardiac glycosides on cation transport and the enzyme activity, namely, glycosides which are potent inhibitors of cation transport are also potent inhibitors of the enzyme activity, and weak inhibitors of cation transport are also weak inhibitors of the enzyme activity (Dunham, 1961). It is significant also that the inhibition of cation transport in the red blood cell by cardiac glycosides is antagonized by increases in K^+ in the external environment, and that the binding of ouabain to the enzyme is also antagonized by potassium (Schwartz, 1969).

Since this enzyme fulfills all the stated requirements for cation transport, it seems probable that the (Na^+-K^+)-ATPase enzyme system described above is responsible for the transport of Na^+ and K^+ across cell membranes.

Several authors have postulated a mechanism for this coupled cation transport. One such hypothesis, proposed by Albers et al. (1968) is shown in Figure 1. When Na^+ is attached to the enzyme at the intracellular site, a high energy phosphate bond can be formed on the enzyme via ATP (step 1). This high energy bond results in a conformational change of the enzyme protein which enables the Na^+ ions to be transported to the outside of the cell membrane (step 2).

At this conformation, the cation sites have a higher affinity for K^+ than Na^+, and therefore, K^+ replaces the Na^+ at the cationic sites (step 3). When K^+ is attached to the enzyme, hydrolysis takes place with the release of inorganic phosphate (step 4). This dephosphorylation of the phosphorylated enzyme allows a second conformational shift such that K^+ is transported to the inside of the cell (step 5). At this point Na^+ displaces the K^+ because the cationic sites now have a greater affinity for Na^+ at this conformation. This completes the transport cycle and the process can then repeat itself.

This model may not be entirely correct but it does demonstrate a plausible mechanism which incorporates much of the present data concerning cationic transport.

(Na^+-K^+)-ATPase activity in cardiac tissue.

Since the agents which specifically inhibit (Na^+-K^+)-ATPase from red blood cells, kidney, and brain are cardiac glycosides, attention was focused on their action on the heart (Na^+-K^+)-ATPase system.

As Bonting et al. (1961) has demonstrated, the (Na^+-K^+)-ATPase enzyme system is widely distributed in animal tissues and is highest in activity in nervous tissue and in tissues concerned with active transport of cations such as the kidney. As this enzyme system would be responsible for maintaining cation gradients, and thereby, cell excitability, and since it is essential that cardiac cells maintain themselves in the excitable state for proper

functioning, one would expect to find relatively high (Na^+-K^+)-ATPase activity in cardiac tissue. However, attempts at isolating the (Na^+-K^+)-ATPase from cardiac tissue by differential centrifugation, which is easily accomplished with tissues such as the crab nerve (Skou, 1957), the electric organ of the Electrophorus electricus (Albers et al., 1963; Albers and Koval, 1962; Bonting and Caravaggio, 1963; and Glynn, 1962) and the brain and kidney of the rat (Skou, 1962) resulted in enzyme preparations with high Mg^{++}-ATPase activity but with little or no (Na^+-K^+)-ATPase activity.

The actomyosin system of the cardiac cells is thought to be responsible for the high Mg^{++}-ATPase activity. The problem, then, in the preparation of the enzyme from cardiac tissue is to reduce the Mg^{++}-ATPase activity and isolate a fraction which has high (Na^+-K^+)-ATPase activity.

Several attempts have been made to isolate a highly active (Na^+-K^+)-ATPase from cardiac tissue (Bonting, 1961; Schwartz, 1962; Auditore and Murray, 1962; Auditore, 1965; Matsui and Schwartz, 1966; Tashima, 1966; Potter et al., 1966; and Portius and Repke, 1967). The addition of deoxycholate and EDTA to cardiac muscle homogenates has resulted in some cases with a $Na^+-K^+-Mg^{++}/Mg^{++}$ activity ratio of 2 or higher (Caldwell et al., 1960; Schwartz, 1962; and Skou, 1962). Previous activity ratios were only slightly greater than one. Storing of the deoxycholate and EDTA tissue homogenates has increased further the activity ratio by lowering the Mg^{++}-stimulated activity more than the

(Na^+-K^+)-ATPase activity (Schwartz, 1962). Sodium iodide (Matsui and Schwartz, 1966), NaN_3 (Auditore, 1965), and LiBr (Potter, 1966; and Portius and Repke, 1967) treatments of various subcellular fractions have also been found to significantly increase the $Na^+-K^+-Mg^{++}/Mg^{++}$ activity ratios of these fractions prepared from cardiac tissue. One such method reported by Matsui and Schwartz (1966) for isolating the (Na^+-K^+)-ATPase system from beef heart tissue with deoxycholate acid and NaI treatment made it possible to obtain an enzyme preparation with high specific (Na^+-K^+)-ATPase activity and a high $Na^+-K^+-Mg^{++}/Mg^{++}$ ratio. However, the method is not practically applicable to smaller animals because of the large quantities of starting material required. A method which produces cardiac (Na^+-K^+)-ATPase preparations suitable for kinetic analysis and drug studies and which is applicable to various animal species has hitherto not been reported.

Species difference in glycoside activity.

Since cardiac glycosides have been shown to greatly influence the cardiac (Na^+-K^+)-ATPase enzyme system in low concentrations, and since this enzyme, as previously mentioned, is presumably responsible for maintaining the cation gradients across cellular membranes, many investigators feel that the inotropic action of the cardiac glycosides is related to its effect on this enzyme system.

There are presently two opposing views of the subject. One is that the positive inotropic changes induced by

cardiac glycosides is a result of the stimulation of the (Na^+-K^+)-ATPase enzyme system. This is supported by the work of several investigators. Lee and Yu (1963) have reported a stimulation of up to 25% of the (Na^+-K^+)-ATPase obtained from guinea pig hearts with a ouabain concentration of 10^{-11} M. Brown (1964) also observed a stimulation of as much as 23% of the enzyme activity obtained from rabbit hearts with a ouabain concentration of 10^{-4} M. However, with only slight alterations in the isolation procedure, he obtained enzymes which were inhibited up to 98% by the same concentration of ouabain. Repke (1963) using guinea pig cardiac tissue also observed a 30% stimulation with a ouabain concentration of 5×10^{-10} M. Palmer (1964) reported a 21% stimulation at 10^{-9} M ouabain in the chicken kidney and has postulated that this type of stimulation could occur with the cardiac enzyme.

In each case of stimulation mentioned above, the range of the concentrations of the drug which caused the stimulation was rather narrow and the inhibition of the enzyme occurred with higher concentrations of ouabain. This biphasic action of cardiac glycosides on (Na^+-K^+)-ATPase was considered by these authors to be related to the biphasic action of these drugs on the heart.

Several investigators have reported inhibition of the enzyme system with concentrations of ouabain greater than 10^{-8} M (Auditore and Murray, 1962; Repke *et al.*, 1965; Schwartz and Laseter, 1964; and others). In addition, Repke *et al.* (1963) have reported a 38-62% inhibition of the

(Na^+-K^+)-ATPase activity of guinea pig cardiac muscle at inotropic doses of ouabain (0.8 μM to 2.7 μM). Thus, the question of whether an increase or a decrease in enzyme activity is related to the positive inotropic effect is unresolved.

It has been shown that there is a considerable species variation in sensitivity to the cardiac glycosides, and that this difference cannot be explained in terms of different rates of metabolism among species (Herrmann and Repke, 1964).

If the (Na^+-K^+)-ATPase is the primary site of action of these compounds, the observed in vivo specie sensitivities to the glycosides may be correlated with the in vitro enzyme sensitivities to these drugs. Such a correlation was reported by Repke (1965). He prepared (Na^+-K^+)-ATPase from several animal species and found a significant correlation between the enzyme sensitivities and the reported in vivo responses to the cardiac glycosides. He also observed a correlation among the toxicity of various cardiac glycosides and their ability to inhibit the cardiac (Na^+-K^+)-ATPase (1965). However, in these studies, different methods of enzyme preparations were used with each species, and the (Na^+-K^+)-ATPase activities reported were relatively low. Further comparisons of in vitro and in vivo sensitivities using preparations with higher (Na^+-K^+)-ATPase activity are needed. Such studies were performed in our laboratory and are reported as a portion of this thesis.

Allen and Schwartz (1969) has demonstrated a difference in the binding of the cardiac glycosides to the enzyme in different species. He found that the (Na^+-K^+)-ATPases prepared from beef and dog cardiac tissues were much more sensitive to ouabain inhibition than enzymes prepared from rat cardiac tissue. When comparing the amount of H^3-labeled glycoside bound to the enzyme, he found that rat enzyme preparation was capable of binding 2 to 3 times more drug per unit of enzyme activity than beef or dog enzyme preparations. Also, by washing the H^3-labeled glycoside enzyme preparations with unlabeled ouabain (10^{-3} M) and then measuring the changes in radioactivity, he found that the binding of the H^3 glycosides to the (Na^+-K^+)-ATPases of the sensitive species was considerably stronger than the binding of the labeled glycoside to the insensitive species.

It seems reasonable to assume, therefore, that the differences in the inhibitory effect of cardiac glycosides on cardiac (Na^+-K^+)-ATPase activity could be due to the variability in binding of the drug to the enzyme.

Purpose of the present study.

It was the purpose of this study to examine further the role of (Na^+-K^+)-ATPase in cardiac function and to explore the primary site of action of the cardiac glycosides.

The objectives of this investigation were as follows:

1. To establish a method for the isolation of a cardiac enzyme preparation with high (Na^+-K^+)-ATPase activity and low Mg^{++}-ATPase contamination from a relatively

small amount of tissue so that the method could be applicable to small animals. Such an enzyme preparation is necessary for kinetic studies and for the comparison of the enzyme among different animal species.

2. To compare the kinetic behavior of cardiac (Na^+-K^+)-ATPase obtained from various animals. Since our method of isolation was different from those previously described, we needed to determine the specific ion requirements for the enzymes from all the species studied.
3. To compare ouabain sensitivites of cardiac (Na^+-K^+)-ATPase preparations obtained from various animals. The species difference in <u>in vivo</u> sensitivity to ouabain is well known (Barnes and Eltherington, 1964), and Repke's group has demonstrated that the difference in sensitivity is not accounted for by the difference in the capability of animals to metabolize or detoxicate the drug (Herrmann and Repke, 1964). Since the effect of ouabain on enzyme activity is dependent upon the concentration of K^+ present (Ahmed and Judah, 1965) it would be reasonable to assume that an enzyme with a greater affinity for K^+ would be less susceptible to ouabain inhibition. Such a relationship was examined by measuring the inhibition of enzyme activity by ouabain at various K^+ concentrations.
4. To correlate the <u>in vitro</u> enzyme sensitivity to ouabain with observed <u>in vivo</u> sensitivity to the drug in various species. This was accomplished by comparing the amount of ouabain necessary to elicit measurable physiological

changes in the animal *in vivo* with that necessary to inhibit (Na^+-K^+)-ATPase *in vitro*.

5. To examine the role of biotransformation in modifying the effect of infused ouabain. Quantities of ouabain necessary to produce specific physiological changes in male and female rats were compared. Ouabain sensitivity, *in vivo*, was further studied in female rats which were treated with Na-phenobarbital preceding ouabain infusion to induce *de novo* synthesis of protein (Conney, 1967).
6. To determine if (Na^+-K^+)-ATPase activity is inhibited *in vivo* during the action of ouabain. Enzymes prepared from ouabain infused animals in which specific physiological changes were observed were compared to the enzymes prepared from control animals.
7. To determine whether the inhibition of the enzyme system is correlated with the inotropic effects of ouabain. (Na^+-K^+)-ATPase activities of the enzyme preparations obtained from dogs which had demonstrated specific increases in cardiac contractile force after the infusion of ouabain were compared with those from control animals.
8. To determine the effects of prolonged increases in cardiac contractile force on the (Na^+-K^+)-ATPase. Significant cardiac enhancement in contractile force was maintained in the dog by isoproterenol infusion, or prolonged stimulation of the stellate ganglion. The (Na^+-K^+)-ATPase activity of enzyme preparations obtained from these animals were compared with those obtained from control and ouabain infused animals.

METHODS

Isolation of the (Na^+-K^+)-ATPase.

Ventricular muscle was obtained from female dogs (12-19 kg), female guinea pigs (360-500 g) and male Sprague-Dawley rats (210-250 g). Pig and sheep heart were obtained fresh from a slaughter house and frozen and stored. They were thawed prior to use. Ten to twelve guinea pig or rat hearts were pooled when necessary. In some studies the dog hearts were frozen and thawed prior to use on the following day. There was no significant difference in (Na^+-K^+)-ATPase activity of those hearts frozen and thawed and those that were prepared immediately.

A. The method for the isolation of a microsomal fraction from rabbit heart described by Auditore and Murray (1962) was applied to rat heart. Fifteen grams of cardiac muscle obtained from rats were homogenized in 60 ml of a solution containing 0.25M sucrose, 5.0 mM histidine HCl, 5.0 mM EDTA, and 0.2% sodium free deoxycholate (DOC) adjusted to pH 6.8 by 0.5M Tris solution. This homogenate was centrifuged at 12,000 x g for 15 minutes. The supernatant was centrifuged again at 35,000 x g for 30 minutes and the resulting sediment of the second centrifugation was resuspended in a 0.25M sucrose solution containing 5.0 mM histidine HCl, and

1.0 mM EDTA (pH 7.0). This solution was then centrifuged at 35,000 x g for 30 minutes, and the sediment was resuspended in 5.0 ml of the resuspending solution mentioned above.

B. Approximately 2.5 grams of guinea pig or rat heart muscle were homogenized in 5 volumes of a solution containing 0.25M mannitol, 5.0 mM EDTA, 30 mM histidine HCl and 0.1% DOC (pH 6.8). A microsomal fraction was then prepared as in method A using a resuspending solution containing 0.25M mannitol, 1.0 mM EDTA, 30 mM Tris HCl buffer (pH 7.4).

In some instances the enzyme suspension was further aged for 24 hrs with 0.1% DOC at 2°C.

C. A microsomal fraction was prepared as described in method A starting from 2.5 g of rat heart muscle and using a 0.32M sucrose solution (without added histidine, EDTA, or DOC) as the homogenizing solution and as the resuspending solution. The final suspension was stored frozen for 48 or 72 hrs, or treated with 0.1% DOC for 48 or 72 hrs at 2°C or dialyzed against distilled water for 24 hrs at 2°C before use.

D. Approximately 2.5 grams of rat heart muscle were homogenized in 5 volumes of a solution containing 0.32M sucrose and 1.0 mM EDTA. This homogenate was centrifuged at 12,000 x g for 15 minutes. The supernatant was then centrifuged at 100,000 x g for 30 minutes and the resulting sediment of the second centrifugation was resuspended in a solution containing 0.32M sucrose, 1.0 mM EDTA and centrifuged again at

100,000 x g for 30 minutes. The sediment was then resuspended in 5 ml of the above mentioned resuspending solution.

E. A microsomal fraction was isolated as in method D with 2.5 grams of rat heart muscle using a 0.32M sucrose solution previously treated with amberlite CG 120 H+ form to remove Ca^{++}. Frozen and thawed enzyme preparations and enzyme preparations treated with 0.1% DOC for 24 hrs at 2°C were examined for enzyme activity.

F. A microsomal fraction was isolated as in method D with 2.5 g of rat heart tissue using a homogenizing solution containing 0.25M sucrose, 5.0 mM histidine HCl, 5.0 mM EDTA, 0.2% DOC (pH 6.8). A resuspending solution containing 0.25M sucrose, 5.0 mM histidine HCl, and 1.0 mM EDTA, with a pH of 7.0 was also used. The final suspensions were either frozen for 24 hrs or aged in 0.1% DOC for 24 hrs at 2°C before they were assayed for activity.

G. A microsomal fraction was isolated as in method D with 2.5 g of rat heart tissue using a solution containing 0.32M mannitol, 5.0 mM histidine HCl and 1.0 mM EDTA, as the homogenizing and resuspending solution. Enzyme preparations were frozen for 24 hrs, kept in resuspending solution for 24 hrs at 2°C, or treated with 0.05% and 0.025% DOC for 24 hrs at 2°C before the assay.

