MICROSOMAL ATPase ACTIVITY IN THE MYOCARDIUM

by

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ABSTRACT

Myocardial microsomal (Na^+, K^+) - ATPase has been studied in several animal species which included, rabbit, sheep, rat, dog and human. Various methods of purifying (Na^+, K^+) - ATPase by membrane disrupting compounds, sucrose density gradient centrifugation, Sephadex gel columns, glass bead column and disc electrophoresis have been investigated. Conditions for homogenizing, keeping tissue and microsomal preparations were studied with dog myocardium. Sub-cellular fractions have been examined electron microscopically and biochemically for enzyme and nucleic acid content. The distribution of (Na^+, K^+) - ATPase within the four chambers of the rat and human heart has been studied.

A (Na^+, K^+) - ATPase has been identified in both human biopsy and post mortem myocardial tissues. Some of its characteristics are described. Post mortem hearts did not require treatment to'unmask' (Na^+, K^+) - ATPase activity. The in vitro effects of diuretics, catecholamines and some other drugs of cardiological interest have been tested on human myocardial (Na^+, K^+) - ATPase. All the diuretics studied were non-competitive inhibitors of (Na^+, K^+) - ATPase in vitro. The effects of cysteine on diuretic inhibition showed that the inhibitory mechanism is possibly different for each diuretic.

Catecholamines were non-competitive inhibitors of (Na^+, K^+) - ATPase with

the exception of 3, 4 - dihydroxymandelic acid. Certain structural requirements of catecholamines were found to be necessary for the inhibitory action on (Na^+, K^+) - ATPase.

Feeding digoxin to rats resulted in a transient increase in (Na^+, K^+) - ATPase in the atrium and right ventricle after one week. Feeding potassium chloride increased myocardial (Na^+, K^+) - ATPase activity but sodium chloride did not. Myocardial (Na^+, K^+) - ATPase activity remained unaffected after treating rats with diuretics. Ephedrine, spironolactone and atropine had no effect on rat myocardial (Na^+, K^+) - ATPase in vivo. The effects on microsomal Mg^{++} - ATPase are quoted but their relation to (Na^+, K^+) - ATPase is obscure.

The specific activity of myocardial (Na^+, K^+) - ATPase remained unchanged in rats with right ventricular hypertrophy. No difference in myocardial (Na^+, K^+) - ATPase was found between human post mortem cardiac and non cardiac cases.

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ABBREVIATIONS AND NOMENCLATURE

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ADP	adenosine 5' - diphosphate
ATP	adenosine 5' - triphosphate
CTP	cytidine 5' - triphosphate
ITP	inosine 5' - triphosphate
GTP	guanosine 5' - triphosphate
PEP	phosphoenolpyruvate
PK	pyruvate kinase
LDH	lactate dehydrogenase
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
Pi	inorganic orthophosphate
g	taken as the mean g_{av} at the centre of the centrifuge tube
ATPase	adenosine - 5' - triphosphatase
(Na^+, K^+) - ATPase	ATP phosphohydralase (EC 3.6.1.3), stimulated by sodium and potassium ions and sensitive to ouabain
Mg^{++} - ATPase	ATP phosphohydralase (EC 3.6.1.4), Mg^{++} ion stimulated ATPase
VMA	vanillylmandelic acid
Km	the substrate concentration giving half maximal velocity (the Michaelis constant)
Ki	the dissociation constant of inhibitor-enzyme complex

EXPLANATION OF METHOD USED TO NUMBER TABLES AND FIGURES

The tables and figures in each chapter are given the chapter number followed by the number of the table or figure in that chapter. For example, the first table used in Chapter V is Table V, 1. The first figure in Chapter III is Fig. III, 1.

CHAPTER I

INTRODUCTION

The electrical activity of excitable tissue is dependent on the unequal distribution of sodium and potassium ions between the intracellular and extracellular compartments. The maintenance of the unequal cation distribution is dependent on a metabolically driven active transport process (Dean, 1941; Hodgkin and Keynes, 1955; Glynn, 1956 and Adrian, 1962). The role played by sodium and potassium ions in the electrical activity of cardiac cells has been extensively studied by Hoffman and Cranefield (1960).

Under normal conditions the concentration of potassium ions is higher in the cell than outside while the concentration of sodium ions is higher outside than inside the cell. The cell membrane is selectively permeable to ions. It is approximately fifty times more permeable to potassium ions than sodium ions. However, sodium ions do enter the cell and are continuously removed to maintain a low intracellular sodium ion concentration. During depolarization of the cardiac cell the membrane permeability to sodium ions increases (Hodgkin and Katz, 1949). Influx of sodium ions causes a depolarization of the cell membrane. Recovery, repolarization, follows by an 'active' extrusion of the sodium ions to the intracellular concentration prior to depolarization.

The active transport of sodium ions from inside to outside the cell requires

the presence of potassium ions outside the cell. As sodium ions are transported out of the cell potassium ions are moved in so that the movement of sodium ions is linked to that of potassium ions (Steinback, 1952; Harris, 1954; Glynn, 1956; Post and Jolly, 1957).

Experiments by Caldwell (1956 and 1960) and Caldwell et al. (1960) on nerve and by Dunham (1957) Whittam (1958) and Hoffman (1960) with erythrocyte membranes, have shown that the energy source for the active transport of cations is ATP. ATP cannot be replaced by other nucleotide triphosphates (Hoffman, 1960). In 1957, Skou, located an ATPase enzyme in sub-microscopic particles isolated from the peripheral nerves of the crab. In the presence of Mg^{++}, Na^{+} and K^{+} ions the ATPase enzyme hydrolysed ATP to ADP and orthophosphate. Skou (1957) suggested that the ATPase may be involved in the active extrusion of sodium from the nerve fibres. Further investigation of the ATPase by Skou (1960) showed its possible relationship to the active linked transport of Na^{+} and K^{+} ions across the nerve membrane. Sodium, potassium ion stimulated ATPase, (Na^{+}, K^{+}) - ATPase, has, since Skou's work in 1957, been located in many different tissues where Na^{+} + K^{+} transport is known to occur and from many animal species. An extensive study by Bonting et al., (1961) showed that (Na^{+}, K^{+}) - ATPase was present in 29 out of 36 tissues from the cat. Only tissue with a very low cell density did not contain the enzyme. The (Na^{+}, K^{+}) -

ATPase enzyme system has been located in the myocardium from cat (Bonting et al., 1961); rabbit (Skou, 1962 and Auditore and Murray, 1962); rat (Schwartz, 1962 and Tashima et al., 1966); calf (Schwartz, 1962); guinea pig (Lee and Yu, 1963); and of the toad (Kennedy and Nayler, 1965).

The basic properties of the enzyme are listed below and provide evidence for the involvement of (Na^+, K^+) - ATPase in the active transport of Na^+ and K^+ ions across the cell membrane. A more detailed account can be obtained from the reviews by Skou, (1964 and 1965).

Location of (Na^+, K^+) - ATPase in the cell

The first requirement of a transport enzyme is that it should be located in the cell membrane (Skou, 1961). The enzyme is located in a subcellular fraction obtained by differential centrifugation which contains the cell membranes. The erythrocyte membrane also contains (Na^+, K^+) - ATPase (Hoffman, 1960, Whittam, 1962 and Glynn, 1962). In myocardium histochemical evidence has shown that (Na^+, K^+) - ATPase is located in the plasma membrane (Wollenburger and Schulze, 1966). Emmelot and Bos (1962) and Emmelot et al. (1964) showed that rat liver (Na^+, K^+) - ATPase was located in the plasma membranes and excluded the possibility of contamination due to mitochondria. Wheeler and Whittam (1964) examined the subcellular fractions of rabbit kidney cortex and concluded that

(Na^+, K^+) - ATPase activity is essentially a membrane bound enzyme. Using guinea pig brain Hosie (1965) made a very careful morphological study of subcellular fractions and found that the external membranes were rich in Na^+ plus K^+ ion stimulated, ouabain sensitive, ATPase activity. Wallach and Kamat (1964) and Kamat and Wallach (1965) using an antigen / antibody technique located (Na^+, K^+) - ATPase activity obtained from Ehrlich ascites tumour cells with a fraction identical with that of cell surface antigens.

It would appear that in most tissues, including the myocardium, the transport ATPase is located in the cell membrane.

The Affinity of (Na^+, K^+) - ATPase for Mg^{++}, Na^+ and K^+ ions

The enzyme has an absolute requirement for Mg^{++} ions. Although not apparently inhibitory in excess (Auditore and Murray, 1962) the optimal magnesium ion concentration has been found to be equimolecular with the ATP substrate concentration.

Na^+ ion requirement

The addition of Na^+ ions besides Mg^{++} ions usually leads to a small increase in ATPase activity. As it is extremely difficult to remove all the endogenous ions present in the preparation, even after exhaustive washing procedures (Goldfarb and Rodnight, 1969), it is difficult to know whether this is a genuine stimulation

by a single ion or not. The increased activity with sodium ions varies in different tissues. The optimal concentration of sodium ions for myocardial tissue, when K^+ ions or another monovalent cation are added, is about 100 mM (Schwartz, 1962, Auditore and Murray, 1962).

K^+ ion requirement

The addition of potassium ions, when magnesium ions are present, leads, in most cases, to a very small increase in activity but in some tissues, including the myocardium, there may be no increase (Skou, 1962).

When Mg^{++} ions and Na^+ ions are present the addition of potassium ions leads to a considerable increase in ATPase activity. The optimal concentration for potassium ions has been found to be in the region of 10 - 20 mM. It would, however, appear that the ratio of sodium to potassium ions is of importance. In myocardium the optimal sodium to potassium ratio is 10 : 1 (Auditore and Murray, 1962 and Gibson and Harris, 1968).

Concentrations of potassium above 20 mM become inhibitory. The inhibition can be partly compensated for by increasing the sodium ion concentration (Skou, 1957 and Auditore and Murray, 1962). Kinetic studies of potassium inhibition by Skou (1960) showed a competitive displacement of sodium ions by potassium ions. A series of kinetic studies on the inhibitory action of potassium ions and sodium ion

activation led Skou (1962) to conclude that there are two separate sites of action, one activated by potassium ions and the other activated by sodium ions.

The two separate affinity sites of sodium and potassium ions have been shown to exist on opposite sides of the membrane of red cells (Whittam, 1962 and Glynn, 1962). A diagram showing the affinity sites is shown in Fig. 1 1. It is noted that the presence of both sodium and potassium ions at each site is required for full activity. Recent work by Masiak and Green (1968) has shown that Na^+ and Mg - ATP binding are at independent sites while K^+ and Mg - ATP also bind at separate sites. They also suggest that it is unlikely that Na^+ and K^+ bind to the phosphorylated enzyme intermediate. Their results support previous studies suggesting that Na^+ and K^+ bind at separate sites in the membrane.

Potassium ions are replaceable and in fact NH_4^+ ions have a greater effect on ATPase stimulation than potassium ions in the presence of magnesium and sodium ions.

Substrate requirements

Maximum enzyme activity is obtained only when ATP is used as a substrate. Some activity is found in the presence of other nucleotide triphosphates (Skou, 1960; Hoffman, 1962 and Gibson and Harris, 1968) but it is small compared to the activity found when ATP is used as substrate.

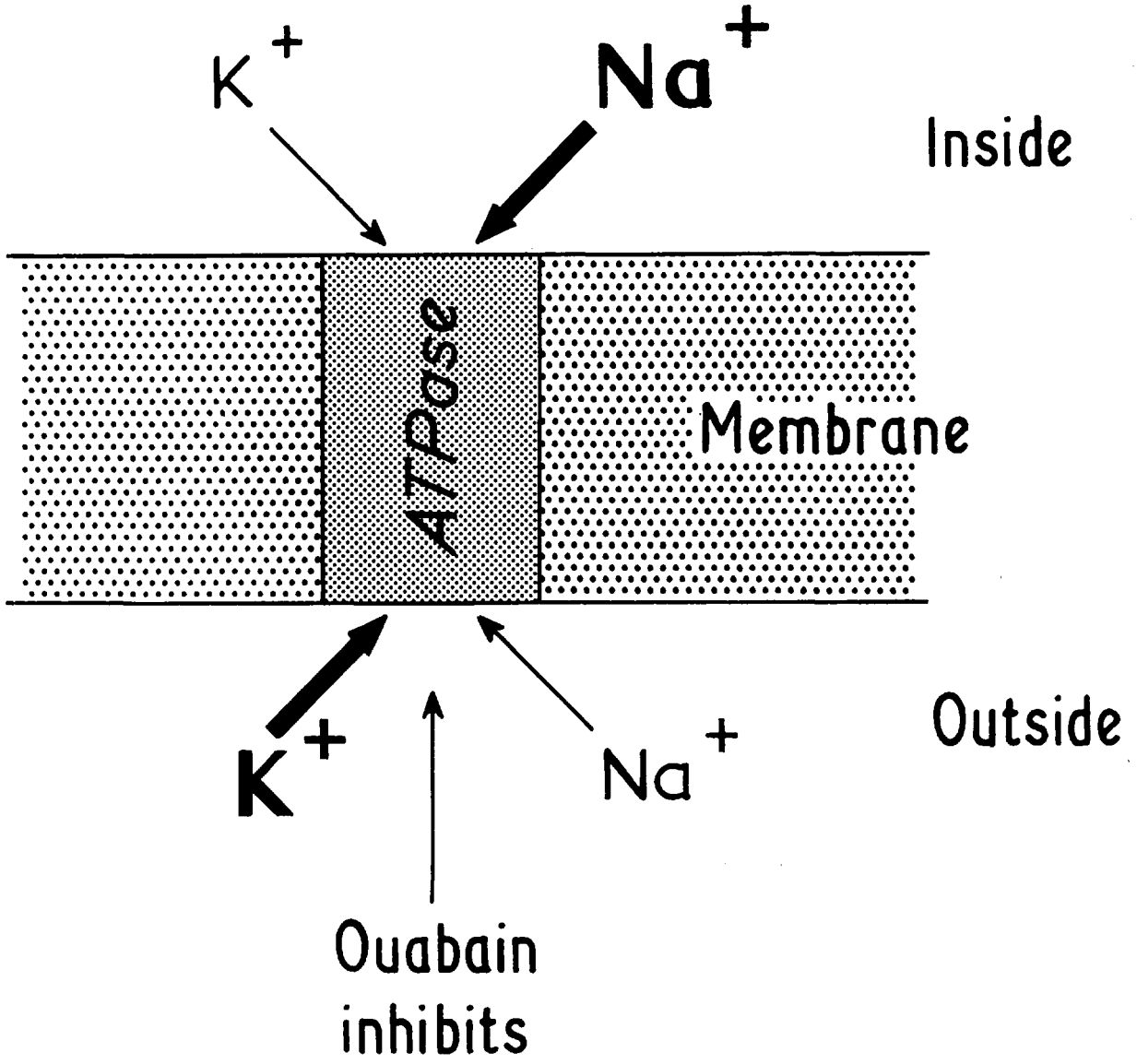


FIG. 1 1 Diagram to show the proposed relationship between the ATPase enzyme system and affinity sites for Na^+ and K^+.

Hydrolysis of ATP

The hydrolysis of ATP is a multistage reaction which is best illustrated as follows,

$$E + ATP \xrightleftharpoons{Mg^{++},\ Na^{+}} E\sim P + ADP \qquad (i)$$

$$E\sim P \xrightarrow{K^{+}} E + Pi \qquad (ii)$$

$E = (Na^{+}, K^{+})$ - ATPase and

$E\sim P$ = the phosphorylated enzyme intermediate

Post and Rosenthal (1962) and Bader, Post and Bond (1968) have reported that sodium ions were required for phosphorylation and potassium ions were required for dephosphorylation. The nature of the phosphorylated intermediate ($E\sim P$) has still to be elucidated. There is some evidence that it is a phosphoprotein (Skou, 1964; Bader et al., 1968 and Glynn, 1965). Rose (1963) and Glynn et al., (1965) excluded the possibility of phospholipid as an intermediate.

It has been suggested that the (Na^{+}, K^{+}) - ATPase enzyme system is the cation carrier molecule (Whittam, 1962 and Hoffman, 1962).

Digitalis glycosides and (Na^{+}, K^{+}) - ATPase

Schatzmann (1953) showed that digitalis inhibited the transport of sodium

and potassium ions in human red cells. This was later confirmed by Glynn (1957) and Whittam (1958) who also demonstrated that it inhibited the membrane ATPase activity. Digitalis glycosides have subsequently been shown to be highly specific inhibitors of the (Na^+, K^+) - ATPase component of the enzyme system (Skou, 1960; Post et al., 1960; Whittam and Wheeler, 1961; Bonting et al., 1962 and Auditore and Murray, 1962).

Bonting et al., 1962 correlated the inhibitory action of digitalis glycosides with cation transport in heart muscle.

Digitalis glycoside had no effect on cation transport when on the inside of squid axons (Caldwell and Keynes, 1959). In red cells ouabain is only an effective inhibitor on the outside of the cell (Whittam, 1962). By increasing the potassium ion concentration the inhibitory effect can be reversed (Glynn, 1957 and Hoffman, 1962).

Fig. I 1 shows, diagrammatically, the possible site for ouabain inhibition.

It would seem, however, that the inhibition by ouabain is due neither directly nor indirectly to the displacement of potassium ions from the potassium binding site in cardiac tissue (Matsui and Schwartz, 1966). Schwartz et al., (1968) suggest that digitalis glycosides may affect ion transport by specifically interacting with an allosteric site on the enzyme.

The specific inhibitory action of digitalis on (Na^+, K^+) - ATPase offers a

possible explanation of the therapeutic action of cardiac glycosides in vivo although it is very difficult to correlate the inhibitory action of (Na^+, K^+) - ATPase with the therapeutic effects. At concentrations known to cause stimulation of myocardial activity digitalis has been reported to stimulate (Na^+, K^+) - ATPase in vitro (Repke, 1963 (a); Brown, 1966; Lee and Yu, 1963 and Palmer et al., 1966) but this has not always been reproducible (Repke, 1963 (b)). Hadju and Leonard (1959) have given an explanation which correlates a decreased intracellular potassium ion concentration with the in vivo effect of digitalis. However, in direct conflict with these studies are the findings of many authors that there is not a potassium loss when therapeutic concentrations of digitalis are given (Klaus et al., 1961 and Bliss and Aldoph, 1963). In toxic doses of digitalis there is a loss of myocardial potassium, which can be reversed by infusion of potassium salts.

Attempts to localize the binding site of tritiated digoxin within the myocardial cell by radioautiography have given contrary findings. In some experiments it was located in the A bands of the sarcomeres (Tubbs et al., 1964 and Smith and Fozzard, 1963) while Spiro and Sonnenblick (1965) showed binding to the sarcotubular structures. Luchi (1966) found that tritiated digoxin was randomly distributed throughout the myocardial cell. None of these experiments has shown specific binding to the plasma membrane where the (Na^+, K^+) - ATPase is thought to reside.