H. A microsomal fraction was isolated as in method D from 2.5 g of rat heart using a homogenizing solution containing 0.25M sucrose, 5.0 mM histidine HCl, 5.0 mM EDTA, and 0.2% DOC with a pH of 6.8 and a resuspending solution containing 0.25M sucrose, 1.0 mM EDTA, 5.0 mM histidine HCl, with a

pH of 7.0. The enzyme preparation was then kept in resuspending solution or 0.05%, 0.025%, and 0.1% DOC for 24 hrs.

I. The method for the isolation of cell membranes from sheep and guinea pig as described by Potter et al. (1966) was applied with some modification to several species. Fifteen grams of guinea pig, dog, sheep or pig heart muscle were homogenized in 9 volumes of a solution containing 1.0 mM Tris-HCl, 1.0 mM EDTA, with a pH of 6.8. This homogenate was then centrifuged at 1,000 x g for 20 minutes. The sediment was then resuspended in the homogenizing solution mentioned above and centrifuged again at 1,000 x g for 20 minutes. The sediment was then resuspended in 1M LiBr (5 ml/one gram of original tissue) and kept at 2°C for 15, 30, 60 and 180 minutes, or overnight (16-20 hrs). These solutions were then centrifuged at 1,000 x g for 20 minutes, resuspended in a solution containing 1.0 mM Tris-HCl, 1.0 mM EDTA (pH 6.8) and centrifuged at 1,000 x g for 20 minutes. The sediments were then resuspended in 1.0 mM Tris-HCl solution (pH 7.6).

J. An enzyme suspension prepared with method H using dog heart muscle was layered on the top of a discontinuous sucrose gradient (0.8, 1.2 and 1.6M sucrose) and centrifuged in a Spinco SW 50 swinging bucket roto at 100,000 x g for 90 minutes. A heavy and a light light fraction were harvested from the interfaces of the 0.8 and 1.2M sucrose and 1.6M sucrose layers, respectively.

K. Ten grams of pig heart muscle were homogenized in 9 volumes of a solution containing 1.0 mM Tris-HCl, 1.0 mM EDTA, and a pH of 6.8. This homogenate was centrifuged at 1,000 x g for 20 minutes. The sediment was then resuspended in the above homogenizing solution and centrifuged again at 1,000 x g for 20 minutes. This sediment was resuspended in 1.0 mM Tris-HCl with a pH of 7.6 (nuclear fraction). The supernatant of the first centrifugation was centrifuged again at 10,000 x g for 20 minutes. After the centrifugation the sediment was resuspended in 1.0 mM Tris-HCl, pH 7.6 (mitochondrial fraction) and the supernatant was centrifuged further at 100,000 x g for 30 minutes. The resulting sediment was resuspended in 1.0 mM Tris-HCl pH 7.6 (microsomal fraction) and the supernatant was designated as the soluble fraction. All these fractions were assayed for enzyme activity.

L. The method for the isolation of a cell membrane fraction from rat liver, described by Neville (1960), was applied to pig heart. Ten grams of pig heart were homogenized in 9 volumes of 1.0 mM $NaHCO_3$. This homogenate was diluted further to a 2% homogenate with the 1.0 mM $NaHCO_3$. This solution was allowed to stand for 10 minutes, passed through a gauze mesh, and centrifuged at 1,000 x g for 10 minutes. Sediments were resuspended in 100 ml of 1.0 mM $NaHCO_3$ and centrifuged at 1,000 x g for 10 minutes. The sediment of this centrifugation was then resuspended in 1.0 mM $NaHCO_3$ or in 0.32M sucrose, and layered on the top

of a discontinuous sucrose gradient consisting of 1.2, 1.4 and 1.6M sucrose, and centrifuged as in method J.

Subsequent 1,000 x g fractions obtained from 15 grams of pig or rat heart muscle were resuspended in 1.0M LiBr, (5.0 ml/gram of original tissue) and kept for 15, 30, and 60 min at 2°C instead of purifying with the sucrose gradient centrifugation. These solutions were washed twice by centrifuging at 1,000 x g for 20 minutes and resuspending in 1.0 mM Tris-HCl containing 1.0 mM EDTA.

M. Ten grams of heart tissue from the rat, guinea pig, dog, sheep, or pig were homogenized with a Dounce ball type homogenizer in 4 volumes of a solution containing 0.25M sucrose, 5 mM histidine HCl, 5 mM EDTA, and 0.2% DOC, adjusted to a pH of 6.8 with 0.5M Tris. This homogenate was then passed through a gauze mesh and centrifuged at 100,000 x g for 15 minutes. The supernatants were centrifuged at 100,000 x g for 60 minutes. The sediments were then resuspended in a solution containing 0.25M sucrose, 5.0 mM histidine HCl, and 1.0 mM EDTA with a pH of 7.0. An equal volume of 2.0M LiBr was then added and the combined solution was kept at 2°C for 60 minutes with constant stirring. This solution was then centrifuged at 100,000 x g for 60 minutes and the sediment was washed twice by resuspending in the above resuspending solution and centrifuging at 100,000 x g for 60 minutes. The final suspension was stored frozen until use.

Assay of protein concentration.

The protein concentration of the enzyme preparation was determined prior to the assay of ATPase activity so that equivalent amounts of protein could be utilized in each enzyme assay.

Lowry's method was used routinely for protein determination (Lowry et al., 1951). 3.0 ml of a solution containing 1.96% Na_2CO_3, 0.01% $CuSO_4$, 0.02% Na^+-K^+ tartrate, and 0.4% NaOH were added to 0.3 ml aliquots of appropriately diluted sample solutions. After ten minutes, 0.3 ml of a 1.0N Folin-Ciocalteu phenol reagent was added and mixed immediately. The absorbance at 750 mμ was measured 30 minutes later.

In some instances when the yield of protein from the isolation procedure was large, the biuret method was used for the protein assay because of its simplicity and greater specificity (Methods in Enzymology, 1957). To 0.2 ml aliquots of each sample were added 0.8 ml of doubly distilled water and 4.0 ml of biuret reagent containing 0.19% $CuSO_4$, 0.75% Na-K tartrate, and 3.75% NaOH. After 30 minutes the absorbance at 540 mμ was determined.

Appropriate standards were prepared for both assays using bovine serum albumin fraction V.

Assay of ATPase activity.

The ATPase activity of various enzyme preparations (0.06 mg of protein) was assayed at 37°C in a total volume of 1.0 ml containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM

$MgCl_2$, and 5 mM Tris-ATP, with or without 100 mM NaCl and 15 mM KCl. The reaction was started by adding Tris-ATP after a 5 minute incubation period and was terminated 15 minutes later by the addition of 1.0 ml of ice-cold 15% trichloroacetic acid solution. The mixture was then centrifuged at 1,000 x g for 15 minutes at 2°C. A 1.0 ml aliquot of the supernatant was added to 1.0 ml of doubly distilled water and 2.0 ml of solution containing 4% Fe_2SO_4, 1.0% $(NH_4)_6Mo_7O_{24}.4H_2O$ and 3.3% H_2SO_4.

Mg^{++}-ATPase activity (assayed in the presence of Mg only) was subtracted from the total ATPase activity (assayed in the presence of Na^+, K^+, and Mg^{++})in order to calculate the (Na^+-K^+)-ATPase activity.

In vitro studies on the (Na^+-K^+)-ATPase enzyme system.

In preliminary experiments, various NaCl and KCl concentrations were added to the ATPase incubation mixture to determine the requirements for Na^+ and K^+.

Ouabain was added to the incubation mixture containing $MgCl_2$, KCl, NaCl, Tris-HCl buffer (pH 7.5), and 0.05 mg of protein. After a 5.0 minute preincubation period at 37°C, Tris-ATP was added and the amount of inorganic phosphate released after 15 minutes of incubation at 37°C was determined. ID50's were then determined from the dose response curves which were plotted for each animal species.

The effect of varying KCl concentrations on the percent inhibition of (Na^+-K^+)-ATPase activity by ouabain was also studied. The concentrations of ouabain which were used were

those which resulted in 50 and 75% inhibition of the (Na^+-K^+)-ATPase activity with cation concentrations that gave maximal activity. A constant NaCl concentration of 100 mM and KCl concentrations varying from 0.0 to 15 mM were used in this assay.

In vivo effect of ouabain.

Under pentobarbital anesthesia, the ECG of female dogs, female sheep, female guinea pigs and rats of both sexes were monitored at 2-5 minute intervals using a Grass Model 7 Polygraph recorder. The blood pressure, from the common carotid artery, was also recorded continuously using a blood pressure transducer. Ouabain was administered by a slow intravenous infusion using a Harvard infusion pump. A rate of ouabain infusion was chosen so that atrioventricular (A-V) block occurred between 45 and 90 minutes of infusion. The total volume infused was approximately 1-3 ml per animal. This rate, when calculated for each individual animal, ranged from 0.59-0.86, 0.58-0.81, 1.7-2.9 and 26-30 µg/kg/min for the dog, sheep, guinea pig and rat, respectively.

The role that biological metabolism may play in determining the ouabain sensitivity was examined by pretreating a group of 5 female rats (weighing between 215-260 grams) with Na-phenobarbital (70 mg/kg, i.p.) 24 hours before ouabain infusion (56-70 µg/kg/min). Doses of ouabain necessary to lower the blood pressure below control levels

in these animals and in animals with no phenobarbital pre-treatment were compared.

To calculate ouabain sensitivity on several cardiovascular parameters in various species, the concentration or dose of ouabain producing an effect in each species was divided by that producing the same effect in the dog. The reciprocal of this value was designated as the relative sensitivity.

(Na^+-K^+)-ATPase activity was examined after ouabain infusion in the dog and compared to enzyme activity of control animals. Six of 12 mongrel female dogs (12-19 kg) were infused with ouabain as described above and ECG readings were taken every 5-10 minutes. After increases of 13-42% in the P-Q intervals were observed, the animals hearts were immediately removed by opening the chest wall in the midline, tying off the vessels of the heart, and excising. Hearts from the 6 other dogs which were given only Na-pentobarbital were excised in the same manner after periods of anesthesia equaling those of the infused animals.

(Na^+-K^+)-ATPase was immediately prepared as described in method M from the ventricular muscle and frozen (-20°C). Enzyme activities were then determined the following day.

To compare the inhibition of cardiac (Na^+-K^+)-ATPase activity with changes in cardiac contractility induced by ouabain, female mongrel dogs (weighing 12 to 19 kg) were anesthetized in pentobarbital sodium, 30 mg/kg, i.v. The animals were artificially respired with room air using a

Harvard respirator at a rate of 16/min and a tidal volume of 30 ml/kg. Section of the bilateral cervical vagosympathetic nerves was performed in each animal. Cardiac contractile force was monitored continuously with a strain gauge arch sutured parallel to the left side of the anterior descending coronary artery as described by Gatgounis and Walton (1962). Contractile force, ECG and blood pressure from the right common carotid artery were recorded on a Grass model 7 polygraph.

Drugs were administered through a left jugular vein cannula. Ouabain in 0.9% NaCl solution was infused at a constant rate of 13.5 µl/min. The concentration of ouabain was adjusted so that ouabain was administered at a rate of 0.8 µg/kg/min. The infusion was terminated 20 to 100 minutes later when a significant increase in the contractile force was observed. Dogs in which ouabain infusion was terminated with less than a 30% increase (10-28%) in contractile force were arbitrarily grouped as "low dose" ouabain whereas those with more than a 30% increase (31-101%) were designated as the "high dose" ouabain. In four dogs, KCl solution was administered intravenously in an attempt to restore the normal rhythm immediately after the first sign of arrhythmia was observed (Williams et al., 1966). The simultaneous infusion of ouabain (as above) and KCl (10 to 20 mg/min) was continued for an additional 10 to 25 minutes. Control dogs received 0.9% NaCl solution for time periods similar to ouabain. To examine the effect of prolonged enhancement of cardiac contractility on the (Na^+-K^+)-

ATPase activity, isoproterenol was infused at the initial rate of 2.0 μg/min until a 40% increase in contractile force was observed. This level of increased contractile force was maintained for 20 to 25 minutes by adjusting the rate of isoproterenol infusion. In addition, in three dogs, the left stellate ganglion was exposed through the second intercostal space, and the postganglionic inferior cardiac nerve was stimulated for 20 minutes with bipolar platinum electrodes. A frequency of 5 to 10/second was used with 20 msec square wave stimuli. The voltage was adjusted to maintain a 40% increase in contractile force (10-20 v).

The heart was removed immediately upon the termination of drug infusion or stellate stimulation. Approximately 10 grams of the left ventricular muscle were excised from the apex of the heart, avoiding the portion to which the strain gauge was attached, and stored frozen at -20°C for the analysis of ATPase activity.

To examine the effect of ouabain on the (Na^+-K^+)-ATPase of other than cardiac tissue, the (Na^+-K^+)-ATPase activity of kidneys, brain, and skeletal muscle of five control and nine ouabain infused animals was studied. Microsomal fractions from the renal medulla and the left cerebral hemisphere were isolated as in method A. The enzyme preparation from gracilis muscle was obtained by method M as previously described for cardiac muscle. In this series of studies, 10 μM dithiothreitol (2,3-dihydroxy, 1,4-dithiol butane) was

added to all solutions used in the isolation of the enzyme, except the final resuspending solution.

Statistical studies.

The results were analyzed for significance by the student "t" test using a group comparison, and a correlation coefficient performed as described in Snedecor (1956).

Instruments.

Tissue was homogenized with a Dounce ball-type homogenizer (Blassig Glass Specialties) using both L and T pestles. A Potter homogenizer (Thomas Scientific Company) with a teflon pestle was used for the resuspension of the centrifugation pellet.

The 12,000 x g centrifugation was carried out in a Sorvall Superspeed RC2-B centrifuge using a SS-34 type rotor (Ivan Sorvall, Inc.). Centrifugation above 12,000 x g were run in a Beckman Model L Ultracentrifuge using a number 40 and SW 50 type rotors (Beckman Instruments, Inc.).

The Spectronic 20 (Baush and Lomb) spectrophotometer was used in the protein and ATPase assays.

A Harvard Compact Infusion Pump Model No. 975 (Harvard Apparatus Co., Inc.), Statham P 23 AC transducer (Statham Laboratories, Inc.), Walton-Brodie strain gauge, Grass Model 7 Polygraph and Grass Stimulator Model S4KR (Grass Instrument Co.), and a National cautery (National Electronic Instrument Co., Inc.) were used in the in vivo experiments.

RESULTS

Isolation of the (Na^+-K^+)-ATPase.

It can be seen from Table 1 that fresh enzymes prepared by differential centrifugation of sucrose, mannitol, tris, and $NaHCO_3$ homogenates of cardiac tissue from various animal species (methods A, B, D, I, K, L) with or without DOC, were comparatively high in Mg^{++}-ATPase activity and low in (Na^+-K^+)-ATPase activity. The range of Mg^{++}-ATPase activities with these methods was 19-108 μmoles Pi released per mg protein per hour while the range of (Na^+-K^+)-ATPase activities obtained with the same methods was only 0-22 μmoles Pi released per mg protein per hour. The $Na^+ + K^+ + Mg^{++}$ to Mg^{++} activity ratio was never higher than 1.34 (method A). A high ratio is indicative of minimal contamination of the (Na^+-K^+)-ATPase with the Mg^{++}-ATPase activity.

Enzyme preparations which were kept in various concentrations of DOC for 24 to 72 hours (methods B and C) resulted in slightly higher activity ratios by preferentially decreasing the Mg^{++}-ATPase activity. An activity ratio of 3.45 was obtained with rat heart tissue prepared with method C, however, the (Na^+-K^+)-ATPase activity remained relatively low (6 μmoles Pi released per mg protein per hour).

Attempts to purify the enzyme by differential centrifugation and sucrose gradient separation of tris or $NaHCO_3$ tissue homogenates of guinea pig, dog, or pig cardiac muscle (methods J and L) resulted in enzyme preparations with low (Na^+-K^+)-ATPase activity (0-10 μmoles Pi released per mg protein per hour) as compared to the Mg^{++}-ATPase activity (24-53 μmoles Pi released per mg protein per hour). The highest activity ratio (1.43) was found in the low density fraction of dog heart muscle as prepared in method J.