The inotropic action of digitalis in the myocardium has more recently been thought to be mediated primarily through Ca^{++} uptake. Inhibition of the sodium pump causes excess sodium to accumulate intracellularly which potentiates the inhibitory effect of digitalis on Ca^{++} transport and allows the calcium to be available to the myofibril (Lee, 1967).

The scope of the experiments on myocardial microsomal (Na^+, K^+) - ATPase in this thesis

The first part of the experimental work was to establish procedures for separating the membrane (microsomal) fraction from myocardial tissue homogenates. An assessment of the purity of the microsomal fraction by electron microscopic and enzymatic techniques was made (Chapter II).

The assay of (Na^+, K^+) - ATPase enzyme activity was complicated by the presence of a Mg^{++} - ATPase and a suitable method for distinguishing them had to be established. This aspect is described in Chapter III. Various methods of purification of (Na^+, K^+) - ATPase to obtain a cellular fraction free from Mg^{++} - ATPase have been examined. The purification techniques included treatment with deoxycholate and sodium iodide, Sephadex gel columns, glass bead columns, sucrose density gradient centrifugation and disc electrophoresis, all of which are reported in Chapter IV.

(Na^+, K^+) - ATPase had not been reported in human myocardial tissue previous

to this study. The enzyme was located in both human myocardial biopsy specimens and human post-mortem myocardial tissue. A detailed study of human myocardial microsomal (Na^+, K^+) - ATPase was made and details given in Chapter V.

The location of (Na^+, K^+) - ATPase in the myocardium of various animal species was studied. A comprehensive study of the effects of storage conditions on dog myocardial microsomal (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity was made. The rat was selected for in vivo studies. Therefore (Na^+, K^+) - ATPase in the rat myocardium was studied to find optimal assay conditions, the distribution of the enzyme in the heart and the effects of age.

Although the effects of digitalis glycosides on myocardial (Na^+, K^+) - ATPase have previously been studied in vitro the effects in vivo have not been reported. The effects of feeding digitalis together with the effects of feeding sodium chloride and potassium chloride are presented in Chapter VII. Other feeding experiments have been made together with in vitro studies of diuretics (Chapter VIII) and other drugs of cardiological interest (Chapters IX and X) on myocardial (Na^+, K^+) - ATPase.

During hypertrophy of the myocardium there is an increase in the cell membranes. As (Na^+, K^+) - ATPase is located in the cell membrane it was of interest to study the effects of hypertrophy on (Na^+, K^+) - ATPase activity. Rats with right

ventricular hypertrophy were used for this study (Chapter XI).

CHAPTER II

PREPARATION OF MICROSOMAL FRACTIONS

Introduction

In 1946 Claude first reported the separation of 'small granules' from liver homogenates. He later termed these small granules 'microsomes'. Morphologically it was not possible to characterize the particles as they were beyond the resolution of the light microscope. Claude calculated the size of the microsomal particles, by their sedimentation rates, as 60 to 150 mμ in diameter and suggested they were derived from the endoplasmic reticulum.

Porter (1953) also gave further support to Claude's hypothesis that microsomes were derived from the endoplasmic reticulum. However, it was not until the advent of the electron microscope that Palade and Siekevitz (1956) were able to make a thorough examination of the 'microsomes'. They confirmed Claude's original hypothesis that the microsomes were derived from the endoplasmic reticulum.

The characterization of microsomal material in heart was first reported by Hulsmans (1961), although the presence of a sarcoplasmic reticulum had been shown to exist prior to his investigation.

Microsomes are usually understood to mean the subcellular fraction which is isolated by high speed centrifugation after the mitochondrial stage has been removed from a tissue homogenate. Many factors will influence any one component of the

microsomal fraction. Therefore a definition of the preparation, homogenziation process and centrifugation conditions must be clearly stated before a meaningful interpretation and comparisons of experiments using 'microsomes' can be made. de Duve (1963) has even suggested that the term 'microsomes' should not be used but instead the term 'microsomal fraction' be substituted.

The main constituents of the microsomal fraction are; -

(i) endoplasmic or sarcoplasmic reticulum vesicles which can be either (a) smooth surfaced or
(b) rough surfaced if they have ribosomes attached to their surface.

(ii) plasma membrane fragments which according to some authors separate as sheets of material and not as vesicles.

(iii) glycogen granules.

(iv) ribosomes.

(v) any other material not separated at the mitochondrial or lysosomal stages of centrifugation.

Sub-cellular fractionation by the differential centrifugation technique is dependent on many characteristics of the particles (de Duve et al., 1959) the most important of which are size and density. A complete separation of materials by differential centrifugation is not possible and some overlapping of fractions will occur because of the large range of particle size within any one fraction.

The degree of overlapping can be restricted by careful choice of suspending medium and centrifugation times and speeds. The differential centrifugation conditions will, to some extent, reflect the interest in the particular fraction one has chosen to investigate. In deciding on the centrifugation conditions described in this chapter allowance was made for the fact that some microsomal material will be lost due to an overlap with the light mitochondrial fraction. By careful and consistent experimental technique the centrifugation can be standardised so that samples prepared from different tissues can be compared.

The fractions were examined under the electron microscope to check their morphology. The presence of nuclear material was detected by a determination of DNA content of the fractions. The distribution of RNA in the various fractions was measured as in some tissues the microsomal RNA content forms a large part of this fraction.

Succinic dehydrogenase activity was selected as a mitochondrial marker enzyme. Although it has been reported that heart microsomal fractions do not contain glucose - 6 - phosphatase activity (Hulsmans, 1961), glucose - 6 - phosphatase was determined as it has been used as a microsomal marker in many other tissue fractionation studies. (Na^+, K^+) - ATPase activity was determined as this represents the main interest of this work and also it has been found to exist mainly in the microsomal fraction of heart tissue, (Auditore, 1962; Auditore and

Murray, 1962). β glycerophosphatase activity was selected as a lysosomal enzyme marker (de Duve et al., 1955). Lysosomal activity has been reported present in a light mitochondrial fraction of myocardial tissue (Hulsmans, 1961).

Methods

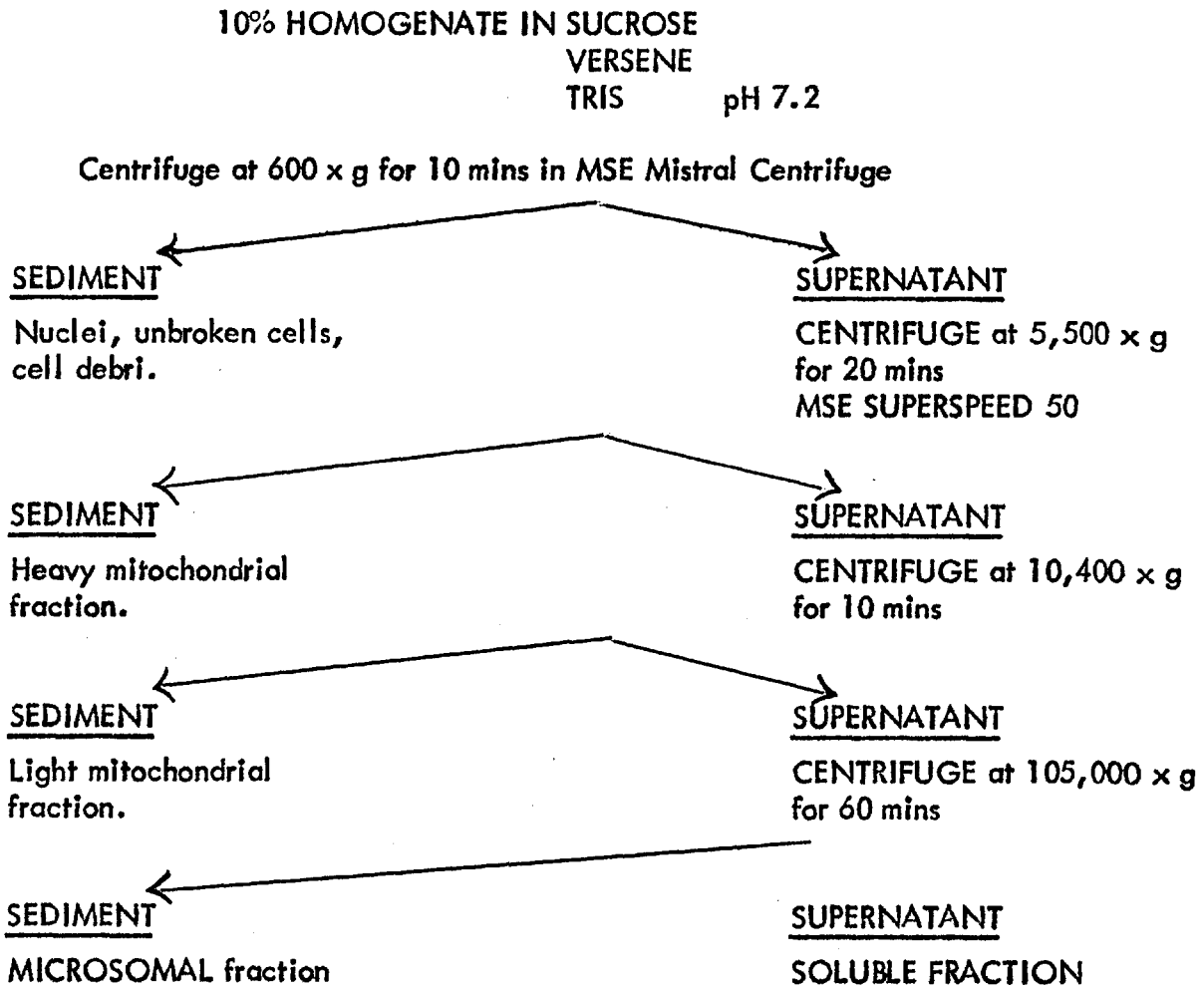
Homogenization of myocardial tissue

Two methods of homogenization have been used throughout the experiments described in this thesis. Unless otherwise stated the first method to be described has been used. At all stages of preparation the sample was kept at 0 to 5°C by surrounding it with ice.

The first method utilized a Potter - Elvehjem (1936) type of homogenizer consisting of a glass mortar and teflon pestle mounted on a stainless steel shaft. The myocardial sample was trimmed of fat and then finely chopped with scissors. Ice-cold sucrose solution (0.25 M) containing 0.002 M EDTA and 0.02 M Tris, pH 7.4, was added to make a 10% homogenate. The chopped muscle was transferred to the glass mortar surrounded by crushed ice. The teflon pestle was mounted in a Black and Decker D 500 drill supported vertically on a stand. The speed of the drill was 2,600 r.p.m. Homogenization was effected by 10 slow, consistent up and down movements of the glass mortar. The gap between the teflon pestle and mortar was 0.004 to 0.006 mm.

Figure II 1.

SCHEME FOR DIFFERENTIAL CENTRIFUGATION OF MYOCARDIAL TISSUE HOMOGENATES



At each stage resuspend sediment in homogenizing medium, centrifuge and add supernatant to previously obtained supernatant.

The second method utilized a MSE homogenizer, supplied by MSE Crawley, Sussex. This method could only be used effectively for the larger specimens of myocardium e.g. from dog and human post mortem specimens. Small samples of muscle were simply caught on the end of the cutting blades and revolved with them without being homogenized. The specimens were trimmed of fat and cut into approximately 5 g portions and placed in ice-cold sucrose solution, described in method 1, to give a 25% homogenate. The sample was transferred to a glass vortex beaker, supplied with the instrument, and surrounded by crushed ice. The beaker was clipped to the instrument and the speed of the blades slowly increased to maximum speed for 1 min.

Centrifugation

During the early stages of this study, when the efficiency of the centrifugation was being studied, the procedure outlined in Fig. II 1 was followed. This method is based on centrifugation procedures described by Appelmans, Wattiaux and de Duve (1955). The nuclei, unbroken cells and contractile material are separated from the homogenate at the first slow speed centrifugation in a MSE Mistral refrigerated centrifuge at 600 x g for 10 mins. The supernatant was carefully removed with a pipette and centrifuged at 5,500 x g in a MSE Superspeed 50 centrifuge for 20 mins giving the heavy mitochondrial

Figure II 2.

SIMPLIFIED

CENTRIFUGATION PROCEDURE FOR PREPARING THE MICROSOMAL FRACTION

10% HOMOGENATE IN
SUCROSE, VERSENE AND TRIS BUFFER 7.2

Centrifuge at 10,400 x g for 10 mins

SEDIMENT

RE-HOMOGENISED IN
½ ORIGINAL VOLUME OF
SUCROSE VERSENE TRIS

SUPERNATANT I

Centrifuge at 10,400 x g for 10 mins

SEDIMENT

DISCARDED

SUPERNATANT II

SUPERNATANT I
COMBINED AND,

Centrifuge at 105,000 x g for 1 hour

SEDIMENT

MICROSOMAL PELLET

SUPERNATANT

SOLUBLE FRACTION
DISCARDED

fraction. The supernatant was removed and centrifuged at 10,400 x g for 10 mins to yield the light mitochondrial fraction, which was extremely small compared to the heavy mitochondrial fraction pellet. The supernatant obtained after removal of the light mitochondrial fraction was centrifuged at 105,000 x g for 1 hr. The pinkish gelatinous pellet was called the microsomal fraction. The supernatant was called the soluble fraction.

At each stage of the above procedure the pellet was resuspended in half the original volume of 0.25M sucrose solution and recentrifuged at the speeds recorded above. A simplified centrifugation procedure Fig.II 2 was used for preparing the microsomal fraction of myocardial tissue from the animal experiments and for the preparation of microsomal fractions from human post mortem tissue.

Determination of Nucleic Acids in Myocardial Homogenate and Subcellular Fractions

Extraction of the nucleic acids, DNA and RNA, from the homogenate and subcellular fractions followed the scheme described by Schmidt and Thannhauser (1945) except that the phospholipid extraction was omitted as this is not desirable, Munro and Fleck (1966). The scheme is outlined in Fig. II 3. The TCA soluble RNA was extracted in supernatants 1 and 2 whilst the precipitate contained the DNA.

The TCA precipitable material was dissolved in 2.5 ml of 0.3 N KOH by

Figure II 3.

EXTRACTION OF NUCLEIC ACIDS FROM MYOCARDIAL HOMOGENATE AND SUB-CELLULAR FRACTIONS

0.4 ml sample was added to 5 ml water and 2.5 ml COLD 10% TCA. Stand in ice for 10 mins

Centrifuged at 2,500 r.p.m. for 10 mins (in Mistral refrigerated centrifuge at 1 - 3°C)

PRECIPITATE	SUPERNATANT
Washed with 5% COLD TCA. Stood for 10 mins	Discarded

Centrifuged at 2,500 r.p.m. for 10 mins (in Mistral refrigerated centrifuge at 1 - 3°C)

PRECIPITATE	SUPERNATANT
2 mls of 0.3 N KOH added and heated at 37°C for 1 hr. Cooled to 0°C then 1.5 ml cold 10% TCA added	Discarded

Centrifuged (as above)

PRECIPITATE	SUPERNATANT
Washed with 1.5 ml cold 5% TCA	(1)

Centrifuged (as above)

PRECIPITATE	SUPERNATANT
Used for the determination of DNA (see text)	(2)

Supernatants (1) and (2) combined and used for the determination of RNA (see text)

heating at 37°C for 1 hr. DNA was determined according to the method of Burton (1956) using the acid extract and reacting it with diphenylamine reagent. The O.D. after 20 hrs incubation at 30°C was measured at 600 mμ. Calf thymus DNA obtained from BDH was used as a standard.

RNA was determined by the method of Ceriotti (1955). To the acid-soluble extract, orcinol reagent was added and the samples placed in a boiling water bath for 40 mins. After cooling the color was extracted with iso-amyl alcohol and the O.D. read at 675 mμ.

Ribose was used as a standard. The conversion factor for ribose to RNA was 23 γ ribose ≡ 100 γ RNA, (Webb, 1956).

Succinic dehydrogenase activity - was measured by the method of Slater and Bonner (1952). The rate of reduction of K_3 Fe $(CN)_6$ was measured by change in O.D. at 400 mμ as a time function at 20°C. KCN was added to the assay to inhibit cytochrome oxidase activity. 0.2 M sodium succinate was used as substrate and the reaction buffered by phosphate buffer pH 7.2 0.2 M.

Glucose 6 - Phosphatase activity - was measured by the method described by Nordlie and Arion (1964). Histidine buffer pH 6.5 0.1 M was used in a 5 ml total volume reaction mixture. 0.3 M G 6 P was used as substrate. The reaction was started, after 5 mins preincubation at 37°C, with the enzyme protein. 1 ml samples were taken from the reaction mixture at 5 min time intervals up to 15 mins

and put into 0.2 ml perchloric acid to stop the reaction. The inorganic phosphate liberated from the substrate G 6 P was determined (See Page 53 for details).

The activity was expressed as the μ moles of inorganic phosphate liberated / mg Protein / hr.

β Glycerophosphatase activity - was determined by the method of de Duve (1955). The method was essentially similar to that described for Glucose 6 - Phosphatase except that the substrate was β glycerophosphate and the reaction was buffered with acetate buffer pH 5.0.

The activity was expressed as the μ moles of inorganic phosphate liberated / mg Protein / hr.

(Na^+, K^+) - ATPase activity - For details of this assay see Chapter III page 49.

Protein - was determined by the procedure described in Chapter III page 54.

Electron microscopic examination of sub-cellular fractions prepared by differential centrifugation

Samples of the pellets from each stage of the centrifugation procedure were placed in either osmium tetroxide or glutaraldehyde. The samples were then processed for electron microscopy at the Department of Anatomy, London Hospital Medical College. Cell fractions were examined from fresh tissue, tissue kept

at + 5°C and tissue kept at - 10°C.

Results

Electron microscopy of sub-cellular fractions prepared from dog myocardium

Dog myocardium was obtained from the Royal Vetinary College and transported to the laboratory packed in ice in a large thermos container. The time between removal of the heart and homogenization was 1½ to 2 hours. The ventricle of one dog was divided into several portions some of which were used fresh, some after storage at -10°C for 24 hours, and some after storage at + 5°C for 24 hours. The samples were homogenized by either method 1 or 2. Differential centrifugation by the method outlined in Fig. II 1 was followed.

Fresh dog myocardium

The nuclear fraction of samples prepared by homogenization methods 1 and 2 appeared similar. Both contained nuclei but very large quantities of contractile material, unbroken tissue and other cell debris. Mitochondria and microsomal like vesicles were also present.

Samples of the heavy mitochondrial fraction were much less contaminated with cellular fragments when prepared by the second method of homogenization process Fig. II 4 compared to the first method, Fig. II 5.