Dialysis of a microsomal fraction obtained from a sucrose homogenate of rat heart tissue (method C) yielded an enzyme preparation with low Mg^{++}-ATPase activity (25 μmoles Pi released per mg protein per hour) and no measurable (Na^+-K^+)-ATPase activity.

LiBr treated cell membranes obtained from tris homogenates of guinea pig, dog, and pig cardiac muscle (method I) were found to have relatively low (Na^+-K^+)-ATPase activity (0-14 μmoles Pi released per mg protein per hour). However, a ratio of 2.94 was obtained with sheep cardiac muscle with significant (Na^+-K^+)-ATPase activity (14 μmoles Pi released per mg protein per hour).

A combined DOC and LiBr treatment of microsomal fractions prepared from sucrose homogenates of rat, guinea pig, dog, sheep, and pig cardiac muscle (method M) yielded enzyme preparations with good $Na^+ + K^+ + Mg^{++}$ to Mg^{++} activity ratios (2.6, 3.9, 2.7, 6.2, and 7.2 for the rat, guinea pig,

dog, sheep, and pig, respectively) and relatively high (Na^+-K^+)-ATPase activity (10-21 µmoles Pi released per mg protein per hour). The ATPase activities of Na^++K^++Mg^{++} to Mg^{++} activity ratios of cardiac muscle enzyme preparations obtained by this method from the various animal species are shown in Table 2. The values represent the mean ± S.E. of five enzyme preparations from different animals in each species. The lower activity ratios observed with the rat and dog were associated with a high Mg^{++}-ATPase activity.

In vitro study of the (Na^+-K^+)-ATPase.

Potassium effects on the (Na^+-K^+)-ATPase activity in the presence of 100 mM NaCl and 5 mM $MgCl_2$ are shown in Figure 2. Enzyme preparations obtained from various species reacted in a similar manner to potassium. Maximal stimulation of (Na^+-K^+)-ATPase activity was obtained at 12 to 25 mM KCl.

In order to explore the nature of the potassium-enzyme interaction, apparent dissociation constants (K_s) for the potassium-enzyme complex were calculated (Webb, 1963). These values were obtained from linear regression lines, fitted by the method of least squares to the double reciprocal (Lineweaver-Burk) plots of the enzyme activity and KCl concentrations for each species. These data were obtained from that portion of the curve ranging from 1 to 25 mM KCl in Figure 2. The K_s, obtained by extrapolation of the regression line to the x-axis intercept, estimates

the affinity of the enzyme for potassium ion. The K_s values obtained were 0.79, 0.92, 1.06, 1.18 and 0.96 mM KCl for the enzyme from dog, pig, sheep, guinea pig and rat, respectively. Thus, the affinity of the cardiac (Na^+-K^+)-ATPase for potassium was similar in these five species.

Effect of ouabain on cardiac (Na^+-K^+)-ATPase activity in vitro.

Ouabain added to the incubation mixture, containing 15 mM KCl, 100 mM NaCl and 5 mM $MgCl_2$, resulted in a different degree of inhibition with the enzyme from each species. Table 3 shows the ID25, ID50 and ID75 for each species calculated from the dose-response curves (Figure 3). It may be observed that the enzymes from the dog, pig, and sheep hearts have roughly similar sensitivities to ouabain; that from the guinea pig was distinctly less sensitive than the dog heart; that from the rat heart was far less responsive to ouabain. Due to the differences in the slopes of the dose-response curves, the relative sensitivity varied somewhat at different levels of inhibition.

The Hunter-Downs plot (Figure 4, A and B) was used to interpret the interaction between ouabain and (Na^+-K^+)-ATPase in the presence of potassium (Hunter and Downs, 1945). In these figures α is the fractional activity in the presence of inhibitor, and [I] is the inhibitor concentration. Competitive inhibition for substrate (KCl) is indicated when the line intercepts both x- and y-axis, whereas non-competitive

inhibition is indicated by a line which intercepts only the y-axis (Webb, 1963). This type of plot may also be used to describe uncompetitive inhibition. The intercept of the line with the y-axis estimates the apparent K_i and the x-intercept the apparent K_s. The precise values can be calculated from regression lines.

In the present study the plots indicated competitive inhibition at potassium concentrations up to 25 mM and noncompetitive inhibition above that concentration. Apparent inhibitor constants (K_i) for ouabain in the dog, pig, sheep, guinea pig and rat, calculated from the values observed at KCl concentrations up to 15 mM KCl, were 0.034, 0.035, 0.035, 0.12 and 2.6 mM ouabain, respectively. The apparent inhibitor constants (K_i) are similar in the dog, sheep and pig, somewhat higher in the guinea pig and several orders of magnitude higher in the rat. The apparent K_s for potassium in these same species was 0.85, 0.80, 0.75, 1.0 and 0.40 mM KCl, respectively. These values for the potassium-enzyme interaction are in good agreement with those obtained previously from Lineweaver-Burk plots.

In vivo study of the (Na^+-K^+)-ATPase.

It was decided to compare the *in vivo* response to a slow i.v. infusion of ouabain, as monitored by the ECG and blood pressure, in the dog, sheep, guinea pig and rat. The parameters selected were maximal increase in blood pressure, subsequent fall in blood pressure, prolongation of the P-Q interval and complete A-V block. In several of the species

we were not able to estimate the dose of ouabain at which each of these effects occurred. In the dog and rat, prolongation of the P-Q interval was linear until the P-Q interval reached 140% of the initial value. After that, the rate of change in the P-Q interval was accelerated and A-V block supervened rapidly in most animals. In the guinea pig and sheep, A-V block took place after a small change in P-Q interval, usually less than a 10% increase. This parameter, therefore, was not used. In the rat and in the sheep, the initial rise in the blood pressure was followed by a gradual fall during the continuous infusion of ouabain. In the dog, the blood pressure increased until cardiac arrest occurred, although slowing of the cardiac rate and arrhythmias made this measurement less reliable in later stages. In the guinea pig, the rise in blood pressure was minimal, therefore, this parameter was also deleted. The only prominent change observed in the guinea pig was a terminal decrease in blood pressure.

The amounts of ouabain in μmoles per kg administered at times when these changes occurred in the four species are summarized in Table 4. Two parameters can be used to compare all four species (the fall in blood pressure and A-V block). From these data it would appear that the dog and the sheep were equally sensitive to ouabain, that the guinea pig was somewhat less sensitive, and that the rat was considerably less sensitive than any of the other species.

The average value for the relative sensitivity, calculated from three parameters, namely, the fall in blood pressure to the initial level, a 20% increase in P-Q interval, and/or A-V block, were 1.0, 0.80, 0.33 and 0.036 in the dog, sheep, guinea pig and rat, respectively.

An unexpected finding was that female rats were more sensitive to ouabain than male rats. As shown in Table 5, female rats require significantly less ouabain than male rats to produce comparable changes in blood pressure and ECG. However, when female rats were pretreated with phenobarbital, significantly larger doses of ouabain were required to produce the same effects. The untreated female rats, however, were still much less sensitive to ouabain as compared to the other animal species.

Correlation of P-Q prolongation and (Na^+-K^+)-ATPase inhibition by ouabain in the dog heart.

The effect of ouabain infusion on cardiac (Na^+-K^+)-ATPase activity is summarized in Table 6, which also shows changes in P-Q intervals observed immediately prior to sacrifice. After the final recording of the ECG, the infusion of ouabain was terminated immediately and the heart rapidly removed. Enzyme was prepared from the hearts of ouabain infused and paired-control animals. The average cardiac (Na^+-K^+)-ATPase activity of six ouabain-treated animals (nos. 2, 4, 6, 8, 10 and 12) was 3.02 ± 0.54 μmoles of Pi released per mg of protein per hour, (mean ± S.E.). The average enzyme activity of the six control animals was

7.25 ± 0.51. A group comparison of the means of the ouabain-infused animals, compared with the controls, revealed a statistically significant difference with a P value of less than 0.001. Mg^{++}-ATPase activity was 2.68 ± 0.39 in six control animals of this series and 2.63 ± 0.39 in the six ouabain-treated animals.

When $(Na^{+}-K^{+})$-ATPase activity was plotted against the percent increase in the P-Q interval observed prior to sacrifice (Figure 5), a significant correlation ($r = 0.91$, $P<.001$) was observed. Thus, with a greater prolongation in the P-Q interval, there was a greater depression of the cardiac $(Na^{+}-K^{+})$-ATPase.

Ouabain, cardiac contractility and ATPase activity.

The effects of ouabain infusion on contractile force, ECG, and blood pressure on female dogs were compared to those with a comparable period of saline infusion. After a constant level of contractile force was established, control activities of each animal were recorded. The infusion of ouabain or of saline was begun after this recording. As observed in a typical experiment (Figure 6A), infusion of saline solution for 53 minutes caused no alteration in cardiac contractile force, ECG or blood pressure. In several experiments (not shown) saline controls were run as long as two hours without a significant effect on these parameters. In 7 control animals sacrificed at the termination of the saline infusion, cardiac $(Na^{+}-K^{+})$-ATPase activity was 23.8 ± 3.1 (mean ± S.E.) and

Mg^{++}-ATPase activity was 9.3 ± 0.8 μmoles Pi/mg protein/hr (Figure 7).

Ouabain was infused until specific increases in contractility were obtained. The concentrations of ouabain infused are collectively referred to as "high dose" (0.065 mg/kg) and "low dose" (0.026 mg/kg) of ouabain. Data which is typical of the "low dose" of ouabain resulting in increases in contractile force ranging from 10-28 percent in this group of animals is shown in Figure 6B. In this dog, the infusion of ouabain at a rate of 0.8 μg/kg/min for 22 minutes produced a 22 percent increase in cardiac contractile force. No change in the ECG was observed in this particular animal although in several experiments, where the increase in contractile force approached 30 percent, a 10 percent prolongation of the P-Q interval was noted. The increase in contractile force was accompanied by a rise in the mean blood pressure. The infusion of the "low dose" of ouabain which produced 26.1 ± 1.8% increase in cardiac contractile force (mean ± S.E. of five experiments) lowered cardiac $(Na^{+}-K^{+})$-ATPase activity moderately (17.2 ± 3.6 μM Pi/mg protein/hr) with no effect of Mg^{++}-ATPase activity which was 7.5 ± 0.8. When the ouabain was infused for a longer period of time, a greater increase in contractile force was observed. In these animals cardiac irregularities marked by extrasystoles or a lengthening of the P-Q interval progressing to atrioventricular (A-V) blockade were characteristic of this concentration of ouabain. A

prominent rise in blood pressure also occurred. One such experiment, after 95 minutes of ouabain infusion (as above), is shown in Figure 6C. In this animal, there was a 61 percent rise in cardiac contractile force. Ouabain caused a disappearance of the "p" wave and a ventricular rhythm characteristic of A-V block. The increase in contractile force and the ECG changes are typical of those animals receiving the "high dose" of ouabain.

The infusion of the "high dose" of ouabain which produced a 54 ± 11% increase in cardiac contractile force (mean ± S.E. of six experiments) lowered the cardiac (Na^+-K^+)-ATPase activity significantly to 12.6 ± 1.7 μmol Pi/mg protein/hr, ($P<0.05$), as can be seen in Figure 7. Mg^{++}-ATPase activity was 7.7 ± 0.7, which was not different from control.

In order to determine whether the biochemical changes could be dissociated from the alterations in cardiac rhythm observed with ouabain infusion, studies were performed as above except that KCl was administered. In four dogs, KCl solution was given intravenously as soon as the first sign of an arrhythmia was observed in the ECG. The ECG returned to normal and remained normal thereafter in spite of the continued infusion of ouabain. As shown in a typical experiment in Figure 6D, with the simultaneous infusion of KCl and ouabain it was possible to obtain a progressive increase in cardiac contractile force without arrhythmias. In this animal, ouabain was infused for 35

minutes and KCl for 10 minutes. At the termination of the experiment there was a 61 percent increase in cardiac contractile force. In four animals with this treatment there was a 40.5 ± 5.3% increase in contractile force (mean ± S.E.). Cardiac enzyme preparations obtained from these animals had significantly lower (11.3 ± 1.1 μmol Pi/mg protein/hr) (Na^+-K^+)-ATPase activity ($P<0.01$). Ouabain and KCl infusion, however, did not change Mg^{++}-ATPase activity in these experiments which was 6.3 ± 0.7 μM Pi/mg protein/hr (Figure 7).

The relationship between the cardiac ATPase activity and the changes in cardiac contractile force due to ouabain infusion is shown in Figure 8 in the form of orthogonal regression lines (the regression line applicable to data in which errors are assumed to occur in both parameters). A significant correlation (correlation coefficient $r = -0.56$; $P<0.05$) was observed when the (Na^+-K^+)-ATPase activity for each animal was plotted against the corresponding enhancement of cardiac contractile force after ouabain infusion, whereas no correlation was observed between cardiac Mg^{++}-ATPase activity and the changes in cardiac contractile force ($r = -0.37$).

Isoproterenol, stellate stimulation and ATPase activity.

Both isoproterenol infusion and stellate stimulation were used as positive controls to determine whether the increase in cardiac contractile force could bring about changes in (Na^+-K^+)-ATPase activity. The infusion of

isoproterenol increased cardiac contractile force rapidly with a simultaneous increase in pulse pressure as shown in a typical experiment in Figure 9A. No change was observed in the ECG. After 22 minutes of infusion of isoproterenol, cardiac contractile force was increased by 29 percent.

The infusion of isoproterenol did not lower the cardiac (Na^+-K^+)- or Mg^{++}-dependent ATPase activities. (Na^+-K^+)-ATPase activity was 27.3 ± 2.2 and Mg^{++}-ATPase activity was 7.7 ± 2.2 μmol Pi/mg protein/hr.

The stimulation of the left postganglionic inferior cardiac nerve also increased cardiac contractile force immediately (Figure 9B). With a fixed voltage, the stimulation gradually decreased. To obtain a similar enhancement of cardiac contractile force, as seen with isoproterenol, the voltage was increased several times during the 20 minute period of stimulation. All experiments were terminated at the time of maximal response in cardiac contractile force. Sympathetic stimulation also did not reduce cardiac (Na^+-K^+)-ATPase or Mg^{++}-ATPase activity although a definite enhancement of the contractile force was observed. (Na^+-K^+)-ATPase activity was 27.4 ± 1.8 and Mg^{++}-ATPase activity was 7.7 ± 1.4 μmol Pi/mg protein/hr.

<u>ATPase activity of kidney, brain and skeletal muscle after ouabain infusion</u>.

In five control and nine ouabain infused dogs, (five "low dose" and four "high dose"), enzyme preparations were prepared from kidney, brain and skeletal muscle after the

termination of ouabain infusion and the ATPase activity was assayed. Neither the $(Na^{+}-K^{+})$-ATPase activity nor the Mg^{++}-ATPase activity of these tissues appeared to be reduced by ouabain infusion (Table 7).

DISCUSSION

Skou (1962) reported that a (Na^+-K^+)-ATPase preparation could be obtained from mammalian brain and kidney by differential centrifugation of a tissue homogenate prepared in a solution containing sucrose and deoxycholate (DOC). Subsequently Nakao et al. (1965) reported that NaI treatment of a microsomal fraction yielded a (Na^+-K^+)-ATPase preparation with low Mg^{++}-ATPase activity. The combination of these two methods yielded a highly specific (Na^+-K^+)-ATPase preparation from rat brain (Robinson, 1967). These methods, however, could be applied satisfactorily only to brain and a few other tissues. The same procedure did not yield satisfactory results for the preparation of the enzyme from cardiac tissue. A survey of the existing methods indicated that these yielded cardiac enzyme preparations which had either a relatively high Mg^{++}-ATPase activity, suggesting much contamination (Bonting et al., 1961; Auditore and Murray, 1962), or low (Na^+-K^+)-ATPase activity (Portius and Repke, 1967). Some isolation techniques required rather long aging periods (Schwartz, 1962; and Tashima et al., 1966) or the presence of high concentrations of NaN_3 in the final incubation mixture (Auditore, 1965). A method by Matsui and Schwartz (1966) yielded

enzyme preparations with relatively high (Na^+-K^+)-ATPase activity and good $Na^+ + K^+ + Mg^{++}$ to Mg^{++} activity ratio but because of the large amount of starting material required for this method it was impractical for a comparative study in smaller animals. Potter et al. (1966) reported that the treatment of a nuclear fraction with LiBr for 18 hours yielded an acceptable enzyme preparation from sheep and guinea pig hearts. However, a satisfactory enzyme preparation with this method was not obtainable with dog heart as the starting material.