The light mitochondrial fraction appeared to contain a mixture of mitochondria

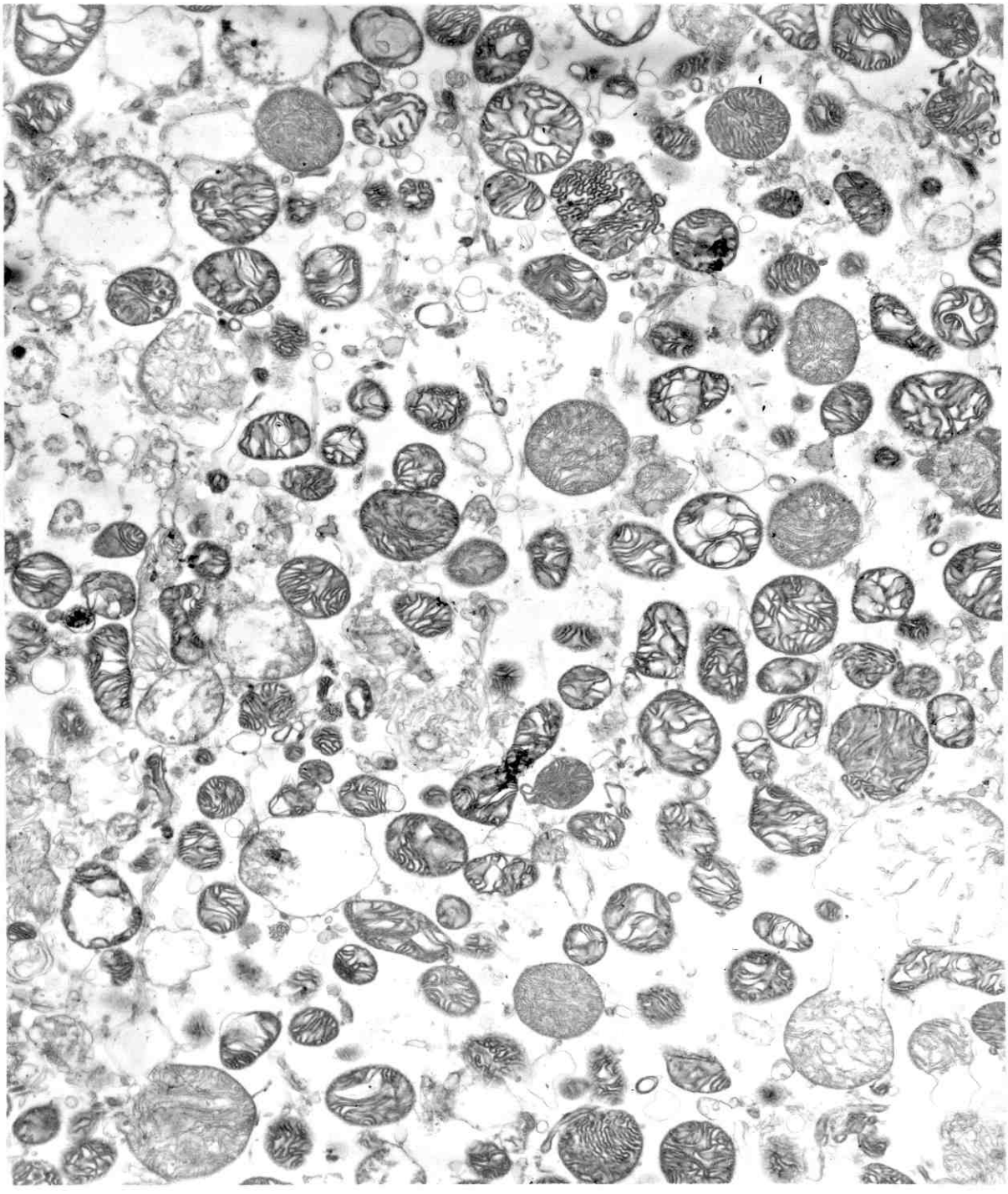


FIG. II 4 Electron micrograph of mitochondrial fraction of fresh dog myocardium prepared with an MSE homogenizer. Magnification x 20,000.

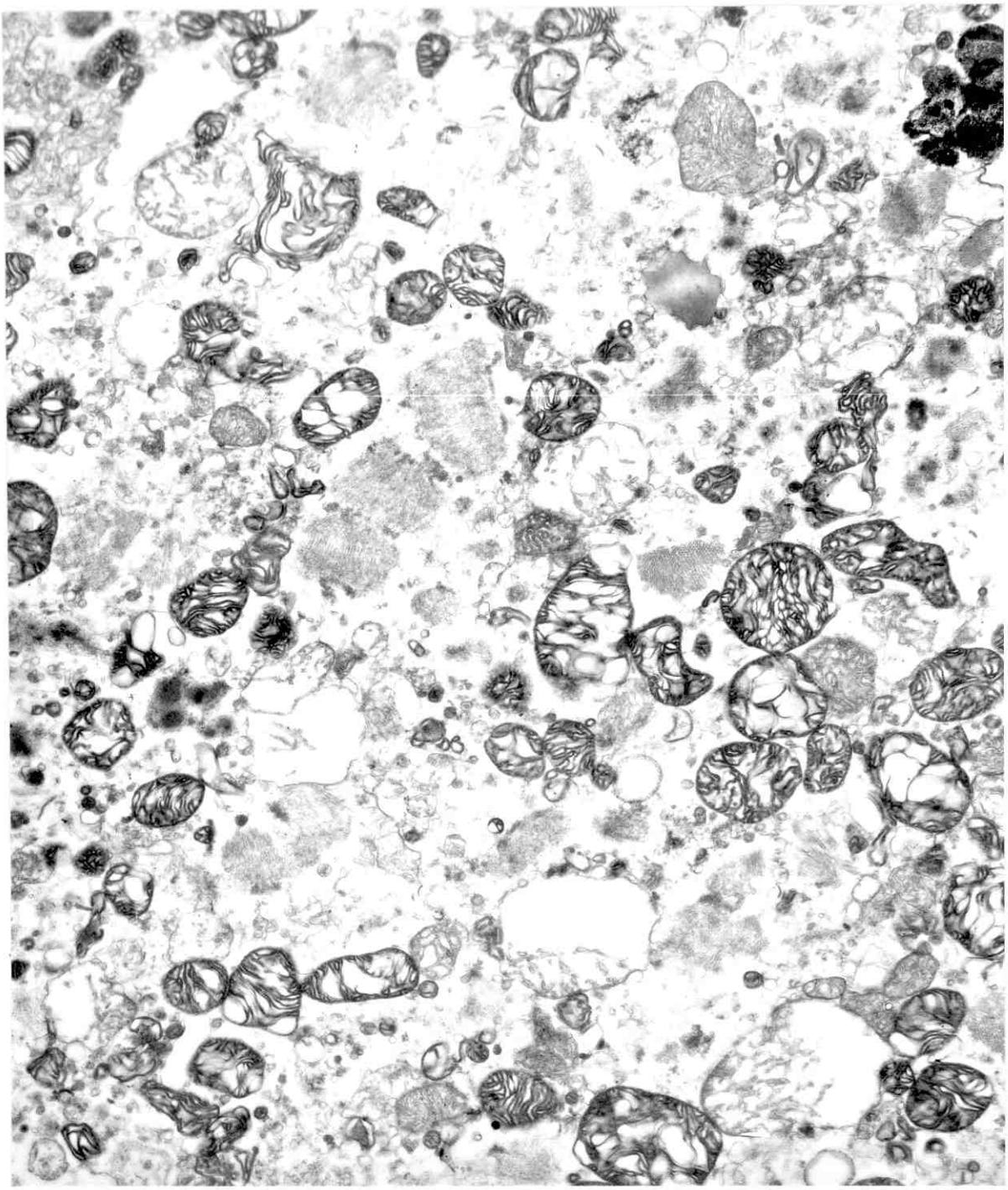


FIG. II 5 Electron micrograph of mitochondrial fraction of fresh dog myocardium prepared with a Potter-Elvehjem homogenizer. Magnification x 20,000.

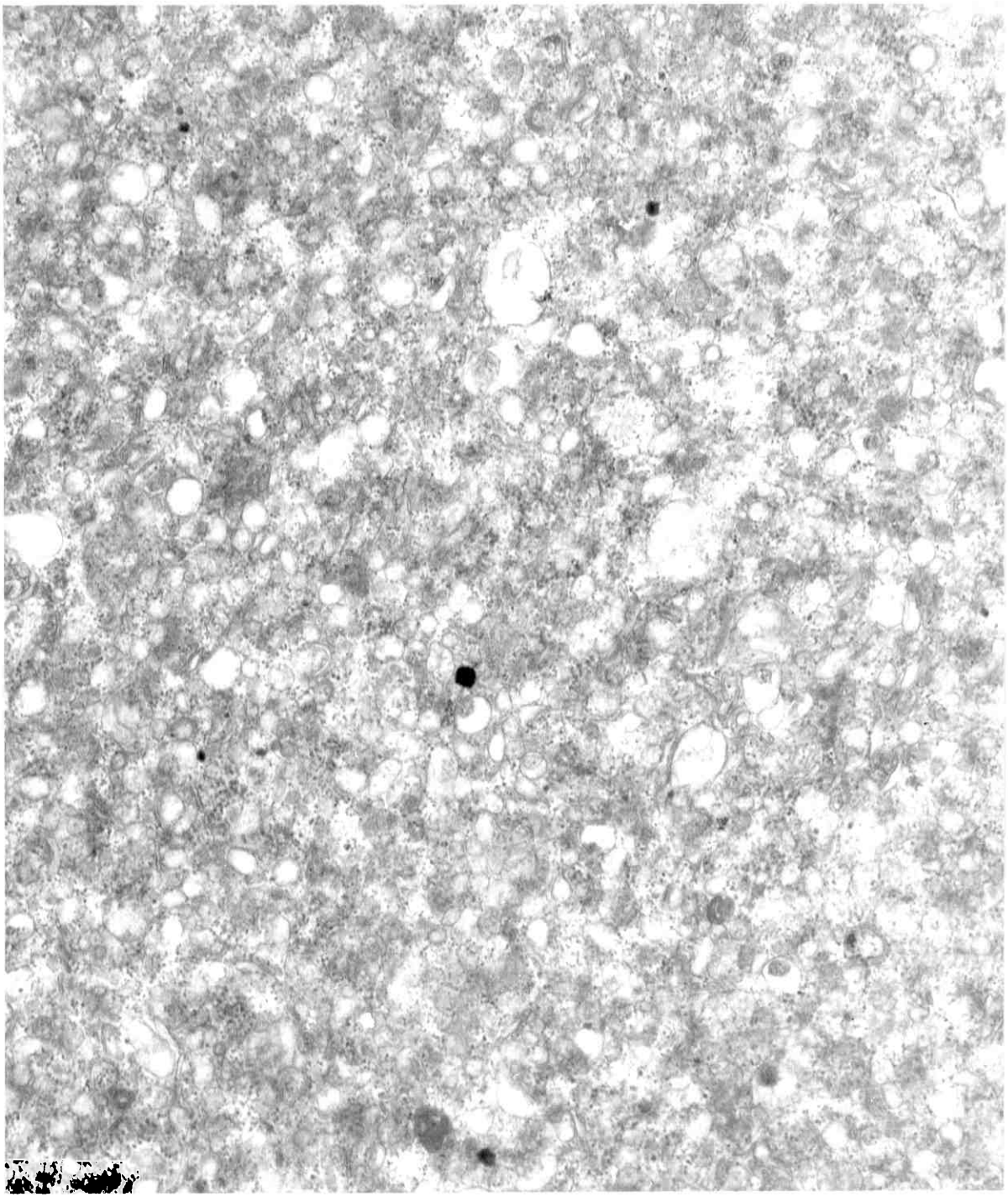


FIG. II 6 Electron micrograph of microsomal fraction of fresh dog myocardium prepared by method 1 homogenization. Magnification x 25,000

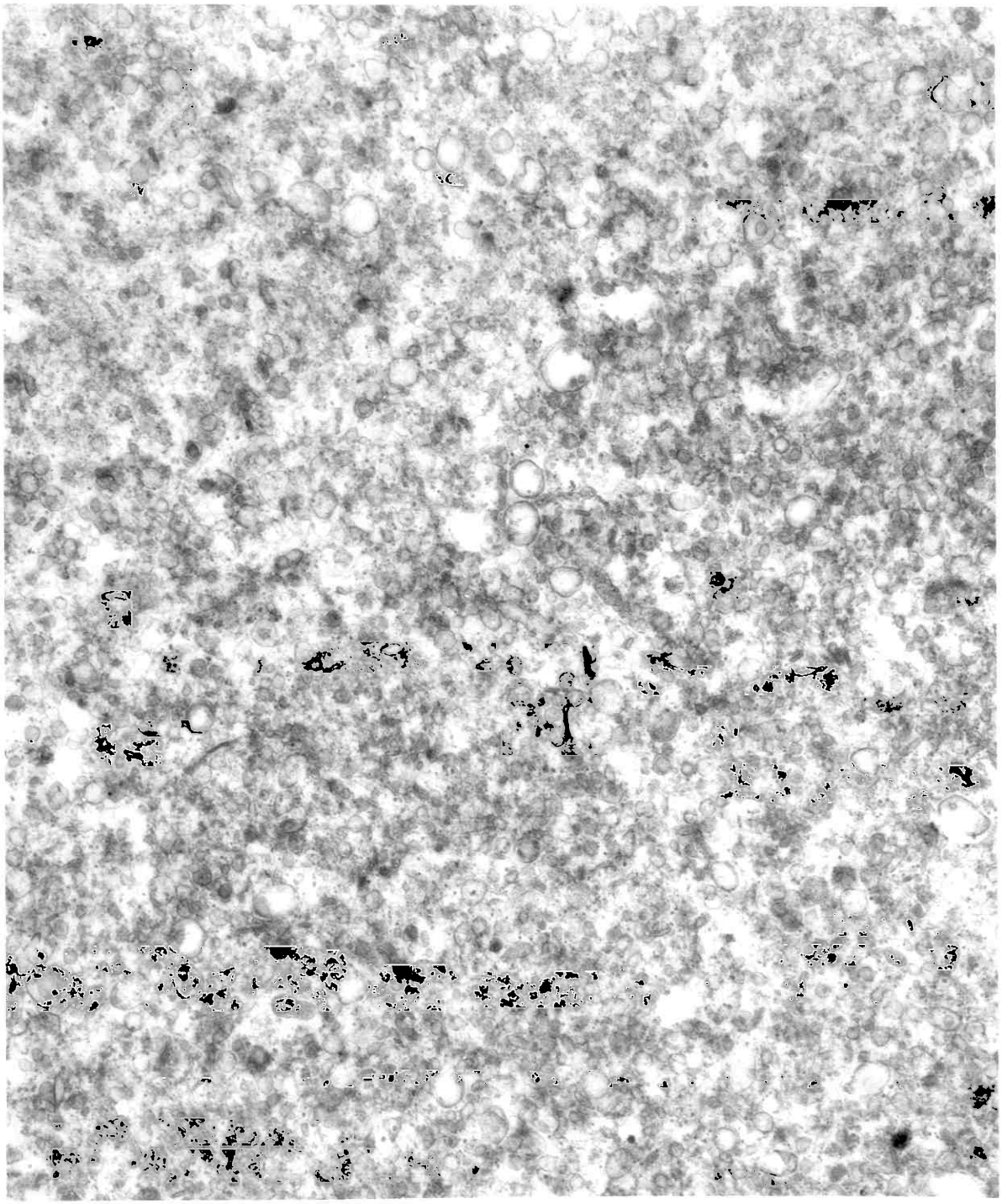


FIG. II 7 Electron micrograph of microsomal fraction of fresh dog myocardium prepared by method 2 homogenization. Magnification x 25,000.

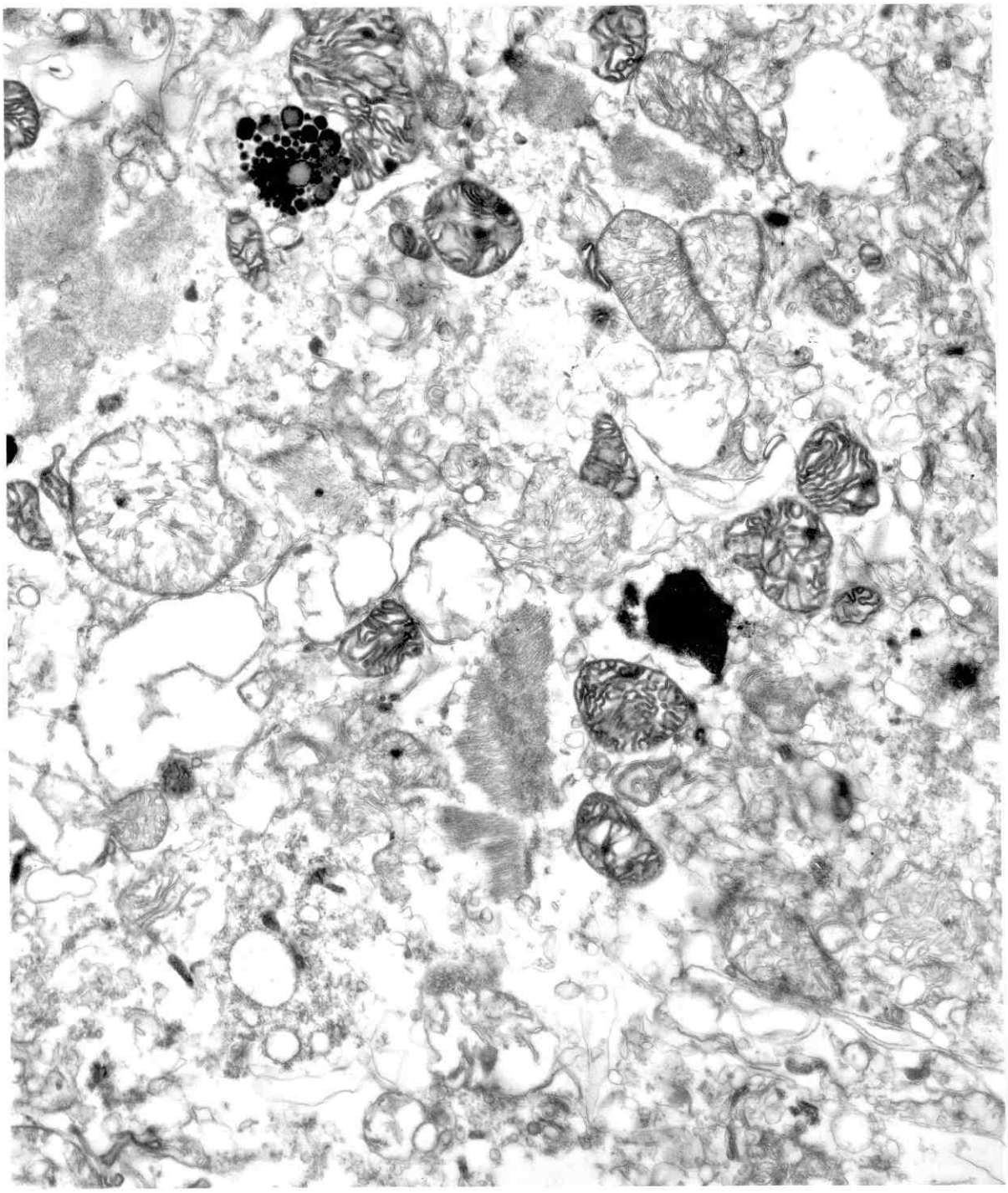


FIG. II 8 Electron micrograph of a mitochondrial fraction prepared from dog myocardium kept at + 5° C for 24 hrs. before homogenization by method 2. Magnification x 20,000.