It was decided to examine the effects of ouabain upon the (Na^+-K^+)-ATPase enzyme system of cardiac tissue of several species. An enzyme preparation was desired which could be obtained from a relatively small amount of starting material and which was high in (Na^+-K^+)-ATPase activity and had a high $Na^+ + K^+ + Mg^{++}$ to Mg^{++} activity ratio. A high activity ratio, as mentioned previously, is indicative of minimal contamination of (Na^+-K^+)-ATPase with Mg^{++}-ATPase activity.

Various homogenizing and resuspending solutions, cellular components, and treatment procedures were examined in the search for a method of isolation which would yield such an enzyme preparation.

Sucrose (methods A, C, D, E, F, H, and M), mannitol (methods B and H), tris (methods I, J, and K), and $NaHCO_3$ (method L) homogenizing and resuspending solutions were utilized because of previous success by Skou (1962), Ahmed

and Judah (1964), and Emmelot and Bos (1962) in isolating the (Na^+-K^+)-ATPase from brain, kidney and liver tissue.

Histidine-HCl (methods A, B, F, G, H, and M) was present in several of the homogenizing and resuspending solutions as a buffer; EDTA (methods A, B, D, F, G, H, I, J, K, and M) as a Ca^{++} chelating agent; and DOC (methods A, B, C, F, H, and M) as a membrane fractionating agent.

Differential centrifugation and sucrose gradient procedures were used to isolate various components of the cell. Large microsomes (methods A, B, and C), small microsomes (methods D, E, F, G, H, and M), nuclei (methods I and K), cell membranes (methods I, J, K, and L), and mitochondria (method K) were all examined for (Na^+-K^+)-ATPase activity.

Enzyme preparations were frozen (methods C, E, and L), kept in DOC (methods C, E, F, G, and H), or LiBr (methods I, L, and M), and dialyzed (method C) for variable time periods. It is believed that freezing the enzyme preparation or storing the enzyme preparation in DOC (Schwartz, 1962) or LiBr (Potter et al., 1966) may cause the cellular fragments to break up into smaller particles and thereby expose more active sites of the (Na^+-K^+)-ATPase enzyme system. Dialysis (Landon and Norris, 1963) of enzyme preparations was done to remove Na^+ and K^+ ions that may be attached to the enzyme system preceding the ATPase assay. These authors claimed that the presence of Na^+ and K^+ attached to the enzyme preparation preceding the assay may reflect

the small amount of stimulation when additional Na^+ and K^+ are added to the incubation mixture.

It was found that method M, which consisted of treating a microsomal fraction of cardiac tissue with LiBr, provided the purest enzyme preparation $Na^+ + K^+ + Mg^{++}$ to Mg^{++} activity ratios of 2.6 to 7.2. The enzyme also had a relatively high $(Na^+ - K^+)$-ATPase activity (Table 2).

In all of the remaining methods the $(Na^+ - K^+)$-ATPase activity was either absent or very low or the enzyme preparations were high in Mg^{++}-ATPase activity (Table 1).

Since method M provided a sufficient quantity of an enzyme preparation with high activity and high $Na^+ + K^+ + Mg^{++}$ to Mg^{++} activity ratio, with a relatively small quantity of starting material, this method was used for the isolation of the $(Na^+ - K^+)$-ATPase enzyme system in this study.

Schwartz (1962) claimed that the freezing of an enzyme preparation resulted in an increase in the activity ratio by preferentially decreasing the Mg^{++}-ATPase activity. However, freezing of the sample is not necessary for low Mg^{++}-ATPase activity with this method, and freezing of the tissue prior to enzyme preparation did not effect enzyme activity. It is possible that LiBr and NaI may react in the same manner with the enzyme preparation to decrease the Mg^{++}-ATPase activity, therefore, the enzyme preparation obtained by the present method may be the same protein fraction obtained by the method of Matsui and Schwartz (1966). However, the present method is simpler, requires

less time for preparation, less starting material and results in a higher yield of protein.

This method yielded enzyme preparations which were quite reproducible. The enzyme preparations obtained from 18 control dogs had a mean (Na^+-K^+)-ATPase activity of 15.1 ± 2.1 (mean ± S.E.) and a mean Mg^{++}-ATPase activity of 6.8 ± 0.7 μmoles of Pi per mg of protein per hour. Dithiothreitol was added to the homogenizing solution in later experiments in an attempt to protect the active sites of the (Na^+-K^+)-ATPase and, therefore, increase enzyme activity.

The in vitro studies with the (Na^+-K^+)-ATPase preparations using this method demonstrated that this enzyme system had cation requirements similar to those of previously reported (Na^+-K^+)-ATPase preparations.

The significant species difference in the inhibition of the (Na^+-K^+)-ATPase by ouabain observed in the present study and in previous studies is of interest. Since the inhibition of (Na^+-K^+)-ATPase activity by ouabain is known to be decreased in the presence of higher concentrations of KCl, the species difference observed could possibly be a result of different affinities of the enzyme preparations for KCl. Enzyme preparations with a high affinity for K^+ would presumably be less sensitive to inhibition by ouabain. Estimates of the K_s for KCl (affinity constant of the enzyme for KCl) were determined for each species. The apparent K_s for K^+ obtained from two different sets of

experiments in the present study are in good agreement and indicate that there is no difference in affinity of the enzyme for K^+ in the five mammalian species studied.

Another possible explanation for the observed species difference to ouabain is that the enzyme preparations obtained from the different animal species have different affinities for ouabain. Enzyme preparations with a high affinity for ouabain would presumably be the most sensitive to that drug. To examine this possibility, the K_i for ouabain (affinity constant of the enzyme for ouabain) was estimated for each animal species. An examination of the K_i values obtained reveals some differences in affinity for ouabain among the animal species studied. The most sensitive species demonstrated the highest K_i values. This data supports the observation by Matsui and Schwartz (1968) that ouabain binds more strongly to the enzyme preparations of the most sensitive species, and may explain the observed species difference to the drug. It should be mentioned, however, that kinetic constants are valid only when the interaction of concern is reversible and at equilibrium. Since the interaction of ouabain and the enzyme system may be irreversible, the precise meaning of the estimated K_i values is debatable.

The interactions of Na^+, K^+, and ouabain with the (Na^+-K^+)-ATPase enzyme system have been the subject of several studies because the inhibition of the (Na^+-K^+)-ATPase activity by ouabain is determined to a large extent by the

amount of K^+ present in the medium. Since the (Na^+-K^+)-ATPase enzyme system is believed to have two specific cation sites (one for Na^+ and one for K^+) it is possible that ouabain may interact with either or both of these sites to cause inhibition of the enzyme system. It could also, however, interact at a completely separate receptor site to cause the observed inhibition.

Conflicting reports on this subject exist in the literature. Kinetic analysis by Ahmed et al. (1966) indicated that ouabain is competitive at both the sodium and potassium sites, whereas, the data of Charnock et al. (1963) and Albers et al. (1968) suggest the irreversible nature of ouabain's action at the potassium site. Matsui and Schwartz (1966, 1968) on the other hand believe that ouabain interacts at a receptor site separate from either cation site.

In the present study we examined the interaction between ouabain and potassium with the enzyme system by constructing Hunter-Downs plots (Figure 4).

The Hunter-Downs plot has several advantages over a Lineweaver-Burk plot in the analysis of the present data. These include the following: 1) Each species can be represented by a single line even though different ouabain concentrations are employed; 2) No extrapolation is required to distinguish between competitive inhibition, noncompetitive inhibition and uncompetitive inhibition; and 3) The entire range of KCl concentrations can be analyzed,

whereas with a Lineweaver-Burk plot, only that portion of data obtained with KCl concentrations lower than 25 mM can be employed.

The present data indicate that the inhibition of (Na^+-K^+)-ATPase by ouabain is enhanced at low KCl concentrations. This is in agreement with previous reports describing the antagonistic relationship between ouabain and potassium in (Na^+-K^+)-ATPase activity from cardiac muscle (Auditore, 1965) and from other tissue (Dunham and Glynn, 1961; Judah and Ahmed, 1964). With KCl concentrations up to 25 mM, there is a linear antagonism of the ouabain inhibition of the (Na^+-K^+)-ATPase activity. Above 25 mM this antagonism is largely independent of KCl concentration. These results may be interpreted to indicate that ouabain inhibits (Na^+-K^+)-ATPase activity competitively with regard to KCl at low KCl concentrations and noncompetitively at high KCl concentrations.

A simple interpretation of the present data is possible. The (Na^+-K^+)-ATPase reaction is believed to occur in at least two steps, namely a Na^+-dependent phosphorylation step and a K^+-dependent dephosphorylation step (Skou, 1965). Although ouabain inhibits both steps, inhibition of the overall (Na^+-K^+)-ATPase reaction is primarily affecting the second, dephosphorylation step (Charnock et al., 1963; and Inturrisi and Titus, 1968). Its effect on ATPase activity would be greater at low KCl concentrations (Figure 10a) where this step has a relatively low velocity. The K^+-dependent dephosphorylation step would then be rate

limiting and a 50% inhibition of this step would then lead to a 50 percent reduction in ATPase activity. At higher KCl concentrations (Figure 10) where the dephosphorylation step had a higher velocity, and the Na^+-dependent phosphorylation step becomes rate limiting, a 50 percent decrease in the K^+-dependent dephosphorylation step may only inhibit a small percent of the ATPase activity. More ouabain would be required in order to reduce overall ATPase activity. Thus, the less ouabain-sensitive phosphorylation step would determine the overall rate of the ATPase reaction, and this would be unaffected by further increases in KCl.

Another possibility that may explain the data presented above is that since a minimal amount of ouabain is bound to the enzyme system in the absence of ATP, it is not being bound during our preincubation period. Binding of ouabain to the enzyme system and enzyme activity are both initiated by the addition of ATP to the incubation mixture. The total (Na^+-K^+)-ATPase activity at the end of 15 minutes, then, is determined largely by the rate and extent of interaction of ouabain with the enzyme system during that time. When lower concentrations of K^+ are present in the incubation mixture the initial rate of binding of ouabain with the enzyme system is essentially unaltered, therefore, enzyme activity at the end of 15 minutes incubation would be expected to be low. When higher concentrations of potassium are present, however, the initial rate of binding

is significantly reduced. This would then lead to an increased enzyme activity during the incubation period.

In order to compare the *in vivo* sensitivity of the various animal species to ouabain, a slow i.v. infusion of the drug of the same volume was administered to each animal species. Specific changes in the ECG and in the blood pressure were used as parameters of drug effect, since they were among the earliest to be observed in the intact animals and could be quantified easily. The relative *in vivo* sensitivity to ouabain in the dog, sheep, guinea pig, and rat observed in this experiment were in good agreement with the *in vitro* (Na^+-K^+)-ATPase sensitivity to the drug in these animals (Table 3).

Repke *et al.* (1965) who compared the effect of specific concentrations of ouabain on (Na^+-K^+)-ATPase activity *in vitro* with quantities of ouabain necessary to produce toxic effects *in vivo* in several species also found a good correlation. Repke (1965) calculated that 40 percent of the (Na^+-K^+)-ATPase activity would be inhibited by a therapeutic dose of the cardiac glycosides, based upon the *in vitro* sensitivity of the enzyme to the drug and the drug concentrations necessary *in vivo* to cause toxicity. In the present experiments, we have demonstrated a significant relationship between *in vitro* and *in vivo* sensitivity in four species.

Female rats were found to be significantly more sensitive to ouabain than male rats. However, after pretreatment

of female rats with phenobarbital, they became significantly less sensitive to the drug. It is reasonable to assume that the phenobarbital may have enhanced the metabolism of ouabain by increasing the de novo synthesis of drug metabolizing enzymes. However, an increase in the excretion rate of the drug is also a possibility which cannot be excluded.

The data presented on Table 6 clearly indicate that in the dog the cardiac (Na^+-K^+)-ATPase activity is inhibited during the infusion of ouabain, whereas the ouabain-insensitive Mg^{++}-ATPase activity is unchanged by drug treatment. A correlation between the prolongation of the P-Q interval and the reduction in cardiac (Na^+-K^+)-ATPase activity (Figure 5) has also been shown. Such a relationship does not, however, imply causality between these parameters. The P-Q interval is a measure of conduction and is merely a useful index of the relative concentration of cardiac glycoside. However, it does suggest a possible relationship between the (Na^+-K^+)-ATPase activity of cardiac muscle and the action of ouabain in vivo.

It was found that the constant intravenous infusion of ouabain produced a progressive increase in cardiac contractile force in open-chested, anesthetized dogs, and that the cardiac (Na^+-K^+)-ATPase obtained from these animals had a lower specific enzyme activity than control animals. The correlation between enzyme activity and contractile force was dose-related, namely, the greater the ouabain-induced increase in contractile force the lower the specific activity

of the enzyme. It is apparent from this data then that (Na^+-K^+)-ATPase is inhibited during the positive inotropic action of ouabain.

It is important to note that the increase in contractile force produced by ouabain is these experiments cannot be accounted for by Starling's law of the heart. When the strain gauge is attached to the ventricular muscle, the muscle fibers to which it is attached are stretched approximately 1.5 times their original length, therefore, changes in cardiac contractility cannot be attributable to changes in fiber length. In addition, the heart rates of animals in which an increase in contractile activity occurred were unchanged or only slightly increased.

The observation that the Mg^{++}-ATPase activity of the enzyme preparation was the same in both drug-treated and control animals supports the reliability of the above findings since Mg^{++}-ATPase thus serves as a control for any preparative procedures which might alter the yield of enzyme and thus effect (Na^+-K^+)-ATPase activity.

The inhibition of (Na^+-K^+)-ATPase was evident prior to the first manifestation of an arrhythmia in dogs infused with the "low dose" of ouabain. The inhibition of enzyme activity was even more prominent in "high dose" ouabain infused dogs in which ECG changes were suppressed by the simultaneous KCl infusion. KCl infusion has been previously shown to restore sinus rhythm without any influence on the inotropic effect of ouabain (Williams et al., 1966; and

Cerqueira-Gomes and Castro-Tavares, 1969). Thus it would appear that the enzyme inhibition is not directly related to the inhibition of A-V conduction, a toxic effect of the cardiac glycosides. However, it should be noted that ventricular muscle was the source of the enzyme preparation in all animals. Nodal tissue was not sampled and it is conceivable that ouabain might inhibit (Na^+-K^+)-ATPase differentially in various areas of the heart.

The enhancement of cardiac contractile force *per se* did not reduce (Na^+-K^+)-ATPase activity as indicated by the isoproterenol and sympathetic nerve stimulation studies. Therefore, it is unlikely that the inhibition of cardiac (Na^+-K^+)-ATPase activity observed in ouabain-infused dogs is the *result* of the positive inotropic effect of ouabain.

One possible interpretation of the present data is that a causal relationship may exist between ouabain-induced inhibition of (Na^+-K^+)-ATPase and the positive inotropic effect of the drug. Such a relationship has recently been suggested by Dransfeld *et al.* (1969). These authors have found that a higher Na^+/K^+ ratio results in a reduced uptake of Ca^{++} by cardiac cell particulate preparations, especially by mitochondria. This is supported by the work of Palmer and Posey (1967) who demonstrated that the addition of 100 mM of NaCl released 1/3 of the Ca^{45} bound to sarcoplasmic reticulum isolated from (rabbit) heart muscle. Thus, Dransfeld *et al.* (1969) postulate that the inhibition of the (Na^+-K^+)-ATPase would result in a higher

intracellular Na^+/K^+ ratio immediately after the excitation of the membrane. This would result, therefore, in an increase in intracellular free Ca^{++} and a resulting augmentation of cardiac contractile force.

Since cardiac glycosides produce their inotropic stimulation by increasing the rate at which tension or force is developed, rather than by prolonging the duration of the contractile process (Wiggers and Stimson, 1927; and Siegel and Sonnenblick, 1963), an increased release of Ca^{++} is a more acceptable mechanism for the action of ouabain than is the inhibition of Ca^{++} uptake as suggested by Lee (1967) or the enhancement of metabolism or catecholamine release as was previously believed.

The observation that the (Na^+-K^+)-ATPase activity of the renal medulla, cerebral hemispheres and gracilis muscle was not influenced by ouabain at a time when cardiac (Na^+-K^+)-ATPase activity was inhibited is an interesting one. Several authors have clearly demonstrated that the (Na^+-K^+)-ATPase of a ouabain-infused kidney has a lower specific enzyme activity when compared with the contralateral kidney from the same dog (Martinez-Maldonado et al., 1969; Hook, 1969; and Nelson and Nechay, 1969). After prolonged ouabain infusion the enzyme preparation from the renal medulla is more sensitive to ouabain than that obtained from the cortex (Martinez-Maldonado et al., 1969; and Hook, 1969). It should be mentioned that in all of these studies, ouabain was infused directly into the renal artery. The

failure to observe inhibition in the enzyme from the renal medulla in the present study may be the failure, under our experimental conditions, to achieve a sufficiently high concentration of ouabain necessary to inhibit the enzyme.

SUMMARY

1. Active (Na^+-K^+)-ATPase preparations with relatively high Na^++K^++Mg^{++}/Mg^{++} activity ratios were obtained from the cardiac tissue of rat, guinea pig, dog, sheep, and pig by treating the microsomal fraction with DOC and LiBr. (Na^+-K^+)-ATPase activity from all these species ranged between 10-21 μmoles Pi released per mg protein per hour, and activity ratios for the rat, guinea pig, dog, sheep, and pig were 2.6, 3.9, 2.7, 6.2, and 7.2, respectively.
2. Maximal stimulation of the (Na^+-K^+)-ATPase activity in the presence of 100 mM NaCl and 5 mM $MgCl_2$ occurred with KCl concentrations between 15 and 25 mM in all species.
3. Calculated dissociation constants for the potassium-enzyme complex indicated that the affinity of the cardiac (Na^+-K^+)-ATPase for K^+ was similar in all five species.
4. A species difference in sensitivity to ouabain was observed <u>in vitro</u>. The cardiac (Na^+-K^+)-ATPase preparations from the dog, sheep, and pig were most sensitive, those from the guinea pig were somewhat less sensitive, while those from the rat were considerably less sensitive to the drug. Inhibitor constants for the ouabain-enzyme complex for the various animal species indicate that enzyme preparations from the dog, sheep, and pig have the

highest affinity for ouabain, the guinea pig has a somewhat lower affinity, while the rat has the least affinity for the drug.

5. The interaction between ouabain and (Na^+-K^+)-ATPase in the presence of K^+ as interpreted from the Hunter-Downs plot indicated competitive inhibition at K^+ concentrations up to 25 mM and noncompetitive inhibition above that concentration.
6. Using specific changes in ECG, and blood pressure as parameters of drug effect, the in vitro order of species sensitivity to ouabain was observed in vivo. The concentrations of ouabain (in mg per liter) necessary to inhibit 25 percent of the enzyme activity in vitro were correlated well with ouabain concentrations (in mg per kg) which were found to elicit specific changes in physiological parameters in vivo.
7. Female rats were found to be more sensitive to ouabain than male rats. However, when female rats were pretreated with phenobarbital, significantly larger doses of ouabain were required to produce the same effect.
8. When (Na^+-K^+)-ATPase activity was plotted against the percent increase in the P-Q interval observed prior to sacrifice, a significant correlation was observed. Thus, with a greater prolongation in the P-Q interval, there was a greater depression of the cardiac (Na^+-K^+)-ATPase activity.

9. Ouabain infusion in the dog in vivo caused significant increases in contractile activity. A significant correlation was observed when the (Na^+-K^+)-ATPase activity for each animal was plotted against the corresponding enhancement of cardiac contractile force after ouabain infusion, whereas no correlation was observed between the cardiac Mg^{++}-ATPase activity and the changes in cardiac contractile force.
10. Prolonged enhancement of contractile force induced by isoproterenol or stellate stimulation did not significantly effect (Na^+-K^+)-ATPase activity.
11. Neither (Na^+-K^+)-ATPase nor Mg^{++}-ATPase activity from the kidney, brain, or skeletal muscle were reduced by ouabain infusion.

Table 1. Cardiac ATPase activities and $Na^+ + K^+ + Mg^{++}/Mg^{++}$ activity ratios obtained from various enzyme preparations.

Method	Animal	Enzyme Prep	ATPase activity (µmol Pi/mg protein/hr) $(Na^+ + K^+)Mg$	ATPase activity (µmol Pi/mg protein/hr) Mg	$(Na^+ + K^+ + Mg^{++}/Mg^{++}$
A	Rat	Fresh	80.8	58.4	1.3
B	Rat	Fresh	79.2	62.4	1.3
	Guinea Pig		81.2	62.4	1.3
	Rat	DOC (0.10%) - 24 hr	8.0	4.8	1.7
C	Rat	Frozen - 48 hr	66.4	57.6	1.2
		- 72 hr	84.0	86.4	1.0
		DOC (0.1%) - 48 hr	8.0	2.3	3.5
		- 72 hr	9.2	7.6	1.2
		Dialysis - 24 hr	16.4	24.8	0.7
D	Rat	Fresh	106.4	108.8	1.0
E	Rat	Frozen - 24 hr	14.4	14.0	1.0
		DOC (0.1%) - 24 hr	0.0	0.0	0.0
F	Rat	Frozen	40.4	33.6	1.2
		DOC (0.1%) - 24 hr	14.4	11.2	1.3
G	Rat	Frozen - 24 hr	81.2	64.4	1.3
			108.8	102.8	1.1
		Sucrose - 24 hr	60.0	45.6	1.3
		DOC (.05%) - 24 hr	0.0	0.0	0.0
		(.25%) - 24 hr	0.0	0.0	0.0
H	Rat	Sucrose - 24 hr	22.2	22.2	1.0
		DOC (.025%) - 24 hr	9.3	5.3	1.8
		(0.05%) - 24 hr	8.9	4.9	1.8
		(0.01%) - 24 hr	7.1	6.2	1.1

Table 1 (cont'd.)

I	Pig	LiBr - 15 min	16.4	14.4	1.1
		- 30 min	8.0	7.2	1.1
		- 60 min	13.2	8.0	1.7
		-180 min	2.8	3.0	0.9
	Dog	- 16 hr	27.6	14.8	1.9
			22.0	14.4	1.5
	Guinea Pig	- 16 hr	18.4	11.6	1.6
	Sheep	- 21 hr	21.2	7.2	2.9
	Pig	- 21 hr	10.4	3.6	2.9
J	Dog	Low density	34.4	24.0	1.4
		High density	27.2	21.6	1.3
K	Pig	Nuclear fraction	28.4	26.0	1.1
		Mitochondria "	21.6	21.6	1.0
		Microsomal "	12.0	13.2	0.9
		Soluble "	6.4	6.0	1.1
L	Pig	Fresh	21.6	19.2	1.3
		Low density layer	39.2	49.2	0.8
		Medium density layer	24.8	28.0	0.9
		High density layer	16.0	15.2	1.1
		LiBr - 15 min	10.0	7.6	1.3
		- 30 min	9.6	7.2	1.3
		- 60 min	7.6	3.6	2.1
	Rat	Fresh	19.2	23.2	0.8
		LiBr - 15 min	18.0	15.6	1.2
		- 30 min	17.2	13.1	1.3
		- 60 min	13.2	10.5	1.3

Table 2. The cardiac (Na^++K^+)-activated ATPase activity in various mammalian species

Animal Species	ATPase Activity			Na^++K^++Mg^{++}/MG^{++} Ratio
	Na^++K^++Mg^{++}[a]	Mg^{++}[b]	Na^++K^+[c]	
	µmol Pi/mg protein/hr			
Dog	16.0 ± 2.4[d]	5.6 ± 0.8	10.4 ± 0.8	2.7 ± 0.3
Pig	19.6 ± 5.2	2.4 ± 0.4	17.2 ± 3.3	7.2 ± 1.1
Sheep	20.0 ± 2.8	3.2 ± 0.8	16.8 ± 2.0	6.2 ± 0.4
Guinea Pig	23.6 ± 3.2	6.4 ± 0.8	17.2 ± 2.6	3.9 ± 0.7
Rat	38.0 ± 7.2	17.2 ± 4.4	20.8 ± 1.3	2.6 ± 0.4

[a]The ATPase assay was carried out at 37°C in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, 50 mM Tris-HCl buffer and pH 7.5 and contained 60 µg of enzyme protein in a final volume of 1.0 ml.

[b]NaCl and KCl were omitted from the above incubation mixture.

[c](Na^++K^+)-activated portion of ATPase activity.

[d]The mean ± S.E. of five experiments.

Table 3. The concentration of ouabain to inhibit 25, 50 and 75% of the $(Na^+ + K^+)$-activated ATPase activity in vitro[a]

Animal Species	ID25	ID50	ID75
	μM	μM	μM
Dog	0.14	0.79	5.6
Pig	0.22	0.73	6.8
Sheep	0.24	1.0	6.3
Guinea Pig	0.40	2.5	10
Rat	5.6	180	1000

[a]Ouabain, enzyme and the salt mixture were preincubated five minutes at 37°C in the absence of ATP prior to ATPase assay. The concentrations of cations were KCl, 15 mM; NaCl, 100 mM; and $MgCl_2$, 5 mM. Dose-response curves for each animal species were constructed from at least 20 determinations (five preparations assayed at four different dose levels in each species).

Table 4. The doses of ouabain required to produce changes in the blood pressure and in ECG in various species

In the dog, the dose is that required to produce half-maximal response in blood pressure.

Species	Blood Pressure		P-Q Interval	
	Maximal increase	Fall[a]	20% increase	A-V block
	μmol/kg		μmol/kg	
Dog	0.063 ± 0.004[b]	0.091 ± 0.005	0.043 ± 0.005	0.069 ± 0.007
Sheep	0.098 ± 0.001	0.107 ± 0.012		0.076 ± 0.008
Guinea pig		0.25 ± 0.03		0.23 ± 0.02
Rat	0.58 ± 0.05	1.88 ± 0.26	1.45 ± 0.06	2.40 ± 0.19

[a]The return of elevated blood pressure to control level and/or the beginning of decrease in blood pressure compared to the control level.

[b]The dose of ouabain administered when indicated changes are observed. Mean ± S.E. of five experiments.

Table 5. Sex differences and the effect of phenobarbital pretreatment on the dose of ouabain altering blood pressure and ECG in the rat.

	Blood Pressure		P-Q Interval	
	Maximal Increase (μmol/kg)	Fall[a] (μmol/kg)	20% Increase (μmol/kg)	40% Increase (μmol/kg)
Male	0.58 ± 0.05[b]	1.88 ± 0.26[c]	1.45 ± 0.06[d]	1.81 ± 0.13[c]
Female	0.52 ± 0.03	1.09 ± 0.18[e]	0.95 ± 0.07[f]	1.20 ± 0.10[f]
Phenobarbital-treated females[g]	0.78 ± 0.13	2.82 ± 0.15[e]	2.05 ± 0.15[f]	2.59 ± 0.19[f]

[a]The return of elevated blood pressure to control level.

[b]The dose of ouabain administered at the time changes were observed. Mean of 5 experiments ± S.E.

[c]Significantly different when compared to male, $P<0.05$.

[d]Significantly different when compared to male, $P<0.01$.

[e]Significantly different when compared to untreated female, $P<0.05$.

[f]Significantly different when compared to untreated female, $P<0.01$.

[g]Phenobarbital sodium (70 mg/kg, i.p.) given 48 hr before the experiment.

Table 6. The effect of ouabain infusion on the P-Q interval and cardiac $(Na^+ + K^+)$-activated ATPase activity.

Dog No.	Ouabain (µg/kg)	Percent Increase in P-Q Interval[a]	$(Na^+ + K^+)$-activated ATPase activity (µM Pi/mg protein/hr)
1	-	none	6.8
2	43.9	13	5.3
3	-	none	5.5
4	53.8	28	2.4
5	-	none	8.0
6	64.5	28	4.0
7	-	none	7.2
8	61.2	32	1.6
9	-	none	9.2
10	58.3	33	2.0
11	-	none	6.8
12	79.9	41	2.8

[a]P-Q interval observed immediately before sacrifice was compared with the initial P-Q interval of each animal prior to ouabain infusion. In 12 animals the initial P-Q interval was 61.2 ± 4.3 msec (mean ± S.E.).

Table 7. ATPase activity of kidney, brain and skeletal muscle.

		Control	Ouabain Infused
		µmol Pi/mg protein/hr	
Kidney	$(Na^+ + K^+)^a$	201 ± 78	201 ± 32
	$(Mg^{++})^b$	55 ± 4.5	55 ± 2.8
Brain	$(Na^+ + K^+)$	110 ± 20	92 ± 12
	(Mg^{++})	39 ± 3.3	31 ± 3.4
Skeletal Muscle	$(Na^+ + K^+)$	5.8 ± 0.7	5.7 ± 0.9
	(Mg^{++})	3.7 ± 1.6	2.5 ± 0.4

[a] $(Na^+ + K^+)$-activated ATPase activity

[b] Mg^{++}-dependent ATPase activity

After the termination of saline or ouabain infusion, portions of renal medulla, cerebral hemisphere and gracilis muscle were rapidly excised. The enzyme preparations obtained from these tissue were assayed for ATPase activity. Five control dogs and five "low dose" and four "high dose" ouabain infused dogs were used for this experiment. The data from all ouabain infused dogs were combined since no difference was observed between animals receiving "low dose" and "high dose" of ouabain. Values expressed are mean ± S.E.

Figure 1. The theorectical scheme of Na^+ and K^+ exchange with the dephosphorylation of ATP.

When Na is attached to the enzyme at the intracellular site, a high energy phosphate bond can be formed on the enzyme via ATP (step 1). This high energy bond results in a conformational change of the enzyme protein which enables the Na ions to be transported to the outside of the cell membrane (step 2). At this conformation, the cation sites have a higher affinity for K than Na, and therefore, K replaces the Na at the cationic sites (step 3). When K is attached to the enzyme, hydrolysis takes place with the release of inorganic phosphate (step 4). This dephosphorylation of the phosphorylated enzyme allows a second conformational shift such that K is transported to the inside of the cell (step 5). At this point Na displaces the K because the cationic sites now have a greater affinity for Na at this conformation. This completes the transport cycle and the process can then repeat itself.