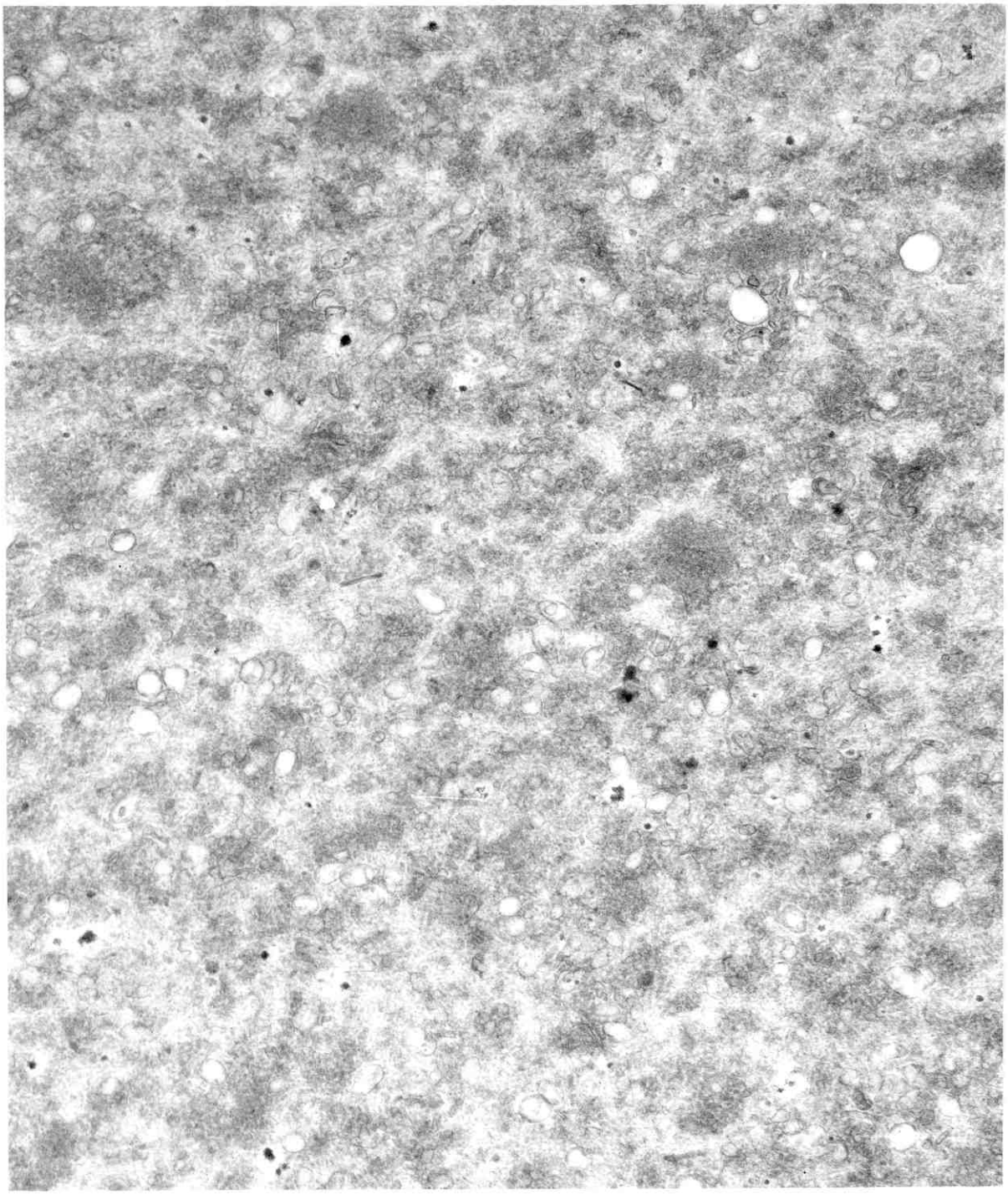


FIG. II 9 Electron micrograph of a microsomal fraction prepared from dog myocardium kept at + 5° C for 24 hrs. before homogenization by method 2. Magnification x 25,000.

and microsomal vesicles. The light mitochondrial fractions obtained from both homogenization procedures had a similar appearance under the electron microscope.

The microsomal fractions Fig II 6 and II 7 were composed of small vesicles. The fractions prepared by methods 1 and 2 homogenization processes were similar and neither contained any mitochondrial contamination nor any other cell component.

Dog myocardium kept at + 5°C for 24 hours before homogenization

Two portions of dog ventricle were kept at + 5°C for 24 hrs. The first portion was homogenized by method 1 and the other by method 2. Electron microscopy revealed very little difference between the two homogenizing processes. The nuclear fraction was heavily contaminated with contractile material, cell debris and cellular components as in the fresh tissue. The heavy mitochondrial fraction Fig. II 8 showed very greatly distended mitochondria although the fraction was still relatively free of contamination. The light mitochondrial fractions were a mixture of the distended mitochondria and vesicular structures. The microsomal fraction resembled that of the fresh material with no apparent contamination by other sub-cellular components Fig. II 9.

Dog myocardium kept at - 10°C for 24 hours before homogenization

Two portions of dog ventricle were kept at - 10°C for 24 hrs. One portion was homogenized by method 1 and the other by method 2. The fractions, except

the heavy mitochondrial fraction, appeared similar to those of fresh myocardium with no difference between the type of homogenization used. However, the heavy mitochondrial fraction Fig. II 10 showed mitochondria which were even more distended than those of the dog myocardial tissue kept at + 5°C.

The microsomal fraction was similar to the previous samples prepared from fresh and tissue kept at + 5°C.

Fresh human myocardial tissue

Human myocardial biopsy specimens were obtained during cardiac operations. The tissue was used within 2 hours of removal. In the intervening period the tissue was kept at 0° to + 5°C in 0.25 M sucrose solution.

The samples were homogenized by method 1 only because the samples were very small, within the range 0.2 to 1 g. Using the centrifugation procedure outlined in Fig. II 1 samples of each stage were prepared for electron microscopy. The results were similar for all fractions to those prepared from dog myocardium by the method 1 homogenization procedure. Figure II 11 shows the microsomal fraction demonstrating the absence of sub-cellular contaminants and the vesicular nature of the microsomal particles.

(Na^+, K^+) - ATPase activity of dog myocardial samples shown in the electron microscopic examination results.

(Na^+, K^+) - ATPase and Mg^{++} - ATPase activities were measured on a

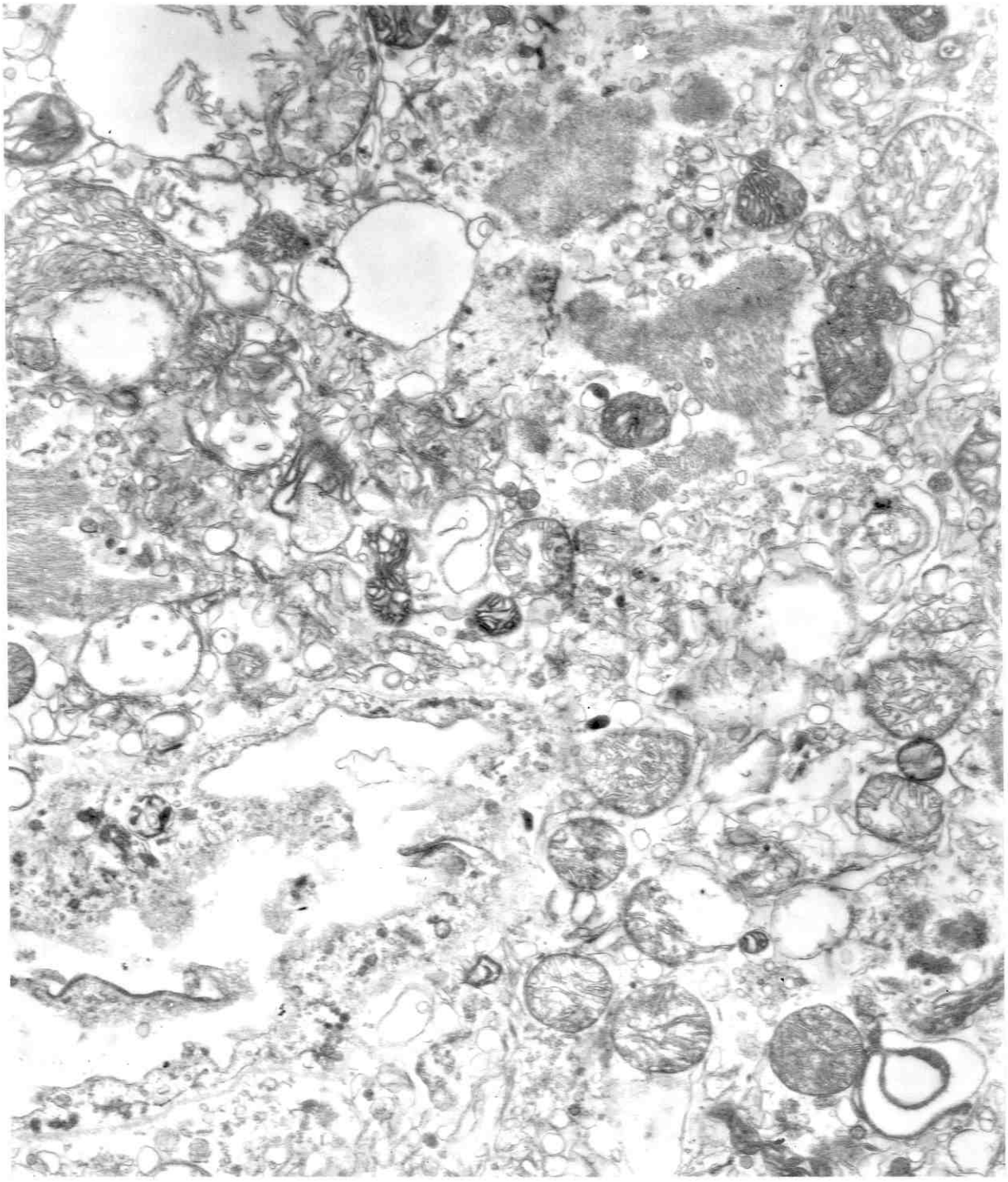


FIG. II 10 Electron micrograph of a mitochondrial fraction prepared from dog myocardium kept at - 10° C for 24 hrs. before homogenization by method 2. Magnification x 20,000.

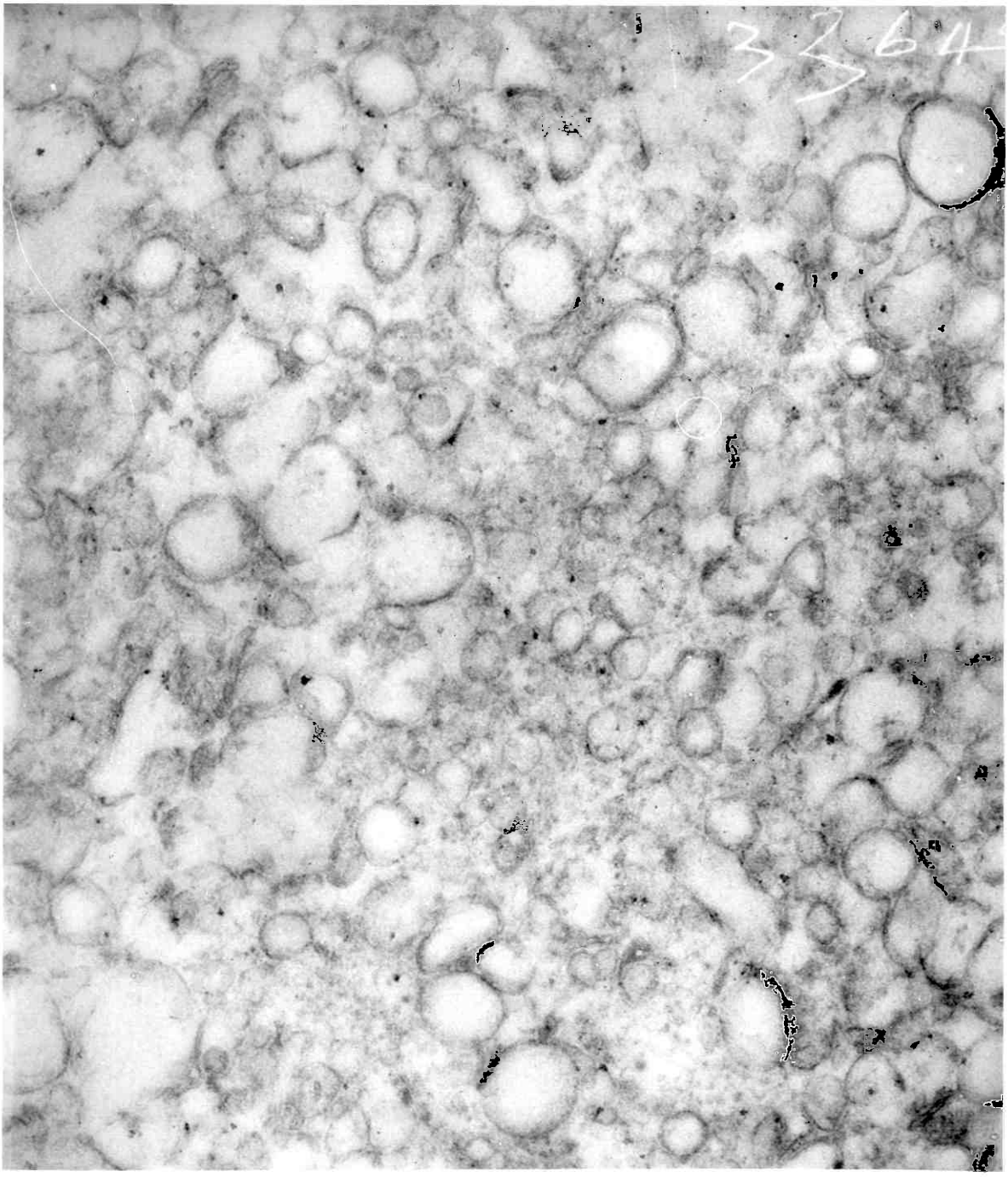


FIG. II 11 Electron micrograph of a microsomal fraction prepared from human biopsy myocardium. Magnification x 25,000.

Table II 1. EFFECTS OF HOMOGENIZER AND KEEPING CONDITIONS ON DOG MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase AND Mg^{++} - ATPase

SAMPLE	POTTER ELVEHJEM HOMOGENIZER			MSE HOMOGENIZER		
	(Na^+, K^+)-ATPase	Mg^{++}-ATPase	R	(Na^+, K^+)-ATPase	Mg^{++}-ATPase	R
FRESH	0.43	1.51	0.35	0.90	0.80	1.12
	1.10	1.70	0.65	0.60	0.65	0.92
Kept at +5°C	2.80	2.23	1.25	2.90	1.51	1.92
for 24 hours	3.23	1.95	1.66	2.81	1.45	1.94
Kept at -10°C	2.21	1.88	1.18	2.05	1.85	1.11
for 24 hours	2.31	2.23	1.06	1.37	1.81	0.76

$$R = \frac{Na^+, K^+ - ATPase}{Mg^{++} - ATPase}$$

The results are from two studies. The upper line of figures in each case representing the first experiment and the lower line the second experiment.

sample of each fraction investigated under the electron microscope. The results are shown in Table II 1. The microsomal (Na^+, K^+) - ATPase activity of the preparation increased with storing at either - 10°C or + 5°C, independent of the homogenizing method. It was also noted that the ratio of $\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$ was higher in the tissues kept for 24 hrs than in the fresh tissue.

Characterization of dog ventricle sub-cellular fractions by enzymatic and other methods

A total protein content of a portion of fresh dog ventricle homogenate was determined and the remaining homogenate fractionated by the differential centrifugation procedure outlined in Fig. II 1. The sub-cellular fractions were suspended in 0.25 M sucrose solution, the volume determined and the protein content assayed. The volume of the soluble fraction was measured and its protein content determined. This then gave the percentage of the total protein content of the homogenate represented by each fraction.

Each fraction was then assayed for: —

DNA content
RNA "
Succinic dehydrogenase activity,
β glycerophosphatase activity,
Glucose 6 - Phosphatase activity,
($Na^+ + K^+$) - ATPase activity.

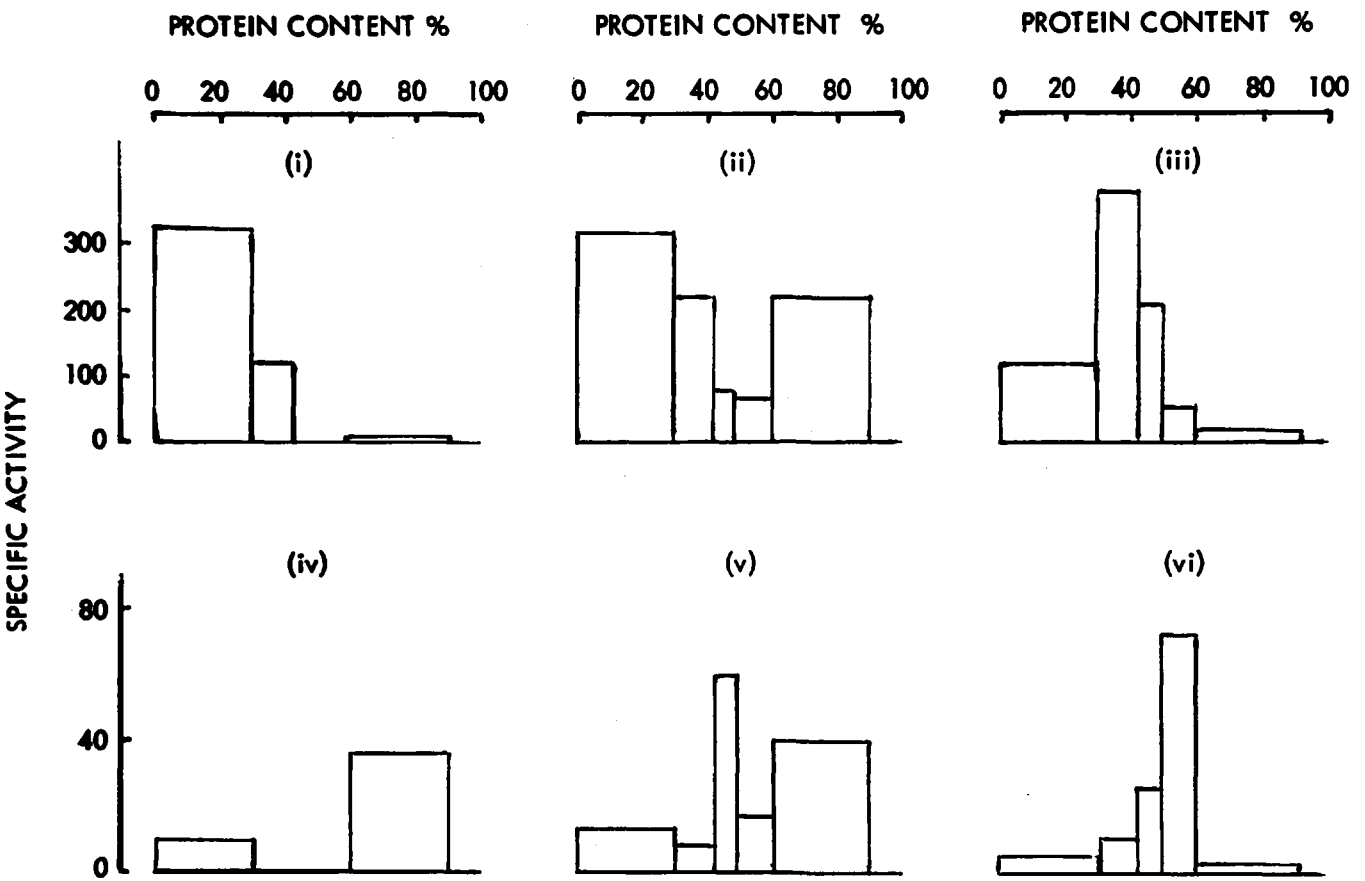


FIG. II 12 Subcellular distribution of RNA, DNA and four enzymes in dog myocardial tissue. (i) DNA (ii) RNA (iii) Succinic dehydrogenase (iv) Glucose 6-Phosphatase (v) Acid B-Glycerophosphatase (vi) (Na^+, K^+) - ATPase.

The results of these experiments are summarised in Fig. II 12. The DNA content was mainly restricted to the nuclear fraction with contamination of the heavy mitochondrial and soluble fractions. RNA was distributed throughout the fractions but mainly concentrated in the nuclear, heavy mitochondrial and soluble fractions.

Succinic dehydrogenase activity was found in all the fractions but most of the activity was concentrated in the heavy mitochondrial and light mitochondrial fractions. Glucose 6 - Phosphatase activity was found in the nuclear and soluble fractions but not in any other fraction.

Acid β glycerophosphatase activity was found in all the fractions, the highest activity was found in the light mitochondrial fraction.

(Na^+, K^+) - ATPase activity was found in all the fractions but the highest activity was found in the microsomal fraction.

Conclusions

It was not intended that this work would determine new methods for isolating microsomal material from myocardial tissue but rather establish a suitable technique and check the fractionation from a morphological and enzymatic view-point. A further consideration was the effects of storage on myocardial tissue and its possible effects on the microsomal extracts. This was important as it was proposed to study

human post-mortem heart which was available only after storage.

The first fraction to be isolated by centrifugation is the nuclear fraction. That the nuclei were restricted to this fraction and not contaminating other fractions was adequately substantiated by lack of nuclei visible under the electron microscope in all but the nuclear fraction and the lack of DNA in all fractions except the nuclear.

The distribution of RNA throughout the fractions followed a pattern similar to that found by Hulsmans (1961) in rat heart and Schwartz (1962) in calf heart sub-cellular fraction. RNA was determined to give a guide to its distribution and its content in microsomal fractions as a measure of purity of this fraction. Mitochondria could be obtained from both homogenization procedures in an intact state although storing the tissue at - 10°C or + 5°C had an adverse effect. No mitochondria could be seen in microsomal fractions under the electron microscope. The second homogenization method yielded a better mitochondrial fraction. The structural integrity of the mitochondria is an important consideration because when they are disrupted or damaged leakage of mitochondrial enzymes will possibly contaminate the microsomal fraction. That the centrifugation technique was adequate to yield a good mitochondrial fraction was further proved by the distribution of the mitochondrial enzyme, succinic dehydrogenase. The activity was found mainly in the heavy mitochondrial fraction, some in the nuclear and

light mitochondrial fraction and very little in the microsomal fraction.

The light mitochondrial fraction contained both mitochondria and small vesicles when viewed under the electron microscope. β glycerophosphatase, a lysosomal marker enzyme, was found to be high in this fraction indicating that at least some of the vesicles were lysosomal. Hulsmans (1961) also reported lysosomal activity in myocardial tissue. Very little lysosomal contamination, by the enzyme method, was found in the microsomal fraction. Morphologically both lysosomal and microsomal material look very similar under the electron microscope hence it was difficult morphologically to detect contamination by lysosomes.

The microsomal fraction, obtained from the differential centrifugation procedure, was relatively pure from a morphological point of view. The appearance of the microsomal fraction seemed to be independent of the preparation, the tissue or the storage conditions studied. Enzymically the microsomal fraction contained little contamination from mitochondria, lysosomes and nuclei.

The (Na^+, K^+) - ATPase activity was higher in the microsomal fraction prepared from dog myocardium kept for 24 hrs after removal from the animal. Keeping the tissues at + 5°C or - 10°C for 24 hrs made very little difference to the microsomal Mg^{++} - ATPase activity. Autolysis or freezing the tissue may have modified the membrane in some way which allowed the enzyme to react with the

substrate ATP. Alternatively a labile protein inhibitor of (Na^+, K^+) - ATPase may have denatured on storage for 24 hrs. Such a protein inhibitor has been found in calf heart tissue, (Schwartz, 1965). In this respect storage may have had a similar effect to deoxycholate, in ' unmasking' (Na^+, K^+) - ATPase from myocardial microsomal preparations (the use of deoxycholate is discussed in Chapter IV).

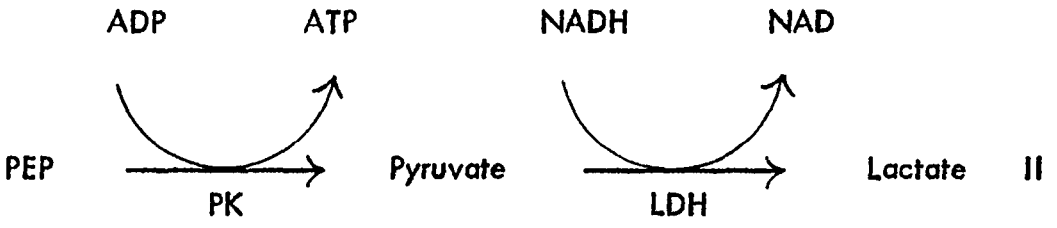
CHAPTER III

ASSAY OF ATPase ACTIVITY

The ATPase enzyme system from microsomal preparations consists of two main components: a Mg^{++} - ATPase and a Mg^{++} plus Na^{+}, K^{+} - ATPase. As it has not been possible completely to eliminate the Mg^{++} - ATPase component both must be measured. This presents problems in choosing a suitable method of assaying the Na^{+} plus K^{+} ion stimulated portion. There are two possibilities (i) to omit Na^{+} and K^{+} ions from one assay to determine the Mg^{++} - ATPase and include Na^{+} and K^{+} ions in another assay to find the total ATPase activity, (ii) addition of a high concentration of ouabain in the presence of Na^{+} and K^{+} ions to one assay mixture, to inhibit the Na^{+}, K^{+} ion stimulated portion. To the other assay mixture ouabain is omitted but Na^{+} and K^{+} ions are present. In both cases the Mg^{++} - ATPase activity must be substracted from the total ATPase activity to obtain the Na^{+}, K^{+} ion stimulated portion of the ATPase activity.

There are two possible methods.by which ATPase activity can be measured. The first method is an optical measurement, the chemical basis of which is outlined in I and II below.

$$ATP \xrightarrow{ATPase} ADP + Pi \qquad I$$



The ADP liberated in I by the Mg^{++} - Na^{+} - K^{+} - ATPase and Mg^{++} - ATPase is used by PEP and PK to form pyruvate (II) which with LDH forms lactate and NAD from NADH. The rate at which NADH forms NAD is measured spectrophotometrically at 366 m μ or 340 m μ. An assay system without Na^{+} and K^{+} ions or plus Na^{+} and K^{+} ions and ouabain is then measured to determine the Mg^{++} - ATPase activity. The difference between the Mg^{++} and Mg^{++} + Na^{+} + K^{+} mixtures is the (Na^{+}, K^{+}) - ATPase activity. The optical method has been used by Schoner et al. (1967) for the determination of ox brain (Na^{+}, K^{+}) - ATPase.

The second method is by measuring the rate at which inorganic phosphate is liberated from ATP (as in I above).

In order to compare the activities of various preparations and different animal tissues a reference unit must be chosen to which the enzyme activity can be related. No completely satisfactory common unit for reference has been found. Measurements of dry weight of tissue or cellular fractions are variable and very dependent on such factors as fatty infiltration and variable protein

content. The common use of protein content is a better criteria than dry weight. Although it has faults it is less open to the errors in dry weight measurement. The protein content of a sample does not always mean that all the protein measured is enzymic. A careful choice of sample preparation will however help in standardizing the protein content. In order to compare with other published data of similar work, protein was determined according to the method of Lowry et al. (1951). An exception to this was the determination of protein peaks from column separations and density gradient separations of microsomal fractions. In these experiments protein was determined by measuring the absorbancy at 280 mμ in a Unicam SP 500 spectrophotometer.

It has been reported (Skou, 1964) that addition of ouabain in high concentration does not inhibit all the Na^+, K^+ stimulated ATPase activity. When Na^+ and K^+ ions are omitted from the reaction a lower activity is found than with Na^+, K^+ plus ouabain.

It has not been possible to remove all the Na^+ or K^+ ions from microsomal fractions even after complex washing procedures, (Goldfarb and Rodnight, 1969). Keeping solutions and obtaining chemicals free from Na^+ and K^+ is very difficult. In studying inhibition or activation by compounds method (i) would be prefered providing Na^+ and K^+ omission gives a suitable and reliable measure of Mg^{++} - ATPase activity. Both methods were studied in this thesis.

Auditore and Murray (1962) found that a 10 sodium to 1 potassium ion ratio was required for maximum (Na^+, K^+) - ATPase activity in rabbit myocardium. A 10 : 1 ratio of sodium to potassium ions was also found to be required for human, Chapter V pages 100 and 106, rat, Chapter VI page 143, and dog myocardium, Chapter VI page 132.

Methods

Optical determination of Na K ATPase activity

In a total volume of 3 mls were, 50 mM tris - HCl buffer pH 7.4; 2 mM ATP; 3 mM $MgCl_2$; 100 mM NaCl; 10 mM KCl; 0.5 mg PEP (Boehringer tricyclohexyl-ammonium salt); 50 μg LDH enzyme (Boehringer, from rabbit muscle), 1 μg PK (Boehringer, from rabbit muscle); 0.5 mg $NADH_2$. The reaction was started by the addition of 0.16 mg of microsomal protein. The decrease in optical density, measured at 366 mμ, was followed for 60 mins. When 10^{-2} M ouabain was added, in addition to the above, the total volume was maintained at 3 mls by adjusting the water volume. The experiments were performed at 20°C.

Measurement of (Na^+, K^+) - ATPase activity by measuring the release of inorganic phosphate from ATP

The total volume of the enzyme assay mixture was 1 ml and contained (unless otherwise stated); 3 mM $MgCl_2$; 100 mM NaCl; 10 mM KCl; 2 mM tris ATP;

50 mM tris - HCl buffer pH 7.4. The assay mixture was pre-incubated at 37°C for 5 min. after which 0.08 mg of microsomal protein was added to start the reaction. The reaction was stopped after 45 min. with 0.2 ml perchloric acid and the inorganic phosphate released was measured. The incubation time was reduced to 30 min. in all the kinetic and inhibition studies (Chapters VIII, IX and X). In some experiments an ATP regenerating system was added to the reaction mixture when the incubation time was extended to 45 min. The ATP regenerating system was: 1 mg of PEP and 1 µg of PK per 1 ml assay volume. The conditions of assay were otherwise those stated above.

Determination of Inorganic Phosphate

Inorganic phosphate was determined by a modification of the method reported by Martin and Doty (1949) based on the method originally described by Berenblum and Chain (1938). 1 ml of the acidified reaction mixture was pipetted into a stoppered tube containing 3 mls of 1.66% ammonium molybdate solution and 4 mls of a 1 : 1 mixture of toluene and n - butanol. The tube was shaken and centrifuged briefly at 2,000 r.p.m. to separate the aqueous and organic phases. 2 mls of the upper solvent layer were removed and put into 3 mls of ethyl alcohol containing 2% sulphuric acid. 0.5 ml of a stannous chloride solution (10g $SnCl_2$ in 25 ml of concentrated HCl) was added to 100 ml of 1 $NH_2 SO_4$. This solution

was prepared fresh each day. 0.5 ml of the diluted stannous chloride solution was added to the ethyl alcohol and toluene : butanol mixture. A blue colour developed which was measured at 750 mμ. The optical density of the standard phosphate in the range 5μg to 20μg was measured. The optical density of the test solutions was read against a graph of the standard solutions to find the inorganic phosphate content.

Protein determination

Protein was determined according to the method of Lowry et al. (1951). To 0.4 ml of protein solution were added, 3 ml alkaline reagent (50 mls of 2% $Na_2 CO_3$ in 1 N NaOH and 1 ml of a solution containing 0.5% $CuSO_4$ in 1% Na tartrate) and 0.4 ml of Folin Ciocalteu reagent (diluted 1 : 4). The optical density of the colour was read at 700 mμ after 20 min. Protein standards were measured with each batch of samples. All protein determinations were in triplicate.

The results of all the ATPase enzyme studies are given as: - micromoles of inorganic phosphate liberated / mg protein / hour.

Results

In all the experiments reported in this chapter dog myocardial microsomal fractions have been used.

Optical assay

Fig. III 1 shows the results of an assay using a dog ventricle microsomal suspension.

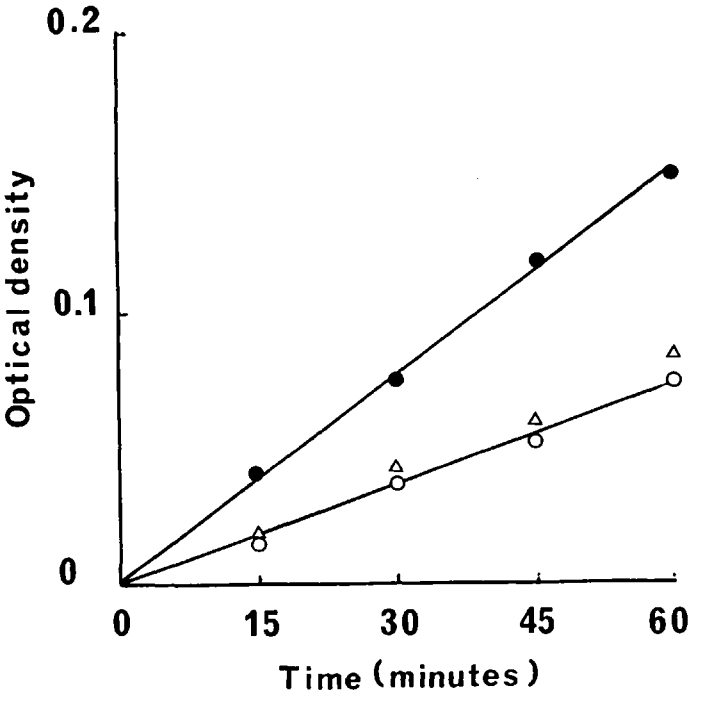


FIG. III 1 Optical determination of (Na^+, K^+) -ATPase activity. ●, complete assay system; ○, assay system with 10^{-2} M ouabain added; △, Na^+ and K^+ omitted from assay.

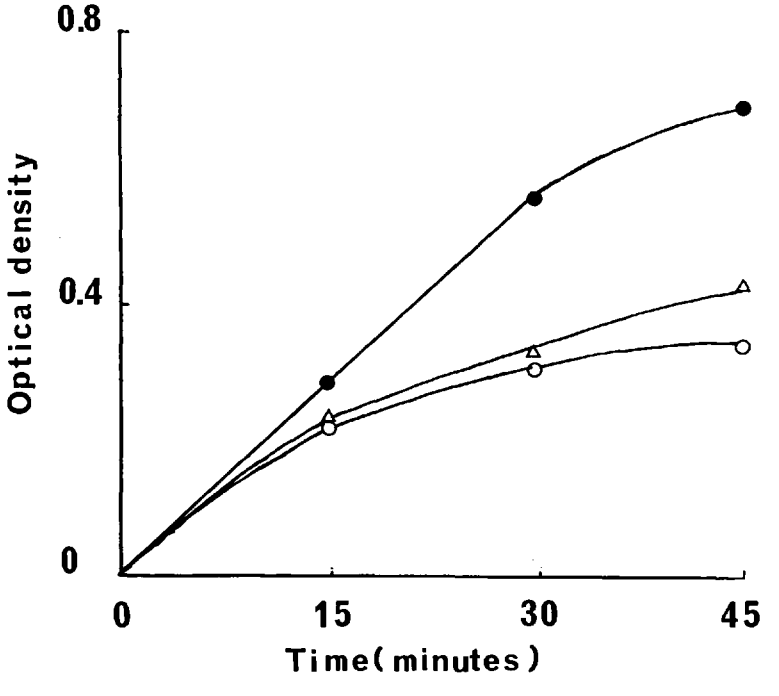


FIG. III 2 Determination of (Na^+, K^+) -ATPase activity by measuring the release of inorganic phosphate. Symbols as in FIG. III 1

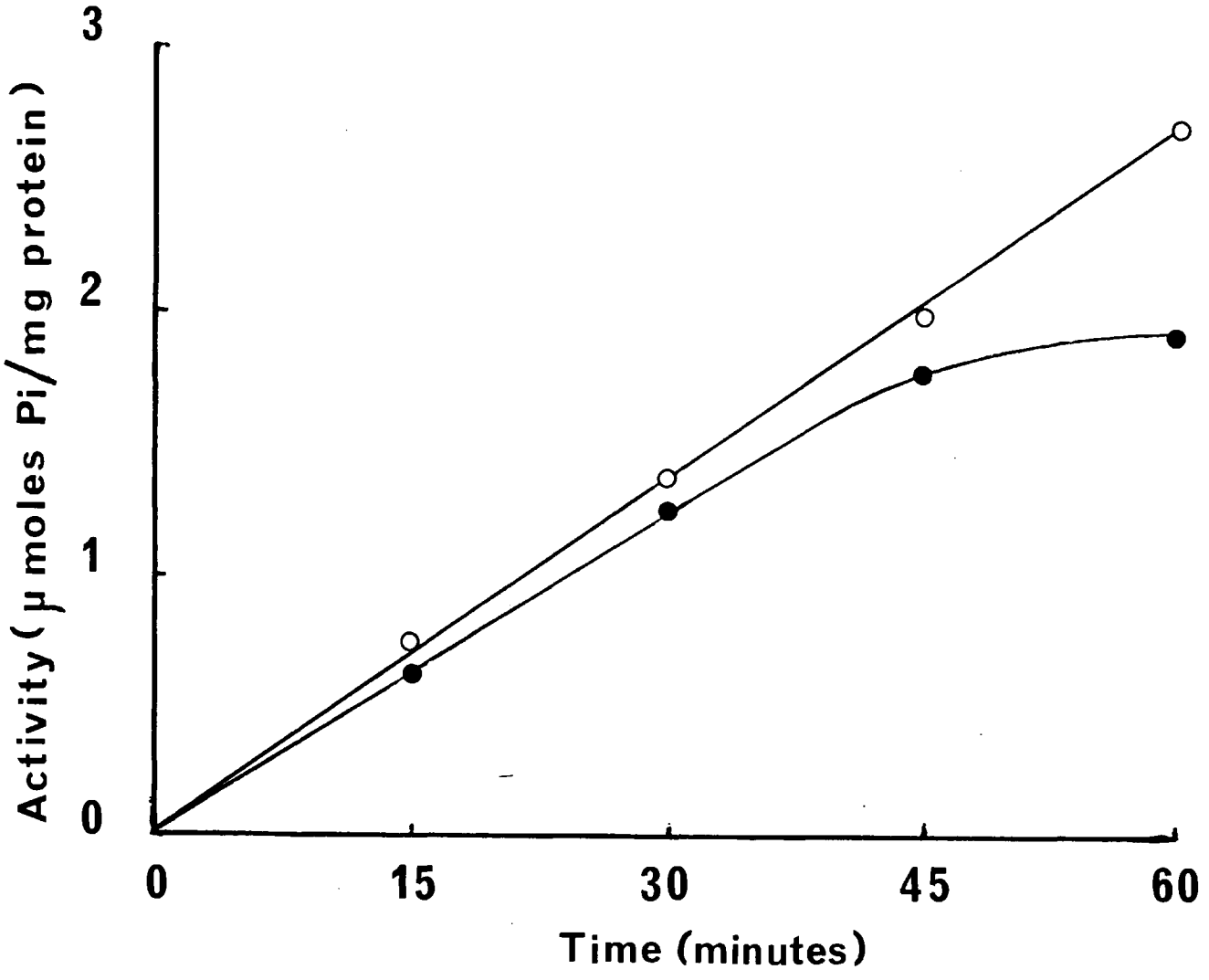


FIG III 3 Time activity relation and the effects of an ATP regenerating system on dog myocardial $\overline{(Na^+, K^+)}$ -ATPase ● no regenerating system; Oplus regenerating system.