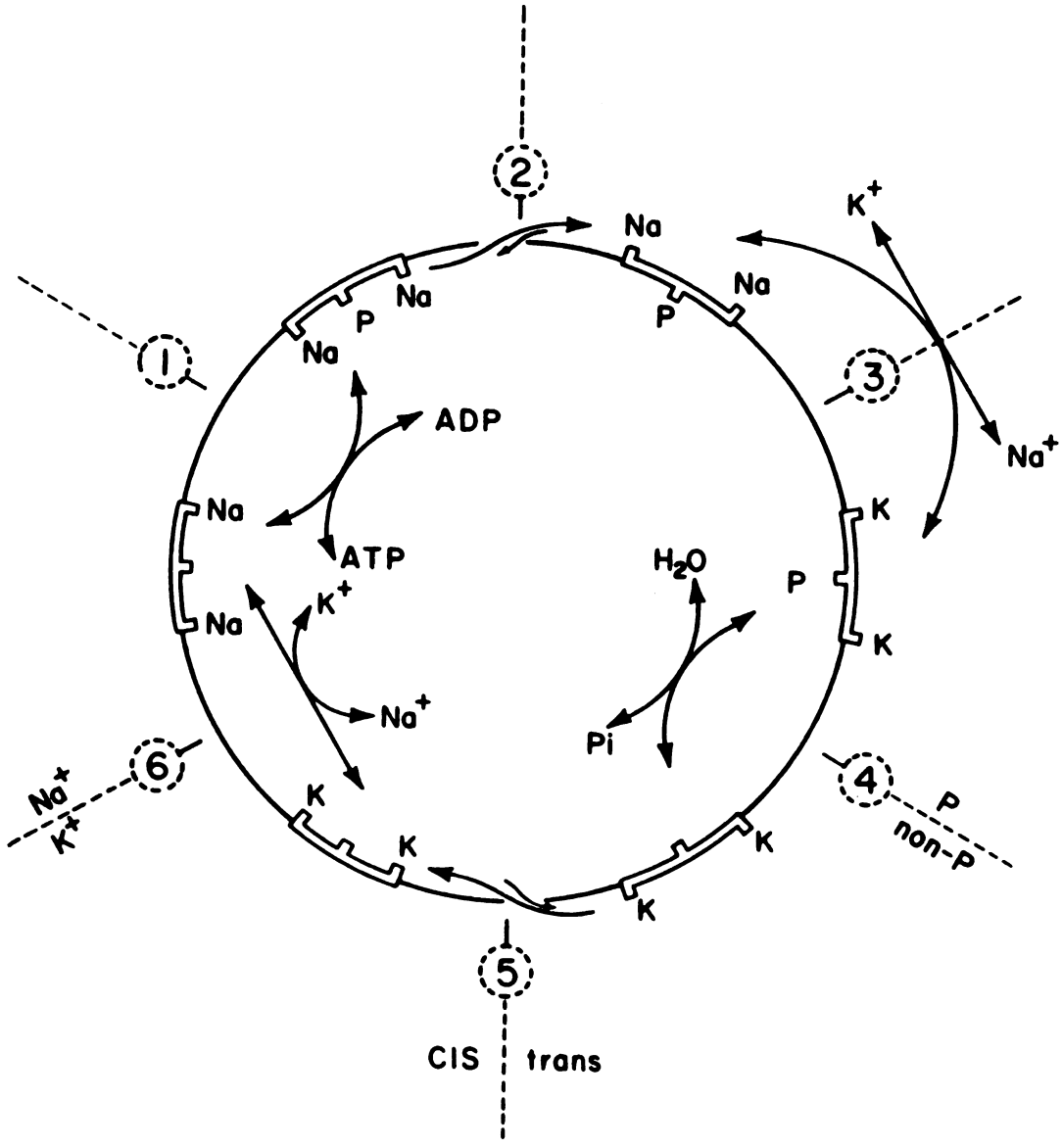

Figure 1. The theoretical scheme of Na^+ and K^+ exchange with the dephosphorylation of ATP.

Figure 2. The effect of potassium concentration on cardiac ATPase activity in various mammalian species.

The effect of potassium concentration on cardiac ATPase activity in various mammalian species. ATPase activity was assayed in the presence of 60 µg of enzyme protein, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, 50 mM Tris-HCl buffer, pH 7.5 and various concentrations of KCl. The incubation was carried out for 15 minutes at 37°C after a 5 minute preincubation period, during with ATP was absent. Mg^{++}-dependent ATPase activity was subtracted from total ATPase activity. Each point represents the mean of five experiments. S.E. for each point ranged from 0.7 to 3.0 µmol of Pi per mg of protein per hr.

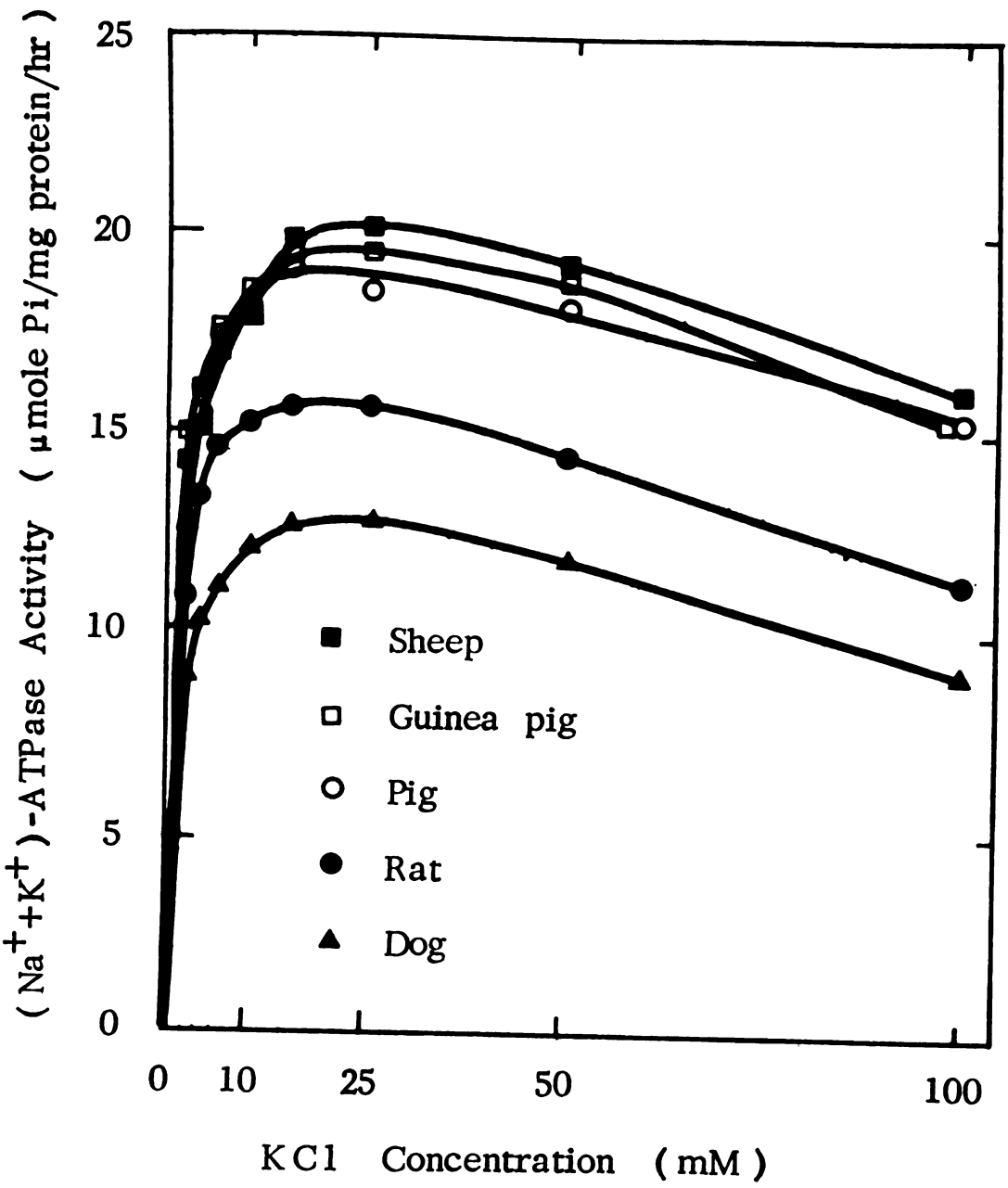

Figure 2. The effect of potassium concentration on cardiac ATPase activity in various mammalian species.

Figure 3. I50 determinations for the various animal species.

I50 determination for the various animal species. ATPase activity was assayed in the presence of 60 µg of enzyme protein, 15 mM KCl, 100 mM NaCl, 5 mM Tris-ATP, 50 mM Tris-HCl buffer, pH 7.5 and ouabain concentrations ranging from $0\text{-}10^{-3}$M. The incubation was carried out for 15 minutes at 37°C after a 5 minute preincubation period, during which ATP was absent. Mg^{++}-ATPase activity was subtracted from total ATPase activity. Each point represents the mean of five experiments. The S.E. for each point was always less than 18 percent. A horizontal line drawn from a point on the coordinate axis representing the point of 50 percent inhibition of the $(Na^{+}\text{-}K^{+})$-ATPase activity for each animal species was extended until it intersected the dose-response curve of each species. The I50 approximation was then determined by extending a vertical line from this point of intersection to the abscissa.

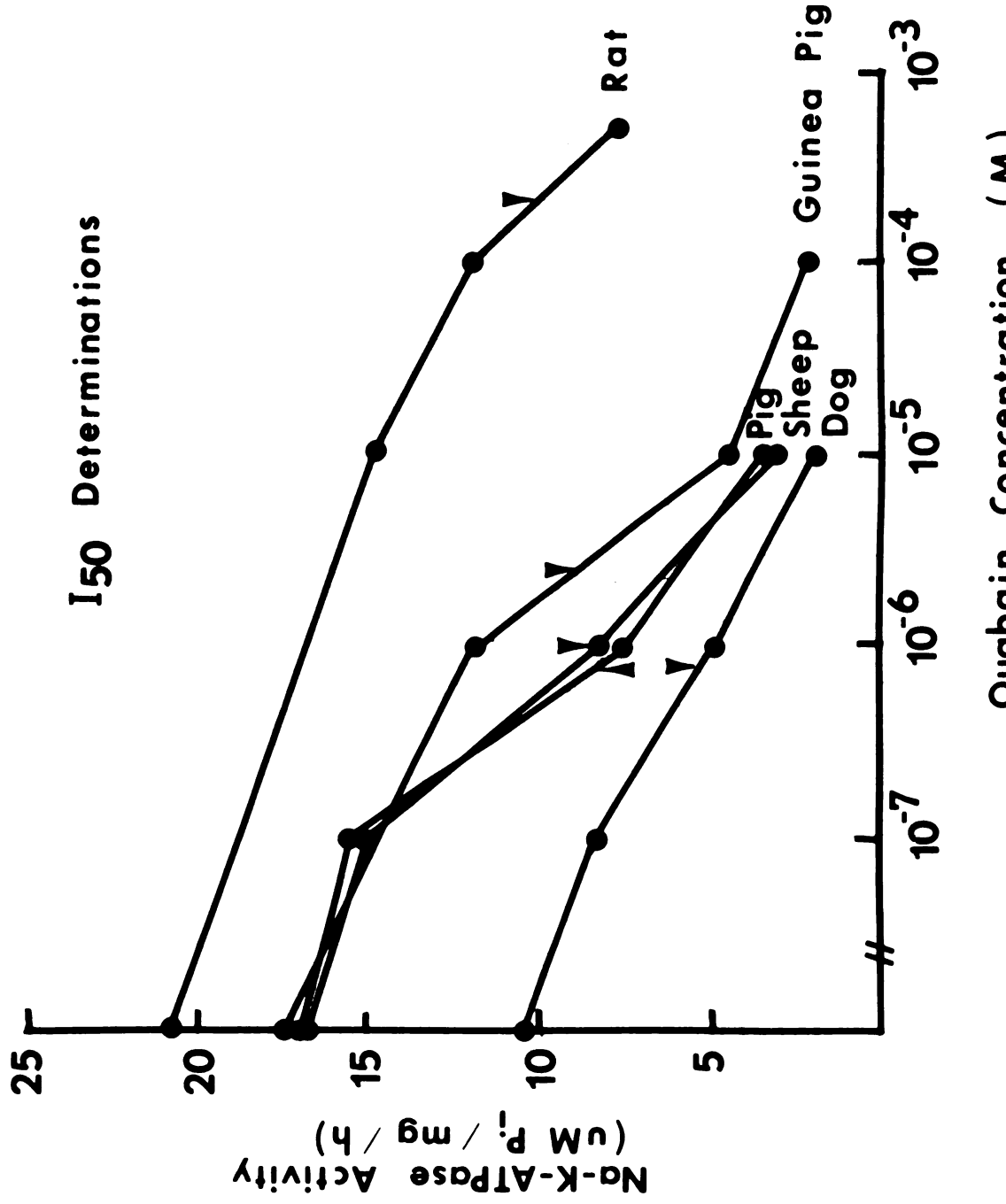

Figure 3. I50 determinations for the various animal species.

Figure 4A. Hunter-Downs plot of the inhibition of the cardiac (Na^+-K^+)-ATPase activity of the guinea pig, sheep, pig, and dog by ouabain under different potassium concentrations.

Hunter-Downs plots of the inhibition of the cardiac (Na^+-K^+)-ATPase activity by ouabain under different potassium concentrations. The experimental conditions were the same as Figure 1, except the incubation mixture was preincubated five minutes at 37°C in the presence of absence of ouabain before the addition of ATP. Ouabain concentrations were: dog, 0.5 and 0.1 μM; pig, 0.5 and 1 μM; sheep, 0.5 and 1 μM; guinea pig, 1 and 5 μM; rat, 10 and 50 μM. α, fractional activity of enzyme preparation inhibited by ouabain; [I], concentration of ouabain in micromoles per liter.

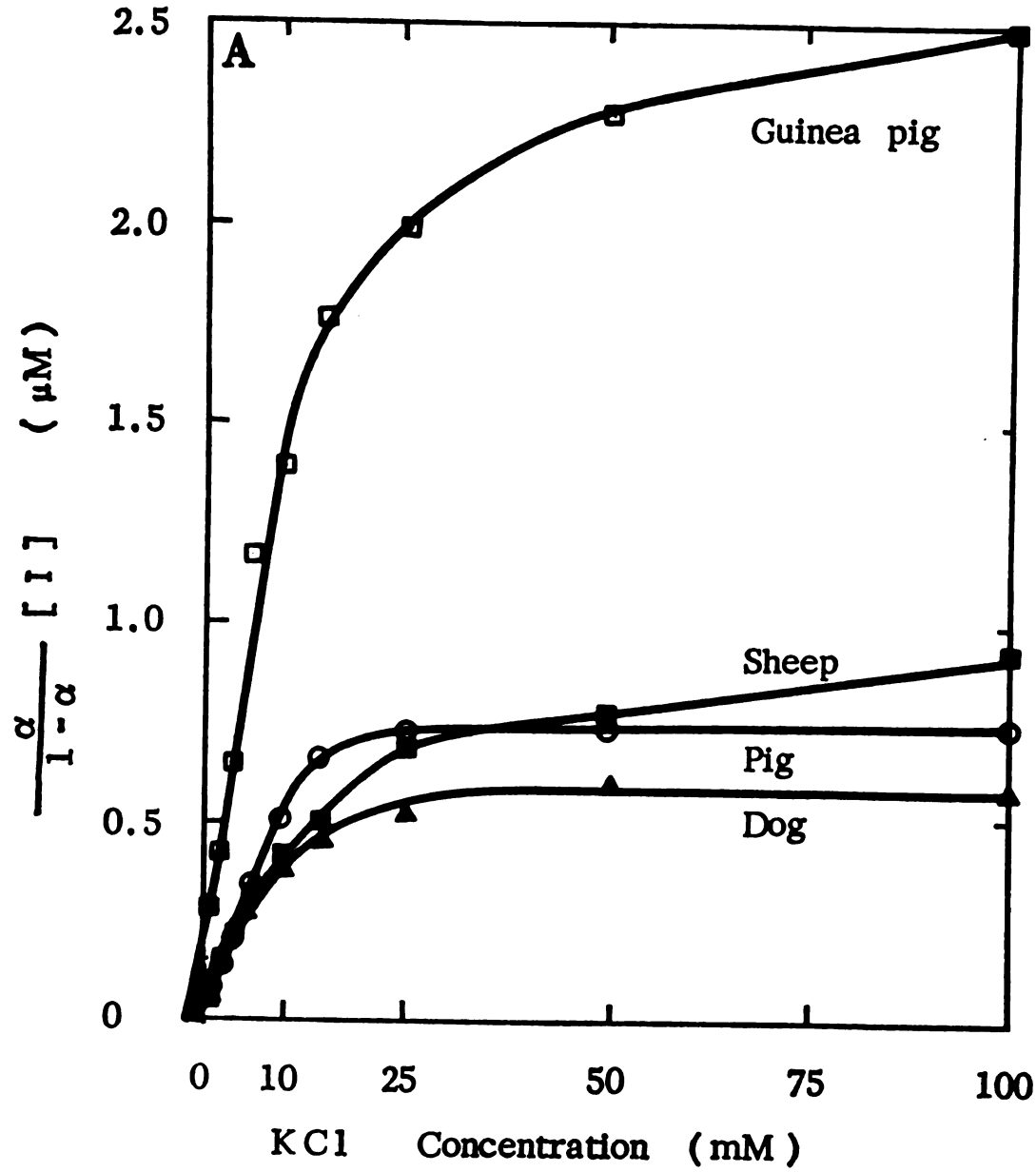

Figure 4A. Hunter-Downs plot of the inhibition of the cardiac (Na^+-K^+)-ATPase activity of the guinea pig, sheep, pig, and dog by ouabain under different potassium concentrations.