The result gives a linear time / activity response up to 60 min. The optical density change after 1 hr. was 0.150 for the complete assay system and 0.075 for one containing 10^{-2} M ouabain. 0.075 optical density therefore represents the change due to the (Na^+, K^+) - stimulated ATPase.

Measurement by release of inorganic phosphate

Fig. III 2 shows the activity / time relation measured by this method. Unfortunately after 45 min. incubation time the inorganic phosphate released was not a linear response. Rendi and Uhr (1964) and Schoner et al. (1966) have shown ADP to be a competitive inhibitor of (Na^+, K^+) - ATPase activity, therefore, an ATP regenerating system was added to convert the ADP released to ATP. Fig. III 3 shows the results of adding such a system. The addition of 10^{-3} M ouabain lowered the ATPase activity slightly below that of the omission of Na^+ and K^+ ions from the assay mixture after 45 mins incubation. In rat, (Chapter VI) human, (Chapter V) and dog (Chapter VI) microsomal preparations the omission of Na^+ and K^+ corresponded to the same Mg^{++} - ATPase activity found by the addition of 10^{-3} M ouabain together with the Na^+ and K^+ ions.

Effects of shaking during incubation of assay samples

Two groups each of 20 tubes were incubated at 37°C for 45 min. One group was shaken in a shaking incubator whilst the second group remained unshaken

during the incubation period. There was no statistically significant difference in either the Mg^{++} - ATPase or the (Na^+, K^+) - ATPase activity between the two groups.

	No. of experiments	Mg^{++} - ATPase		(Na^+, K^+) - ATPase	
		Mean	S.D.	Mean	S.D.
Assay tubes shaken during incubation	15	2.68	0.31	6.21	0.90
Assay tubes not shaken during incubation	15	2.09	0.30	6.13	0.79

Use of the disodium salt of ATP

In solution disodium ATP was more stable than tris - ATP and gave a lower phosphate 'blank' reading. As ADP is an inhibitor of (Na^+, K^+) - ATPase the more stable form would be desirable. Therefore, in most experiments, except the kinetic studies, the disodium salt was used. The presence of disodium ATP was a further influence on the choice of an assay system whereby (Na^+, K^+) - ATPase activity was measured by the difference between the presence and absence of ouabain.

Effects of Inorganic Phosphate on (Na^+, K^+) - ATPase

Fig. III 4 shows the results of adding increasing concentrations of phosphate. Inorganic phosphate had no effect up to 15 µg after which there was inhibition

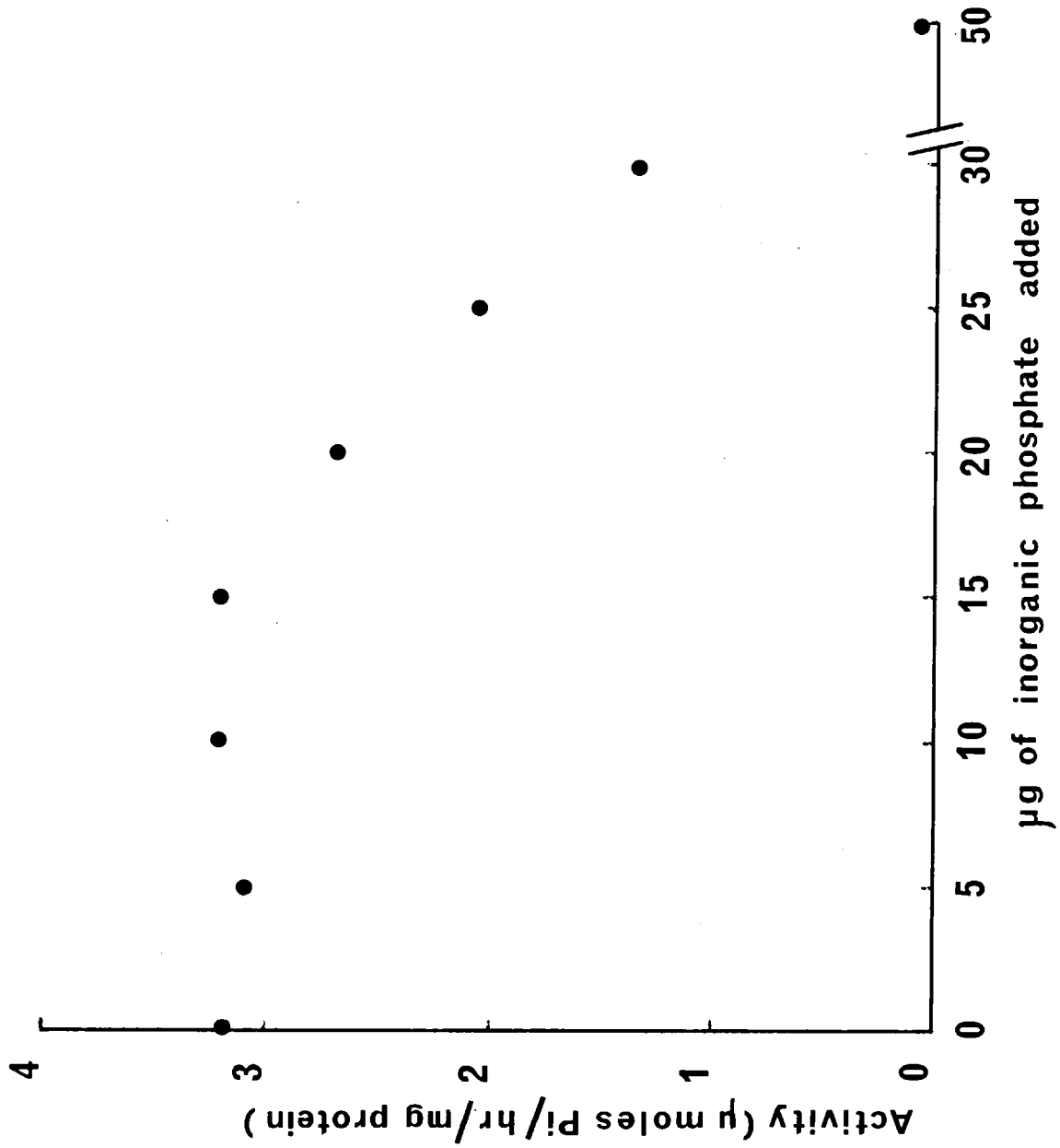


FIG. III 4 The effects of phosphate on (Na^+, K^+) -ATPase activity.

up to 50 µg at which (Na^+, K^+) - ATPase activity was fully inhibited.

Discussion

The measurement of ATPase activity by the release of inorganic phosphate from ATP was considered to be better than the optical determination. Only three assays at a time could be performed using the optical method and the optical density change was lower than that determined by the inorganic phosphate method for the same quantities of microsomal protein. Inorganic phosphate release did not interfere with the reaction mixture under the assay conditions, not allowing more than 15% breakdown of ATP. In the assay method, measuring the release of inorganic phosphate, ADP inhibited (Na^+, K^+) - ATPase activity if incubation was prolonged without the addition of a ATP regenerating system. Addition of an ATP regenerating system converted the ADP to ATP and inhibition was alleviated. In kinetic studies of calf heart (Na^+, K^+) - ATPase Matsui and Schwartz (1966) also used an ATP regenerating system.

Both Mg^{++} - ATPase and (Na^+, K^+) - ATPase are present in the microsomal preparations and so both have to be measured. The most convenient method was found to be by calculating the difference between the ATPase activity in the presence and absence of 10^{-3} M ouabain. 10^{-3} M ouabain was shown completely to inhibit the (Na^+, K^+) - ATPase portion of the total ATPase activity. In most

experiments the addition of 10^{-3} M ouabain gave a slightly lower Mg^{++} - ATPase activity than a system without ouabain, sodium and potassium ions. This can be explained by the presence of endogenous ions present in the preparation, sodium ions present in the disodium salt of ATP, sodium deoxycholate and small quantities of sodium and potassium ions present in solution all of which will have a stimulating effect in the presence of magnesium ions. Stimulation of ATPase activity by ions from these various sources was eliminated by the addition of 10^{-3} M ouabain.

Samples having a low Mg^{++} - ATPase activity and a high (Na^{+}, K^{+}) - ATPase activity could be incubated for 30 mins to give a linear activity / time response without the addition of an ATP regenerating system.

The sodium salt of ATP was used wherever possible as it was more stable than tris - ATP.

CHAPTER IV

PURIFICATION OF MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase

The criteria by which an enzyme can be called pure cannot be strictly applied to (Na^+, K^+) - ATPase. The enzyme system isolated in a microsomal fraction is possibly a group of enzymes rather than a single enzyme (Skou, 1964). The structural integrity of the membrane is required for full activity (Skou, 1964) which creates difficulties in freeing the enzyme from the cell membrane.

Phospholipids appear to play an important role in the enzyme structure (Tanaka and Abood, 1964). Phosphatidyl serine (Fenester and Copenhaver, 1967) and lecithin (Tanaka and Strickland, 1965) have been found to activate the (Na^+, K^+) - ATPase from both kidney and brain. Phospholipase C (Swanson, Bradford and McIlwain, 1964; Skou, 1961; Schatzman, 1962) destroys the activity of (Na^+, K^+) - ATPase.

For the present study purity has been judged by the increase in the ratio of $\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$, the criteria used by Matsui and Schwartz (1966). Furthermore, the (Na^+, K^+) - ATPase portion must remain sensitive to ouabain inhibition.

Before the enzyme can be detected in myocardial microsomal fractions some form of modification to the membrane fragments is required to allow the enzyme and

substrate to react. This is not common to all tissues as for example (Na^+, K^+) - ATPase in erythrocytes can be measured without pre-treatment to liberate the enzyme activity.

Many different methods have been used to cause this modification of the myocardial membrane. Deoxycholate has been commonly used (Skou, 1962 for brain and kidney, Schwartz, 1962 in calf heart). In high concentrations deoxycholate will inactivate the enzyme but at concentrations of about 0.1% it causes an activation. At a concentration of 0.1% deoxycholate Swanson, Bradford and McIlwain (1964) reported that this was not sufficient to sulubilize the phospholipids present but just sufficient to cause the necessary modification of the membrane allowing (Na^+, K^+) - ATPase activity to be liberated. Triton and other surface activating substances have also been used (Swanson et al., 1964 and Somogyi, 1964). Nakao et al., (1963) used NaI to purify (Na^+, K^+) - ATPase. The microsomal fraction was treated with high concentrations of NaI, a "structure disrupting" compound (Tonomura et al., 1962 and Warren et al., 1966) and re-centrifuged at 105,000 x g for 1 hour. The complete purification scheme is shown in Fig. IV 1.

Matsui, and Schwartz, (1966) used a combination of deoxycholate and NaI treatment to purify calf heart (Na^+, K^+) - ATPase.

It is possible that NaI or deoxycholate treatment inactivates a labile inhibitor. A protein inhibitor in heart muscle has been shown to exist, (Skou, 1964 and

Schwartz and Laseter, 1964 (b)). Schwartz (1965) claims the inhibitor is a histone or histone like compound derived from the cell nucleus. A heat stable factor was found in the supernatant of heart muscle homogenates after centrifuging at 250,000 x g for 1 hour (Skou, 1964). When the factor is added to heart muscle enzyme it decreases the activity of Mg^{++}-ATPase more than the (Na^{+}, K^{+}) - ATPase activity.

A further possibility is the formation of Mg^{++} - ATPase from a (Na^{+}, K^{+}) - ATPase by some modification to the enzyme (Skou, 1964) or a conversion of Mg^{++} - ATPase to Na^{+}, K^{+} - ATPase (Skou and Hilberg, 1965). Auditore and Murray (1962) demonstrated that in rabbit ventricle the Mg^{++} - ATPase has a pH optimum of 9.0 whilst the (Na^{+}, K^{+}) - ATPase has a pH optimum of 7.5. Mg^{++} - ATPase is insensitive to ouabain and other inhibitors of (Na^{+}, K^{+}) - ATPase. Mg^{++} - ATPase is also more labile than the (Na^{+}, K^{+}) - ATPase. This would further indicate that Mg^{++} - ATPase and (Na^{+}, K^{+}) - ATPase represent different enzymes.

Further separation of the microsomal fraction obtained by differential centrifugation by sucrose density gradient centrifugation has been reported for cerebral microsomal fractions (Schwartz, Bacheland and McIlwain, 1962) and liver microsomes (Emmelot and Bos, 1962). However, the fractions from the sucrose gradients with (Na^{+}, K^{+}) - ATPase activity also had Mg^{++} - ATPase activity.

Heat treatment at 65°C for 10 min has been reported to inactivate the more labile

Mg^{++} - ATPase whilst the (Na^{+}, K^{+}) - ATPase of a rat brain microsomal fraction still remains active (Somogyi, 1964).

Storage of a deoxycholate-treated calf heart microsomal fraction at -5°C for a number of days lead to a decrease in the Mg^{++} - ATPase activity and an increase in the Na K stimulated ATPase activity (Schwartz, 1962).

Schoner et al. (1967) used a glass bead column to purify (Na^{+}, K^{+}) - ATPase from a microsomal fraction of ox brain. Tsujimoto and Bartlett (1965) suceeded in only a partial fraction action of (Na^{+}, K^{+}) - ATPase from Mg^{++} - ATPase on a CM Sephadex C 50 cation exchange column.

A comparison of the various purification techniques has been made in an effort to find a suitable technique to remove Mg^{++} - ATPase activity from myocardial microsomal fractions. Experiments to determine the effects of storage on microsomal ATPases and its use as a 'purification' method have been listed under the separate animal species investigated.

Methods and Results

The methods and results in this chapter have been combined under their separate headings to avoid confusion in reference from one section to the other.

Deoxycholate treatment

Dog myocardial microsomal fractions were used to establish the effects of

TABLE IV 1

EFFECTS OF DEOXYCHOLATE TREATMENT ON DOG MICROSOMAL (Na^+, K^+) - ATPase AND Mg^{++} - ATPase ACTIVITY

	No. of experiments	(Na^+, K^+)-ATPase	Mg^{++}-ATPase	$\frac{(Na^+, K^+)\text{-ATPase}}{Mg^{++}\text{-ATPase}}$
No Deoxycholate	3	0.43	7.12	0.06
0.1% Deoxycholate	3	3.42	3.12	1.11

TABLE IV 2

EFFECTS OF NaI TREATMENT ON DOG MICROSOMAL (Na^+, K^+) - ATPase AND Mg^{++} - ATPase ACTIVITY

	No. of experiments	(Na^+, K^+)-ATPase	Mg^{++}-ATPase	$\frac{(Na^+, K^+)\text{-ATPase}}{Mg^{++}\text{-ATPase}}$
Untreated Sample	3	0.31	6.53	0.05
Fraction I	3	0.43	7.12	0.06
Fraction II	3	3.42	3.12	1.11

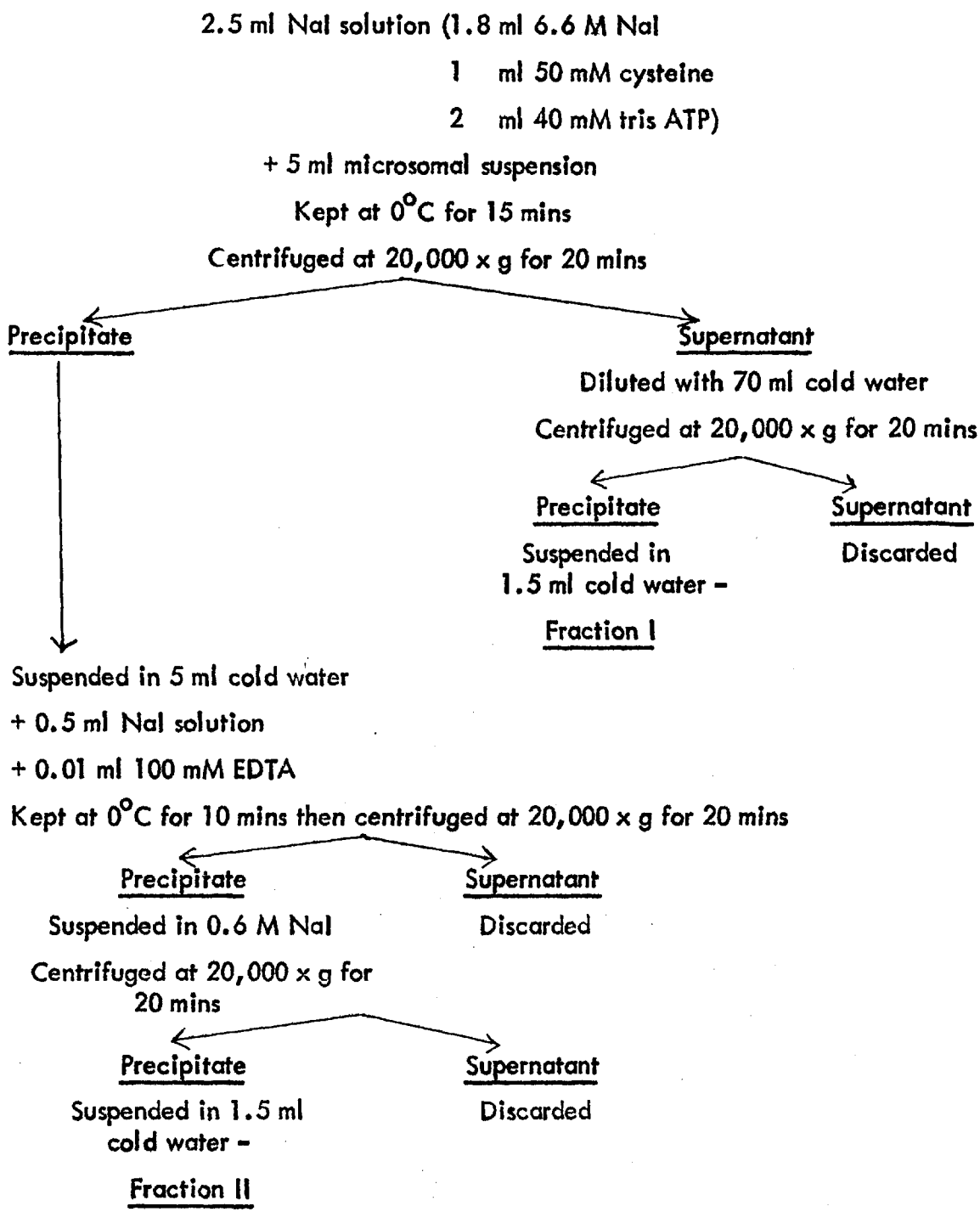
deoxycholate treatment as a method of purifying myocardial (Na^+, K^+) - ATPase enzyme. Microsomal fractions were suspended in either 0.25 M sucrose solution or 0.25 M sucrose solution containing varying quantities of deoxycholate. The suspensions were adjusted to give a protein content of 0.8 mg / ml and then left at -10°C for 24 hours before the enzyme activity was assayed. The effects of a range of deoxycholate concentrations are shown in Fig. IV 2 and IV 3. The concentration that would result in a maximum stimulation by Na^+ and K^+ ions was 0.1%. Table IV 1 shows the results of 0.1% deoxycholate treatment on both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. Deoxycholate treatment increased (Na^+, K^+) - ATPase activity about eight times and simultaneously decreased the Mg^{++} - ATPase activity. The $\frac{(Na^+, K^+ - \text{ATPase}}{Mg^{++} - \text{ATPase}}$ ratio was increased nearly twenty times that without deoxycholate treatment. The stimulatory effect of deoxycholate reached a peak after 20 hours storage at -10°C, after which neither the (Na^+, K^+) - ATPase nor the Mg^{++} - ATPase activity was changed for up to 1 month storage.

NaI treatment

Fig. IV 1 shows the scheme followed for the purification of dog myocardial microsomal suspension by the method reported by Nakao et al. (1963). Fraction 1 (Table IV 2) gave the highest (Na^+, K^+) - ATPase activity and the lowest

FIGURE IV 1

PURIFICATION OF DOG MYOCARDIAL MICROSOMAL FRACTION BY NaI METHOD



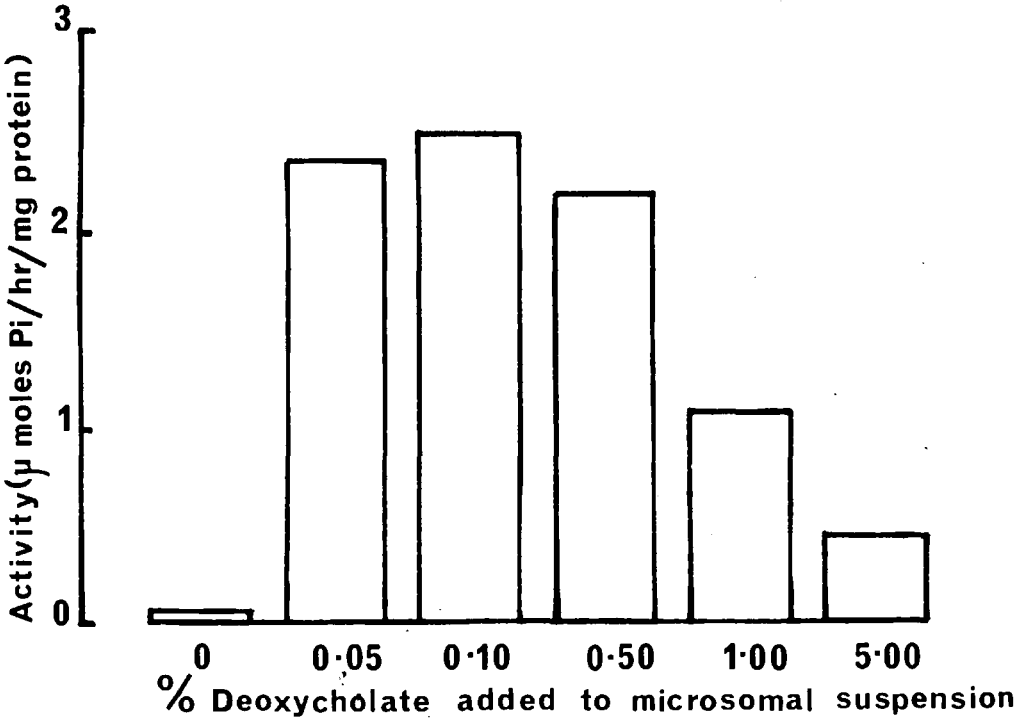


FIG. IV 2 The effects of various concentrations of deoxycholate on dog myocardial microsomal (Na^+, K^+) -ATPase.

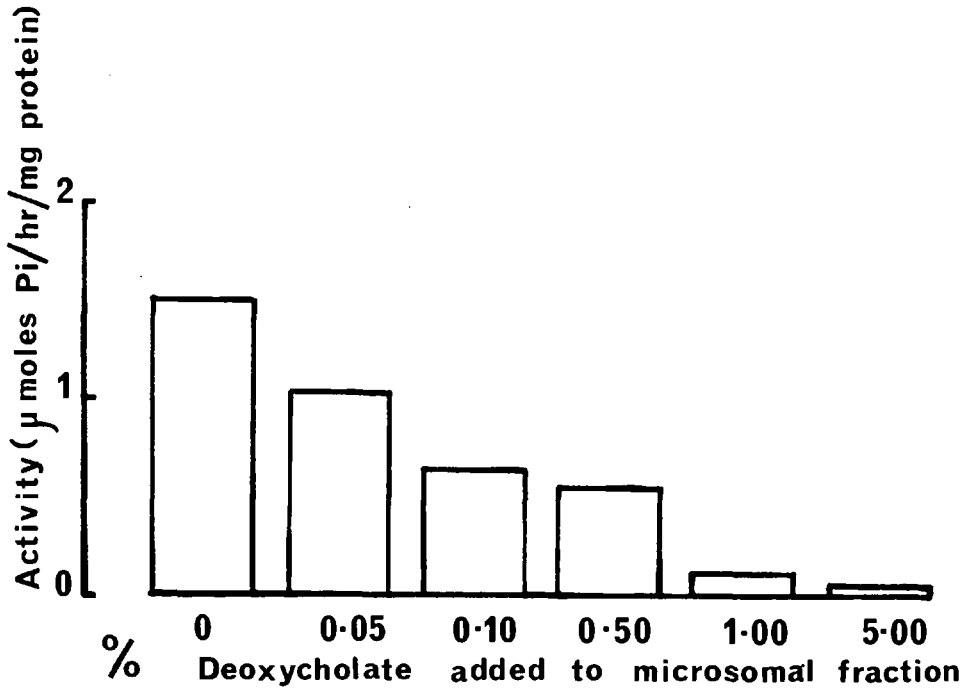


FIG. IV 3 The effects of various concentrations of deoxycholate on dog myocardial microsomal Mg^{++} -ATPase.

Mg^{++} - ATPase. Fraction II still contained a high Mg^{++} - ATPase activity despite further NaI treatment. The optimal (Na^{+}, K^{+}) - ATPase activity attainable by this method did not appear to be greater than by the deoxycholate method. The $\frac{(Na^{+}, K^{+}) - \text{ATPase}}{Mg^{++} - \text{ATPase}}$ ratio was not significantly different between Fraction I of the NaI method and deoxycholate treatment.

Density Gradient Centrifugation

The microsomal fraction cannot be separated by further differential centrifugation (see Chapter II). Density gradient centrifugation, however, allows separation of particles of closely related size and density.

Linear sucrose density gradients were prepared using an MSE density gradient former Type JA 100. Step sucrose gradients were formed by layering sucrose solutions on top of each other from a syringe fitted with a size 16 needle. The sucrose solutions were allowed to flow through the syringe under gravity. The sample in distilled water was carefully layered on top of the gradient. The samples were centrifuged in an MSE 50 Superspeed centrifuge using a 3 x 30 ml swing out rotor type, 59590. The results of the density gradient experiments are shown in Figs. IV 4, 5, 6 and 7. Details of centrifugation times and speeds are given for each experiment. 1 ml fractions of the gradient were collected from the bottom of each tube using an MSE tube piercing device. The gradient flowed from the tube by gravity and the speed

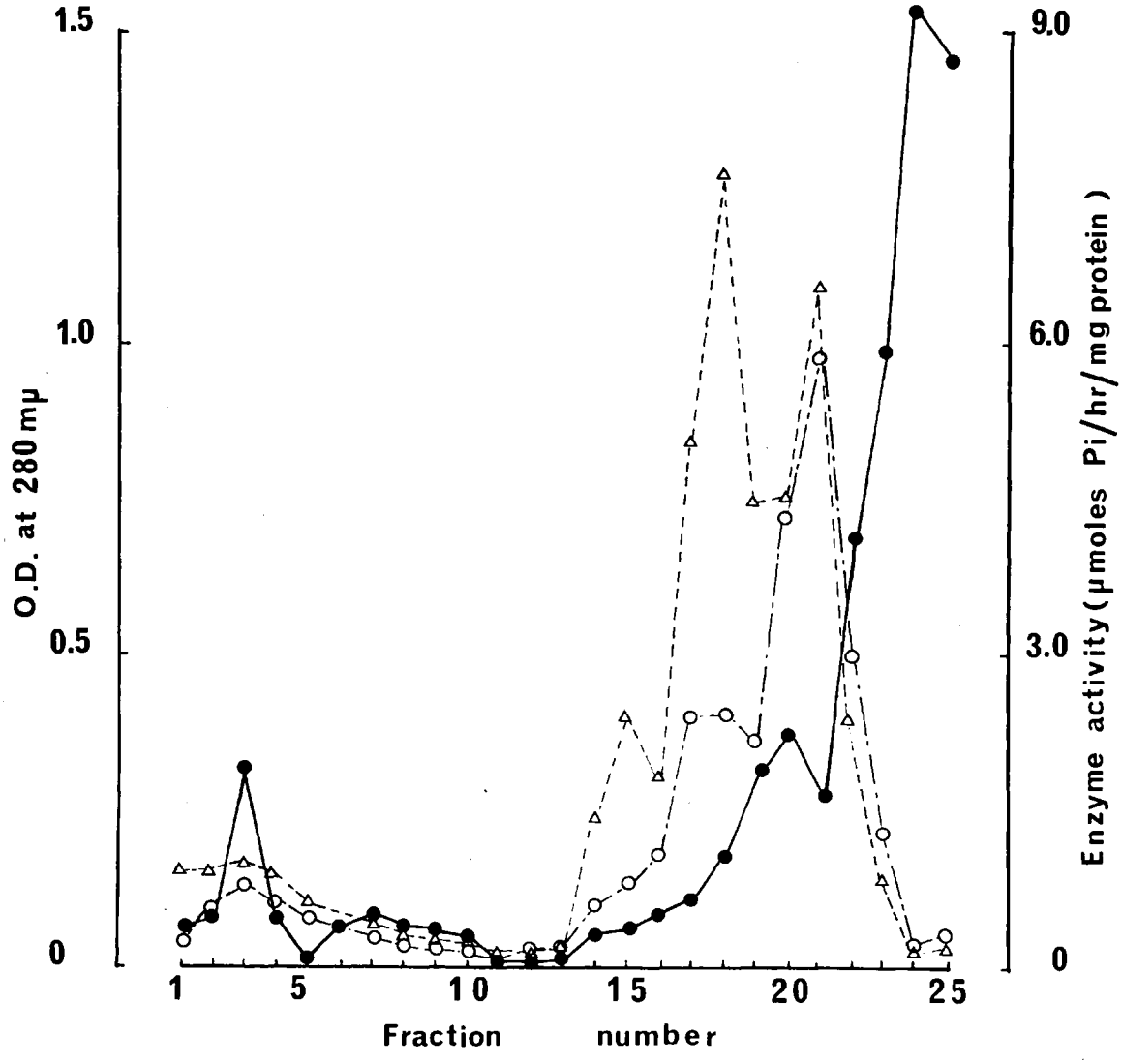


FIG. IV 4 Continuous sucrose gradient 0.6 to 1.5 M sucrose. 10 mg human myocardial microsomal fraction was layered on top of the gradient and centrifuged at 25,000 r.p.m. for 2 hours.
●, protein; —-O—-, (Na^+, K^+) -ATPase; --Δ--, Mg^{++} -ATPase.

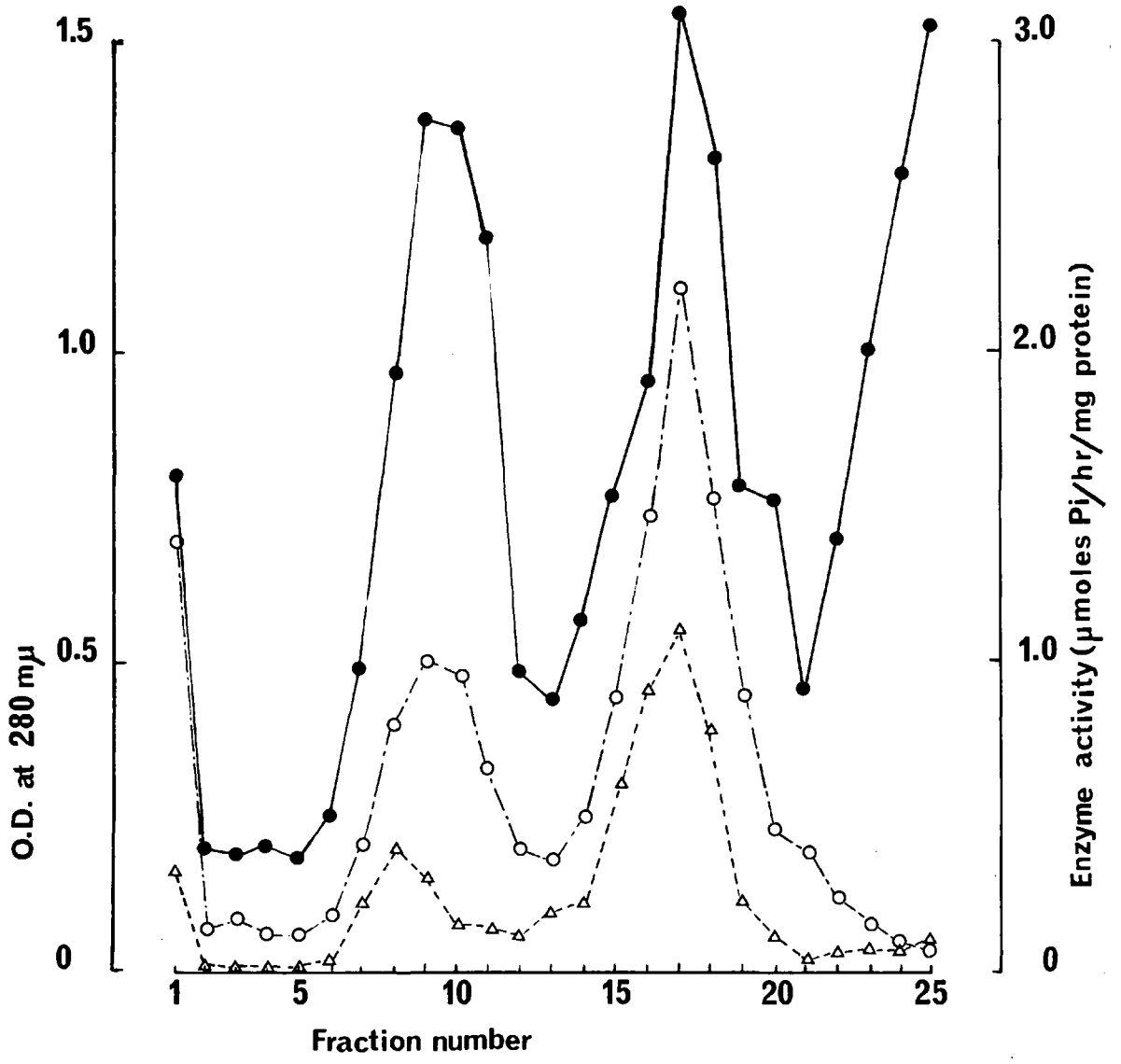


FIG. IV 5 Discontinuous sucrose gradient; 40%, 30%, 10%. 9 mg human myocardial microsomal fraction suspension layered on top of gradient and centrifuged at 25,000 r.p.m. for $1\frac{1}{2}$ hrs.
Symbols as in FIG. IV 4