Figure 4B. Hunter-Downs plot of the inhibition of the cardiac (Na^+-K^+)-ATPase activity of the rat by ouabain under different potassium concentrations.

Hunter-Downs plots of the inhibition of the cardiac (Na^+-K^+)-ATPase activity by ouabain under different potassium concentrations. The experimental conditions were the same as Figure 1, except the incubation mixture was preincubated five minutes at 37°C in the presence of absence of ouabain before the addition of ATP. Ouabain concentrations were: dog, 0.5 and 0.1 μM; pig, 0.5 and 1 μM; sheep, 0.5 and 1 μM; guinea pig, 1 and 5 μM; rat, 10 and 50 μM. α, fractional activity of enzyme preparation inhibited by ouabain. [I], concentration of ouabain in micromoles per liter.

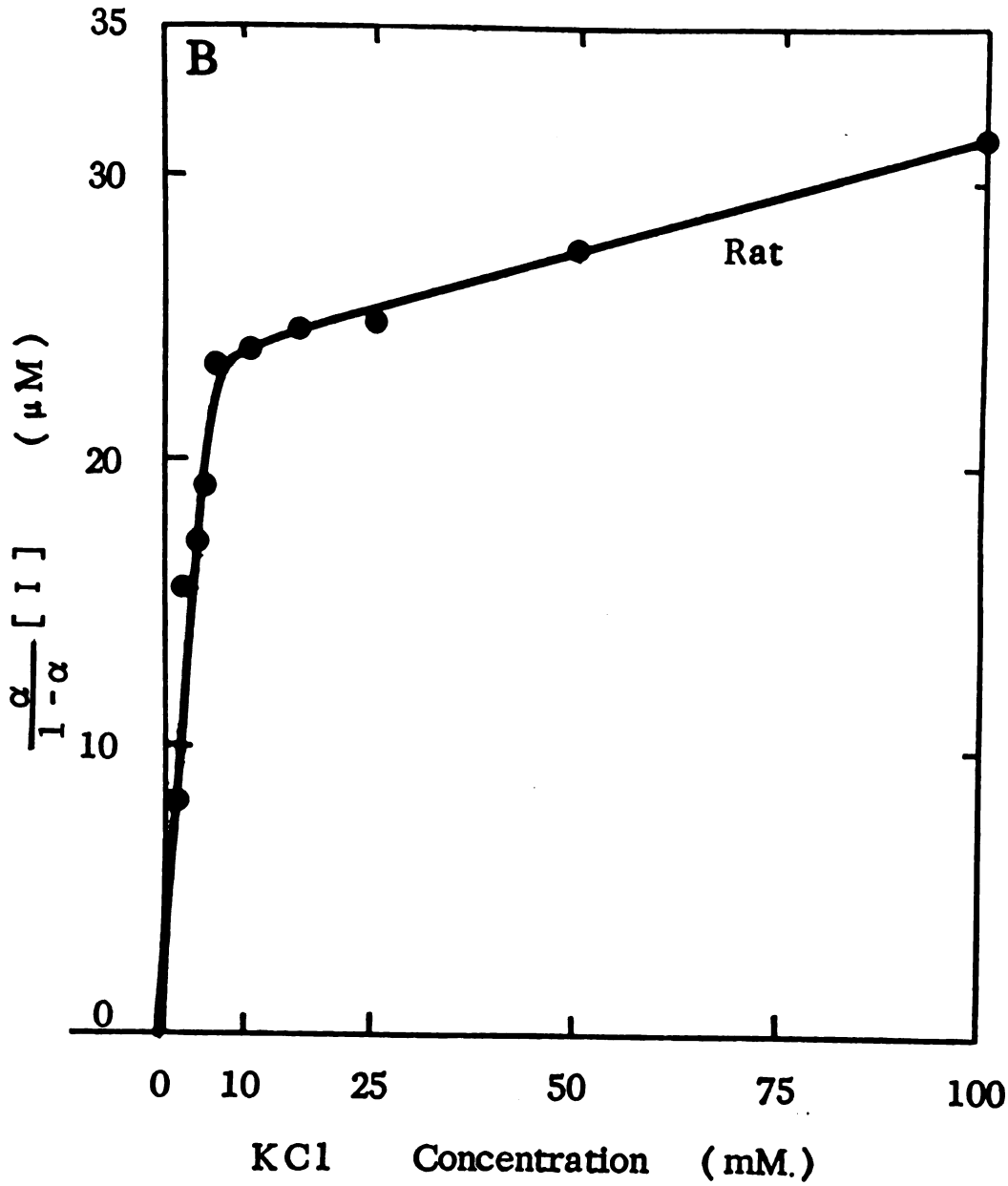

Figure 4B. Hunter-Downs plot of the inhibition of the cardiac (Na^+-K^+)-ATPase activity of the rat by ouabain under different potassium concentrations.

Figure 5. The relationship between ouabain-induced changes in the P-Q interval and cardiac (Na^+-K^+)-ATPase activity.

The relationship between ouabain-induced changes in the P-Q interval and cardiac (Na^+-K^+)-ATPase activity. Ouabain was administered by a slow i.v. infusion to dogs for 20 to 36 minutes. The (Na^+-K^+)-ATPase activity of enzyme preparations obtained from these animals was plotted against the changes in P-Q interval observed prior to sacrifice.

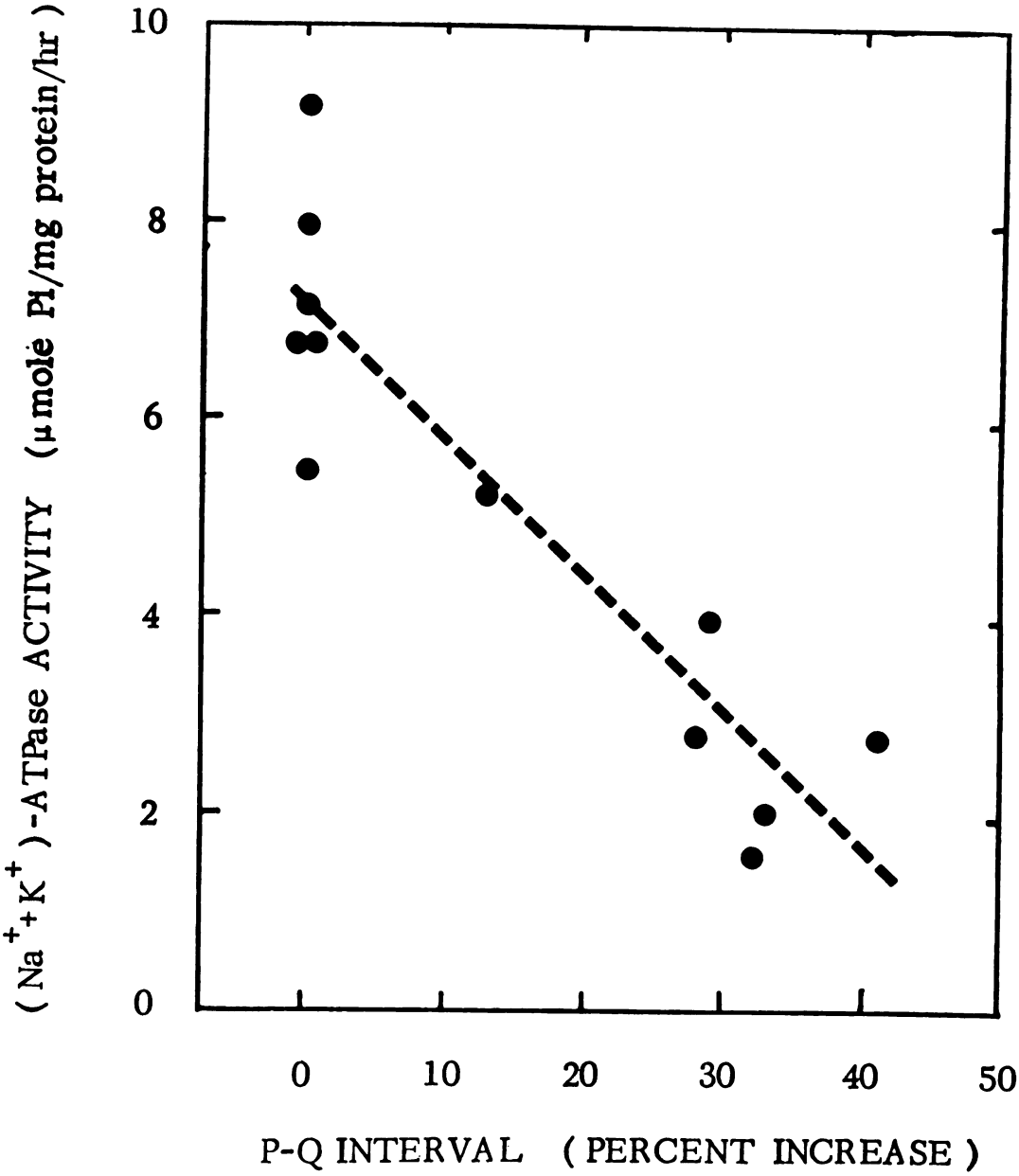

Figure 5. The relationship between ouabain-induced changes in the P-Q interval and cardiac (Na^+-K^+)-ATPase activity.

Figure 6. The effect of ouabain infusion on cardiac contractile force, ECG, and blood pressure.

The effect of ouabain infusion on cardiac contractile force, ECG and blood pressure. The left panel shows the control recordings of cardiac contractile force (CF), ECG and blood pressure (BP) taken in each animal before the start of the infusion. Infusion of saline, (13.5 µl/min, i.v.) or ouabain (0.8 µg/kg/min, 13.5 µl/min, i.v.) was started immediately after the recording and continued for the specified periods. At the end of the infusion, recordings were taken and the animal was sacrificed immediately. A: saline infused control. Female dog, 12.6 kg. B: "low dose" ouabain. Female dog, 14 kg. C: "high dose" ouabain. Female dog, 12 kg. D: "high dose" ouabain + KCl infusion. (After the first 25 minutes of ouabain infusion, arrhythmias were observed. The infusion of KCl, 10 mg/min, i.v., was started immediately and the simultaneous infusion of ouabain and KCl was continued for 10 minutes.) Female dog, 12 kg.

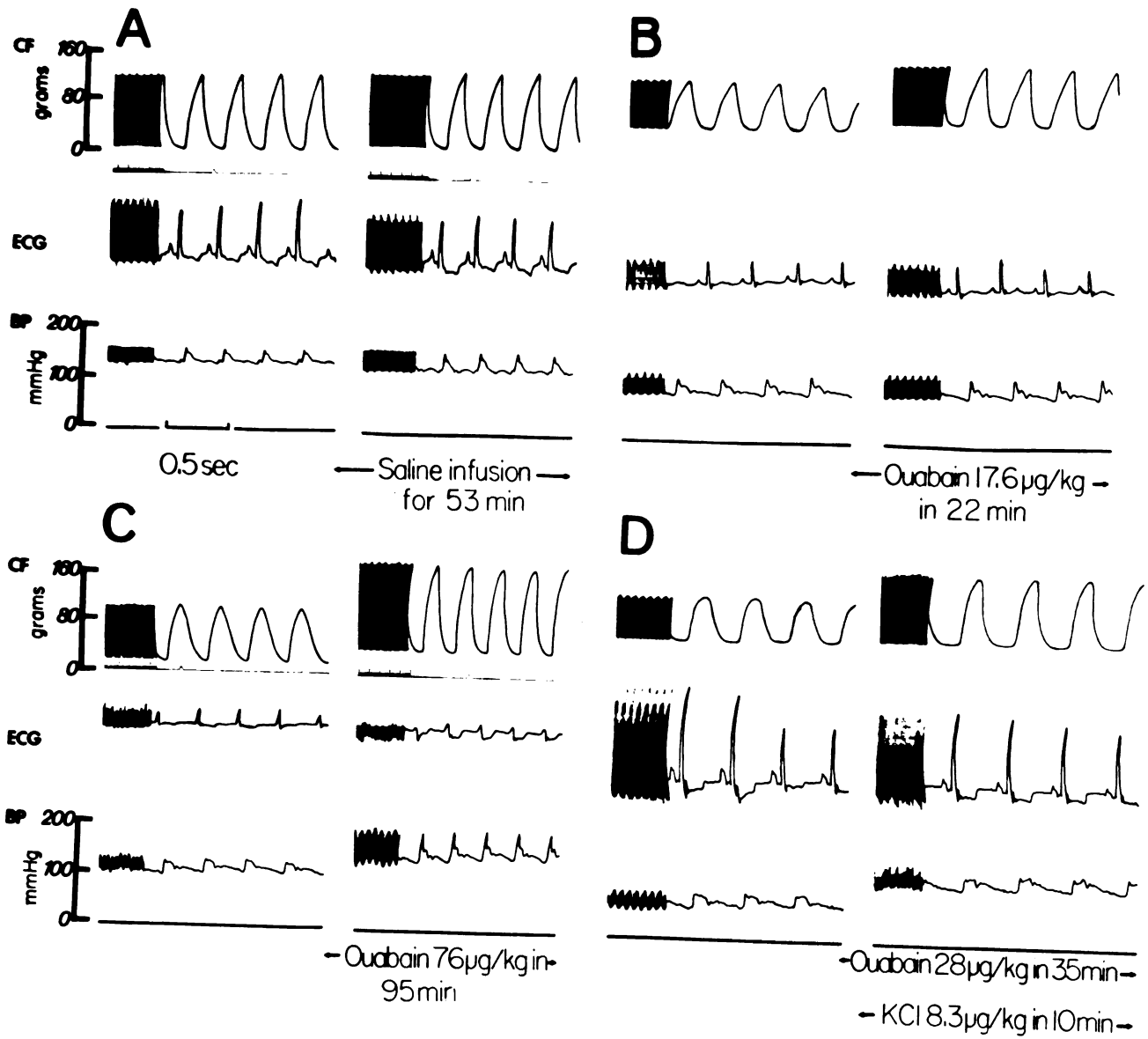

Figure 6. The effect of ouabain infusion on cardiac contractile force, ECG, and blood pressure.

Figure 7. Cardiac ATPase activity after ouabain or isoproterenol infusion or after the prolonged stimulation of the inferior cardiac nerve.

Cardiac ATPase activity after ouabain or isoproterenol infusion or after the prolonged stimulation of inferior cardiac nerve. After the termination of saline, ouabain, isoproterenol infusion of stellate stimulation, ventricular muscle was rapidly excised. The enzyme preparations obtained from these tissues were assayed for ATPase activity. The horizontal bars indicate the S.E. Numbers in parentheses indicate the number of animals.

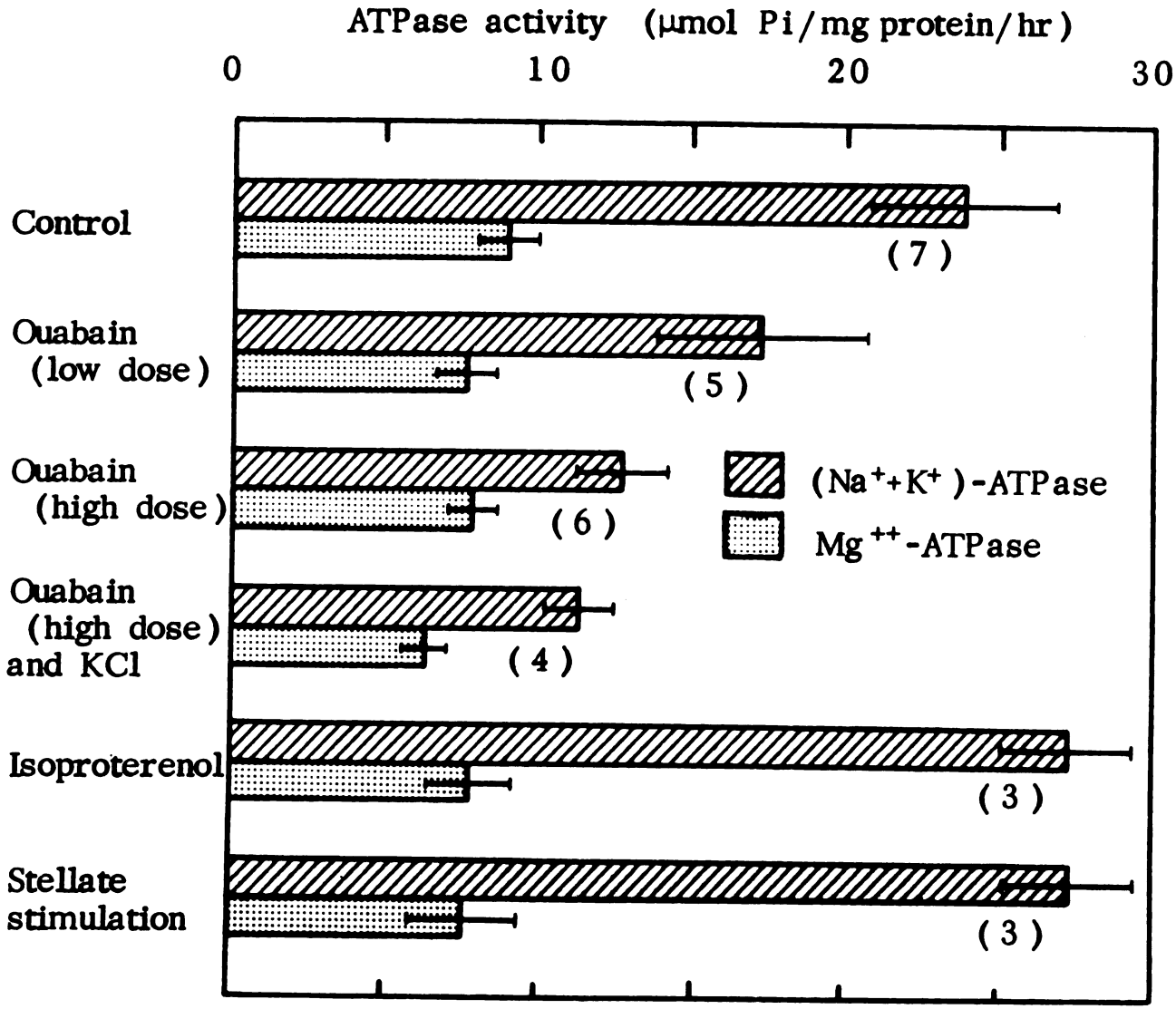

Figure 7. Cardiac ATPase activity after ouabain or isoproterenol infusion or after the prolonged stimulation of the inferior cardiac nerve.

Figure 8. Correlation between the cardiac ATPase activity and the changes in cardiac contractile force after ouabain infusion.

Correlation between the cardiac ATPase activity and the changes in cardiac contractile force after ouabain infusion. The orthogonal regression lines were calculated by least squares method from 18 experiments. The shaded areas show 95 percent confidence intervals for the regression lines.

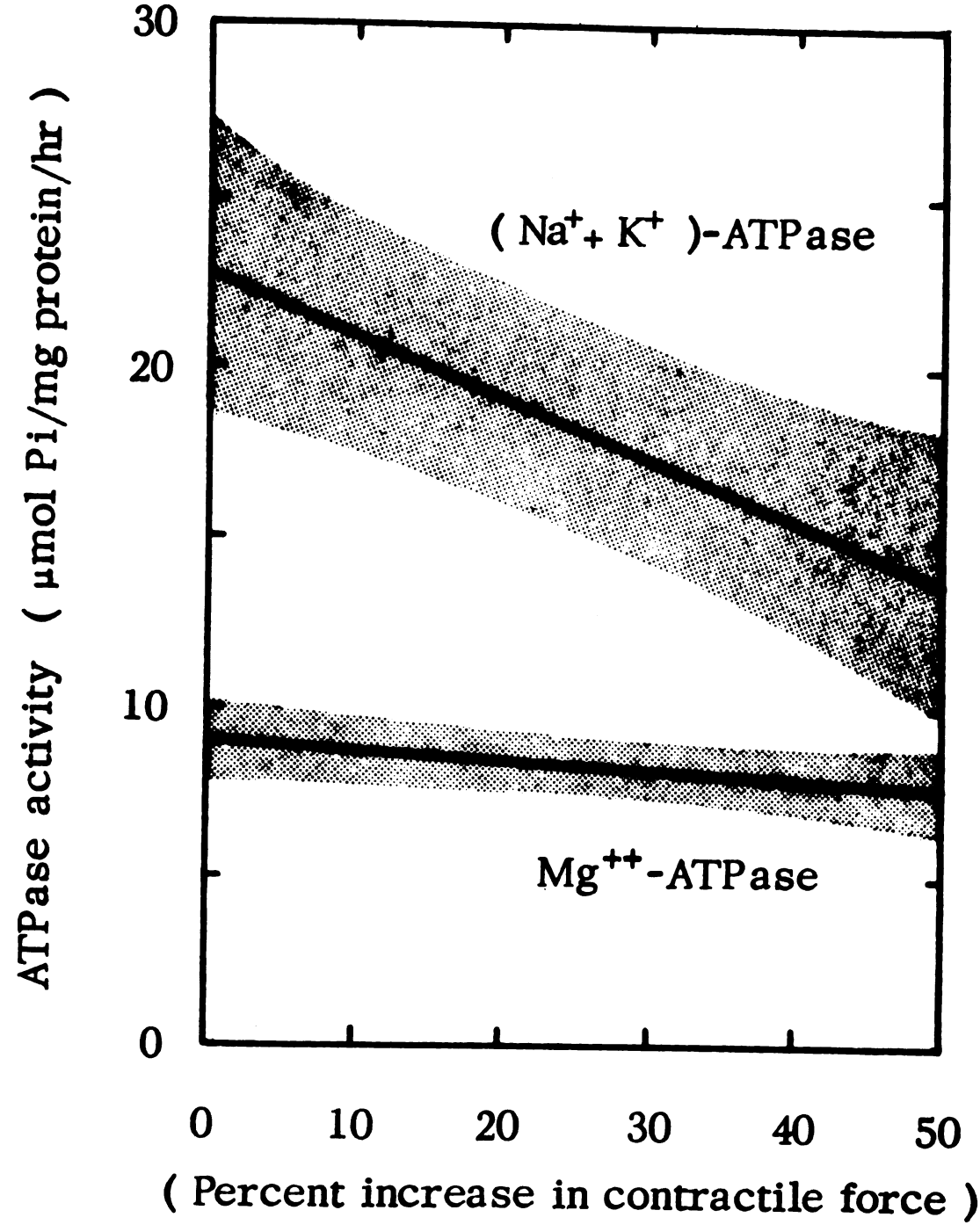

Figure 8. Correlation between the cardiac ATPase activity and the changes in cardiac contractile force after ouabain infusion.

Figure9. Isoproterenol infusion and stellate nerve stimulation.

Isoproterenol infusion and stellate nerve stimulation. See the legend for Figure 1. A: Isoproterenol infusion (isoproterenol was infused to maintain an approximately 40 percent increase in cardiac contractile force. The infusion was terminated after 22 minutes.) Female dog, 12 kg. B: Postganglionic inferior cardiac nerve stimulation (voltage of stimuli was adjusted so that approximately a 40 percent increase in cardiac contractile force was maintained. The stimulation was continued for 20 minutes.) Female dog, 14 kg.

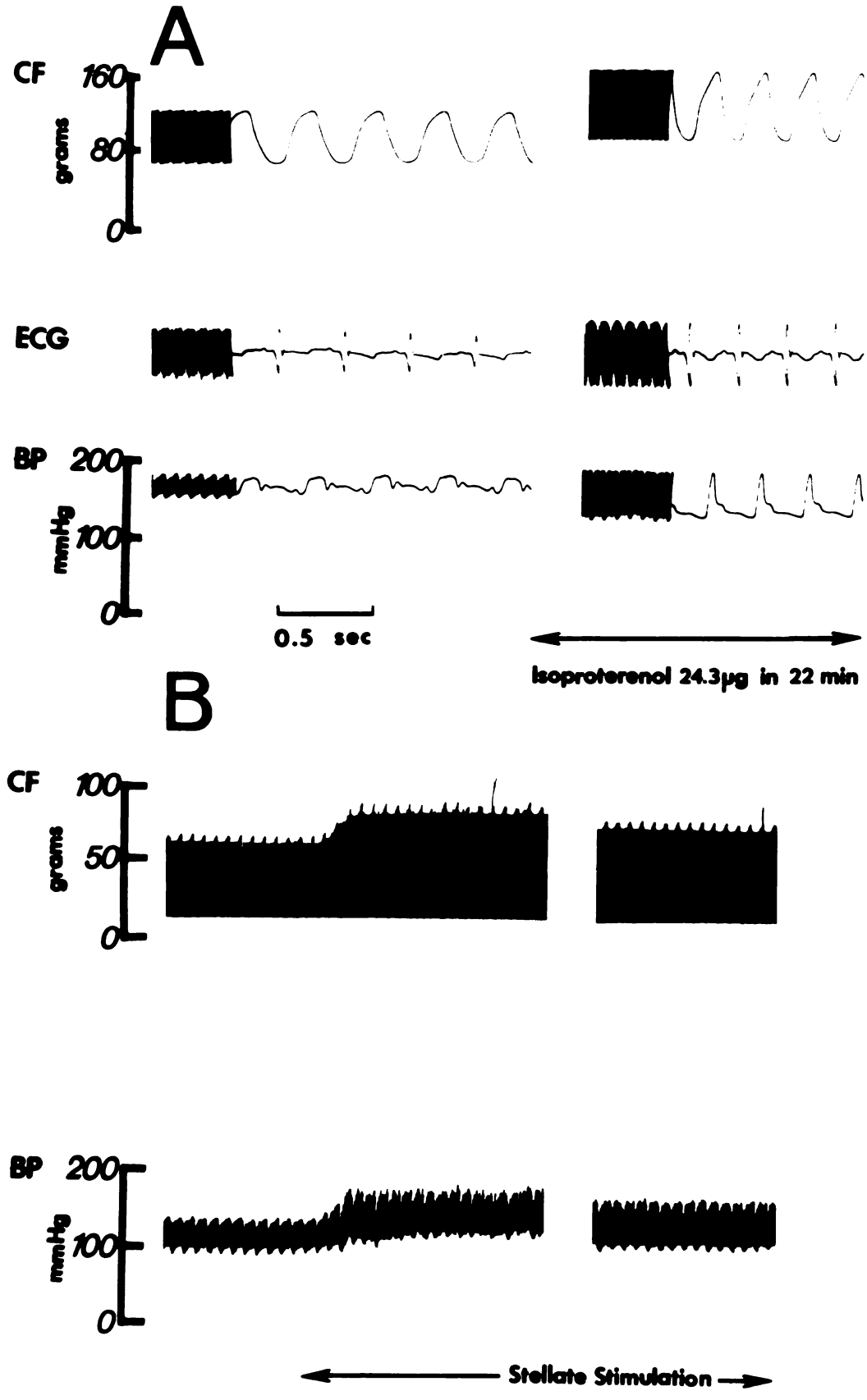

Figure 9. Isoproterenol infusion and stellate nerve stimulation.

Figure 10. Theoretical explanation of the effects of low and high K^+ concentrations on the amount of inhibition of (Na^+-K^+)-ATPase by ouabain.

Theoretical explanation of the effects of low and high potassium concentration on the amount of inhibition of (Na^+-K^+)-ATPase by ouabain. See the discussion section for the explanation of this figure.

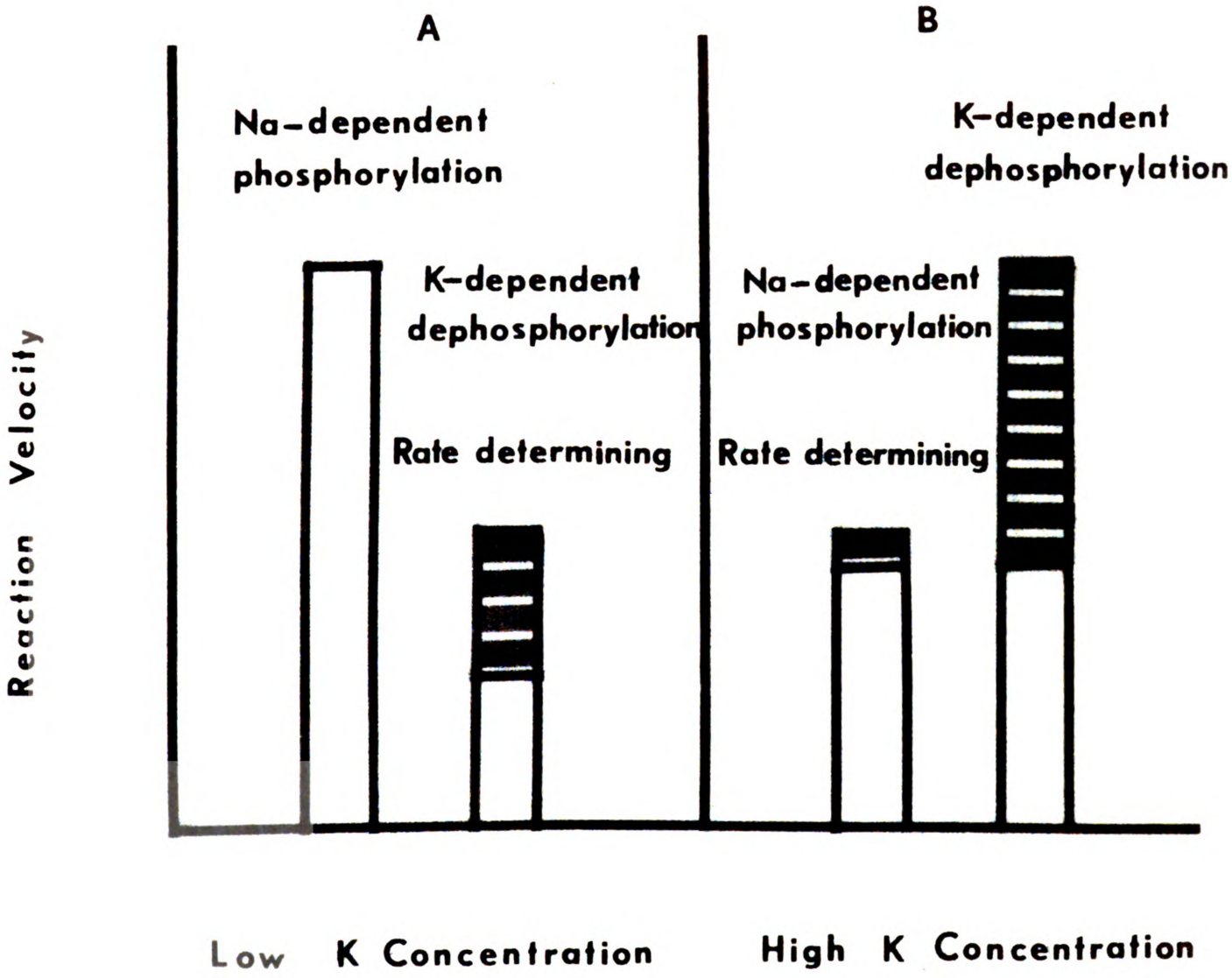

Figure 10. Theorectical explanation of the effects of low and high K^+ concentrations on the amount of inhibition of (Na^+-K^+)-ATPase by ouabain.

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