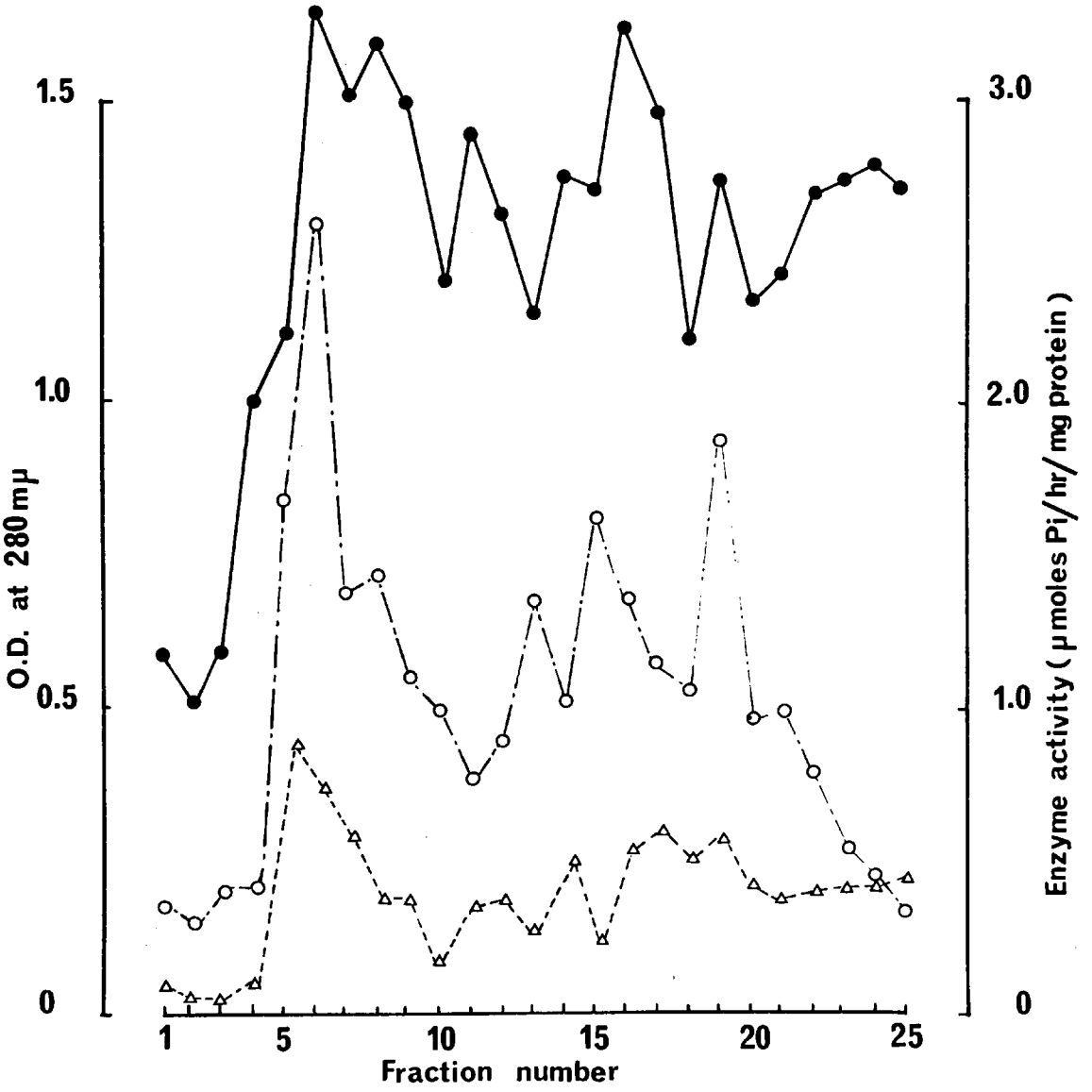


FIG. IV 6 Discontinuous sucrose gradient; 40%, 30%, 20%. 9.6 mg of human myocardial microsomal fraction was layered on top of gradient and centrifuged at 25,000 r.p.m. for $1\frac{1}{2}$ hrs.
Symbols as in FIG. IV 4

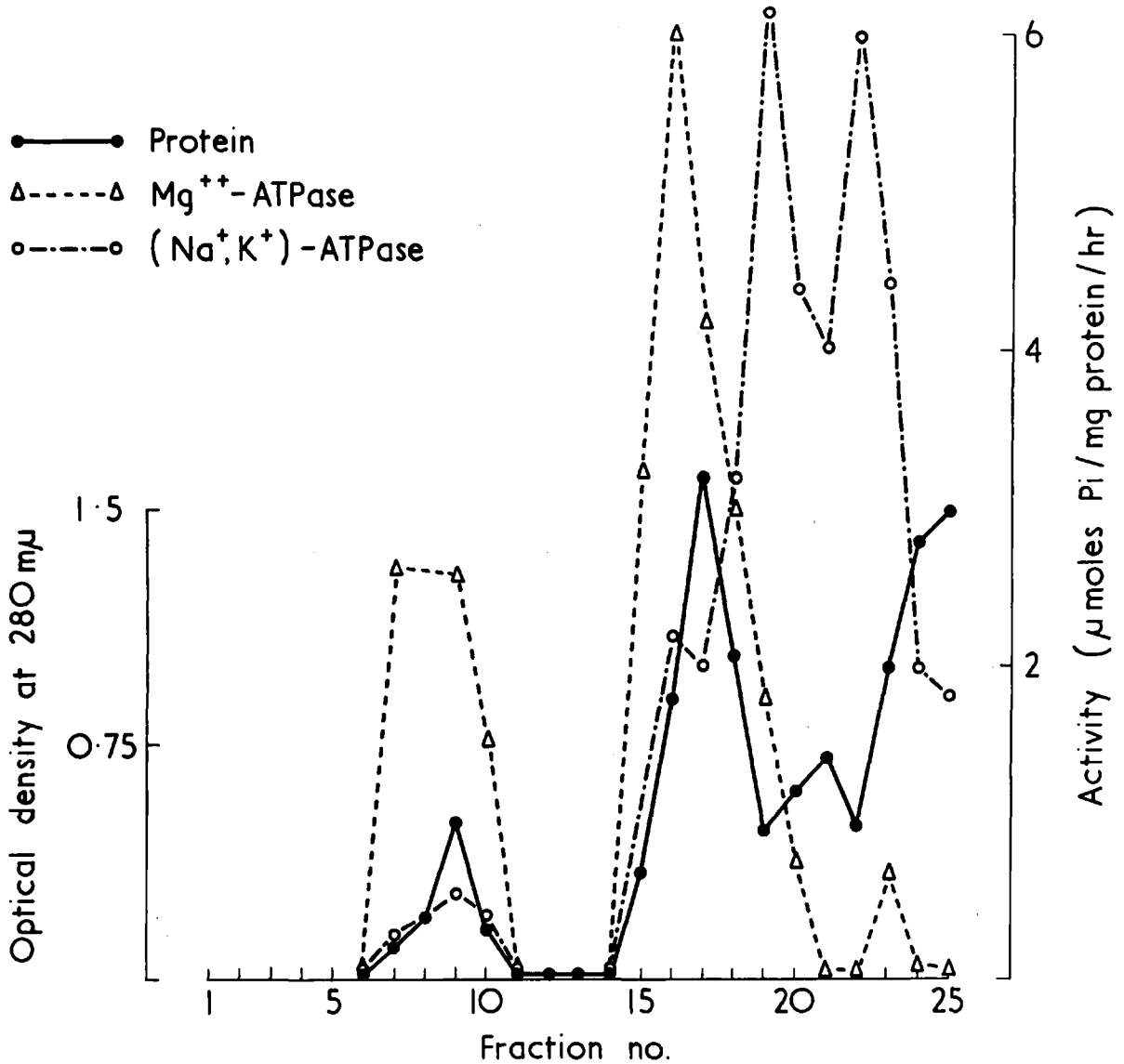


FIG. IV 7 Discontinuous sucrose gradient; 60%, 40%, 20%. 13.8 mg human myocardial microsomal fraction suspension was layered on top of the gradient and centrifuged at 25,000 r.p.m. for 2 hrs.
Symbols as in FIG. IV 4

of flow was controlled by regulating the air flow into the top of the tube. In the results to follow fraction number one will always represent the first fraction from the bottom of the tube. In all the sucrose density gradient studies the fractions from the top of the gradient had a high protein reading but very little (Na^+, K^+) - ATPase or Mg^{++} - ATPase activity. As an inhibitory factor had been reported to be present in heart preparations it was thought that the inhibitory factor may possibly be located in the upper fractions from the gradients.

Upper fractions from the sucrose gradients were, therefore, added to microsomal fractions and the (Na^+, K^+) - ATPase activity measured. Table IV 3 shows the results of these experiments. The upper fractions inhibited both the (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. The inhibitor must be of small molecular size to remain in the upper sucrose layers after $1\frac{1}{2}$ hrs centrifugation.

The Mg^{++} - ATPase activity closely followed the activity of the (Na^+, K^+) - ATPase in most of the sucrose density gradient centrifugation experiments. One notable exception was the 60 : 40 : 20% sucrose density gradient separation (Fig. IV 7). Fractions 19 to 24 had a high (Na^+, K^+) - ATPase activity and a low Mg^{++} - ATPase activity while fractions 15 to 18 had a high Mg^{++} - ATPase activity compared to the (Na^+, K^+) - ATPase activity. No morphological check on the fractions from the sucrose density gradients could be made due to a lack of electron microscopic facilities at this stage.

TABLE IV 3

EFFECTS OF ADDING FRACTION FROM THE TOP OF A SUCROSE DENSITY GRADIENT TO A MICROSOMAL FRACTION SUSPENSION BEFORE SUCROSE DENSITY GRADIENT CENTRIFUGATION

Each figure represents the average of experiments assayed in duplicate.

Fraction added to microsomal suspension	% INHIBITION	
	(Na^+,K^+)-ATPase	Mg^{++}-ATPase
Fig. IV 6 (40:30:20 sucrose gradient)		
No. 24	9.5	9.3
No. 25	10.2	10.5
Fig. IV 5 (40:30:10 sucrose gradient)		
No. 24	12.5	7.0
No. 25	23.5	15.2

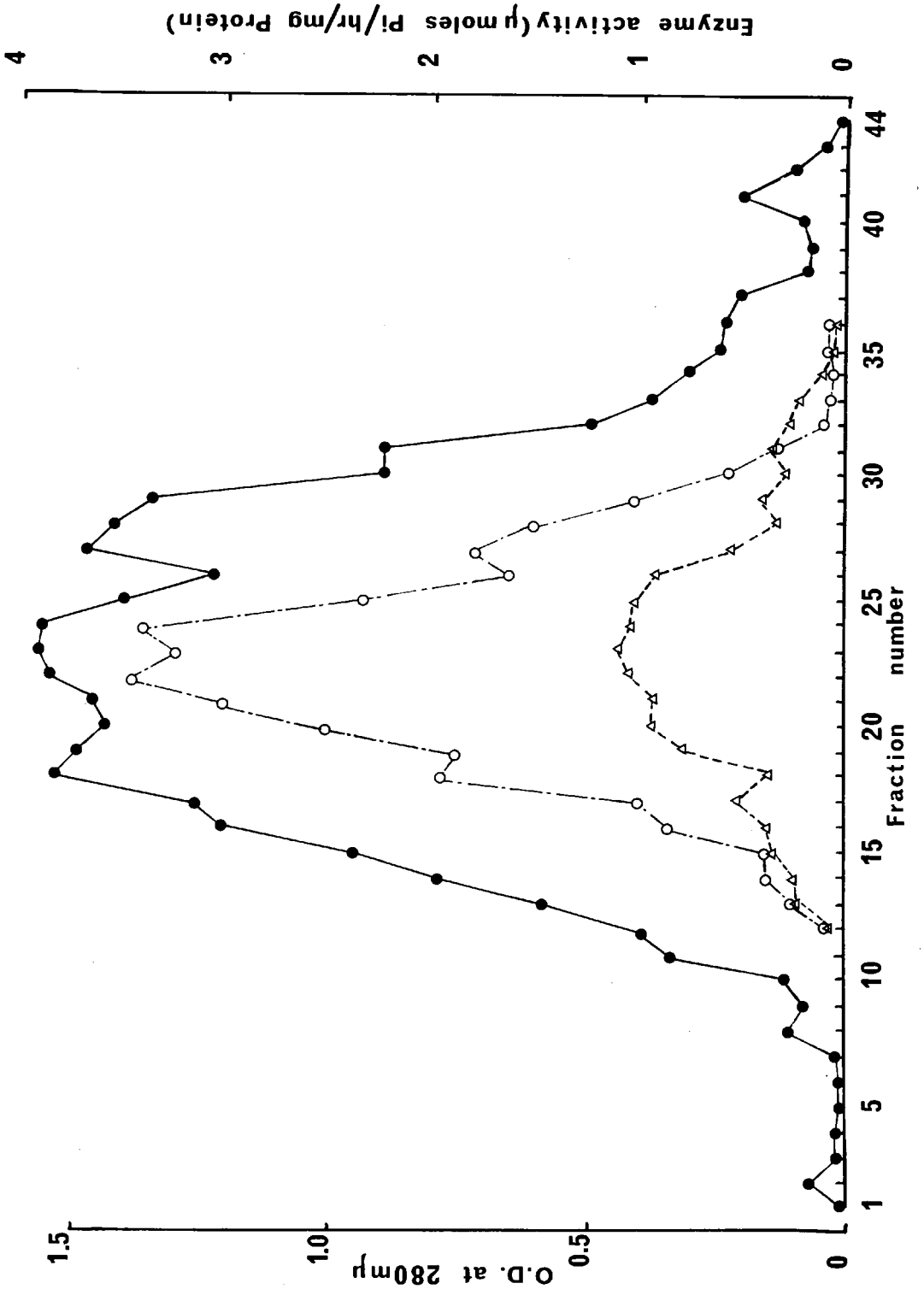


FIG. IV 8 Elution pattern of untreated human myocardial microsomal suspension from a glass bead column.
●, protein measured at 280 mμ; —-O—-, (Na^+, K^+) -ATPase, --Δ--, Mg^{++} -ATPase

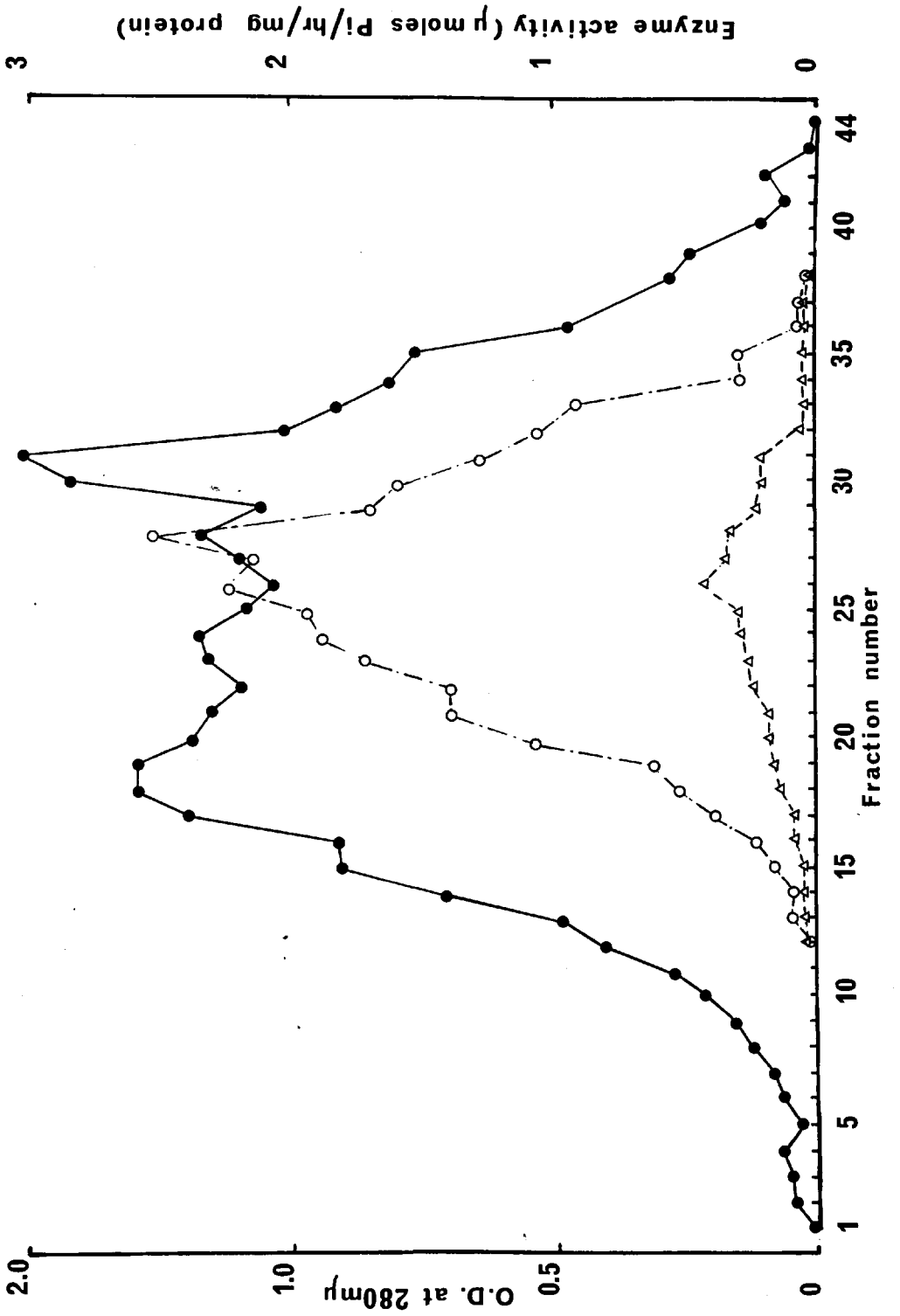


FIG. IV 9 Elution pattern of human myocardial microsomal fraction, pretreated with 0.1% deoxycholate, from a glass bead column. Symbols as in FIG. IV 8

Purification of (Na^+, K^+) - ATPase on a Glass bead column

A Wrights FCJ 60 cm x 2.1 cm column was packed with glass beads, 0.5 mm diameter. The column was well washed with ice cold tris - HCl buffer pH 7.4. Cold water, 5°C, was circulated in a cooling jacket surrounding the column. 2 mls of microsomal suspension with a protein content of 5 mg / ml was passed thrcugh the column and eluted with tris - HCl buffer pH 7.4. The first 60 mls eluted from the column after application of the sample were free of protein. The elute was collected in 1 ml fractions after the first 60 ml was eluted. Samples were collected until no more protein could be detected in the elute. Each 1 ml fraction was assayed for both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. Figs IV 8 and IV 9 show the results of samples passed through the glass bead column.

Although some non ATPase enzyme protein could be removed there was no separation of (Na^+, K^+) - ATPase from Mg^{++} - ATPase activity. In samples pre-treated with 0.1 % deoxycholate before application on the column there was a reduction in Mg^{++} - ATPase but the (Na^+, K^+) - ATPase specific activity was also lowered (Fig IV 9).

Sephadex column separations of microsomal suspensions

Sephadex is a modified dextran. The dextran macromolecules are cross-linked to give a three dimensional network of polysaccharide chains. The degree of swelling

of a particular grade of Sephadex controls the matrix through which filtration occurs. The more compact the gel, the lower the molecular weight of substance retained by the gel. Molecules are therefore eluted from a Sephadex column in the order of decreasing molecular size. Filtration is dependent on size, shape and chemical nature of the substance.

Three Sephadex gels were used to separate microsomal fractions:

	Molecular Weight
i) G - 100, fraction range for proteins	4,000 to 150,000
ii) G - 150, " " " "	5,000 to 400,000
iii) G - 200, " " " "	5,000 to 800,000

The gels were allowed to swell in tris - HCl buffer pH 7.4 for 3 days. In transferring the gel to the column great care was taken to exclude all air bubbles. The gel was well washed with buffer before the sample was applied to the top of the column. Tris - HCl buffer pH 7.4 was also used to elute the sample from the column. 1 ml fractions were collected and the optical density measured at 280 $m\mu$ to indicate protein content. The elution patterns from Sephadex column are shown in Fig. IV 10 for the G - 100, Fig. IV 11 for the G - 150 and Fig. IV 12 for the G -200. The (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities closely followed the first protein peak. Although some non ATPase enzyme protein was removed from the

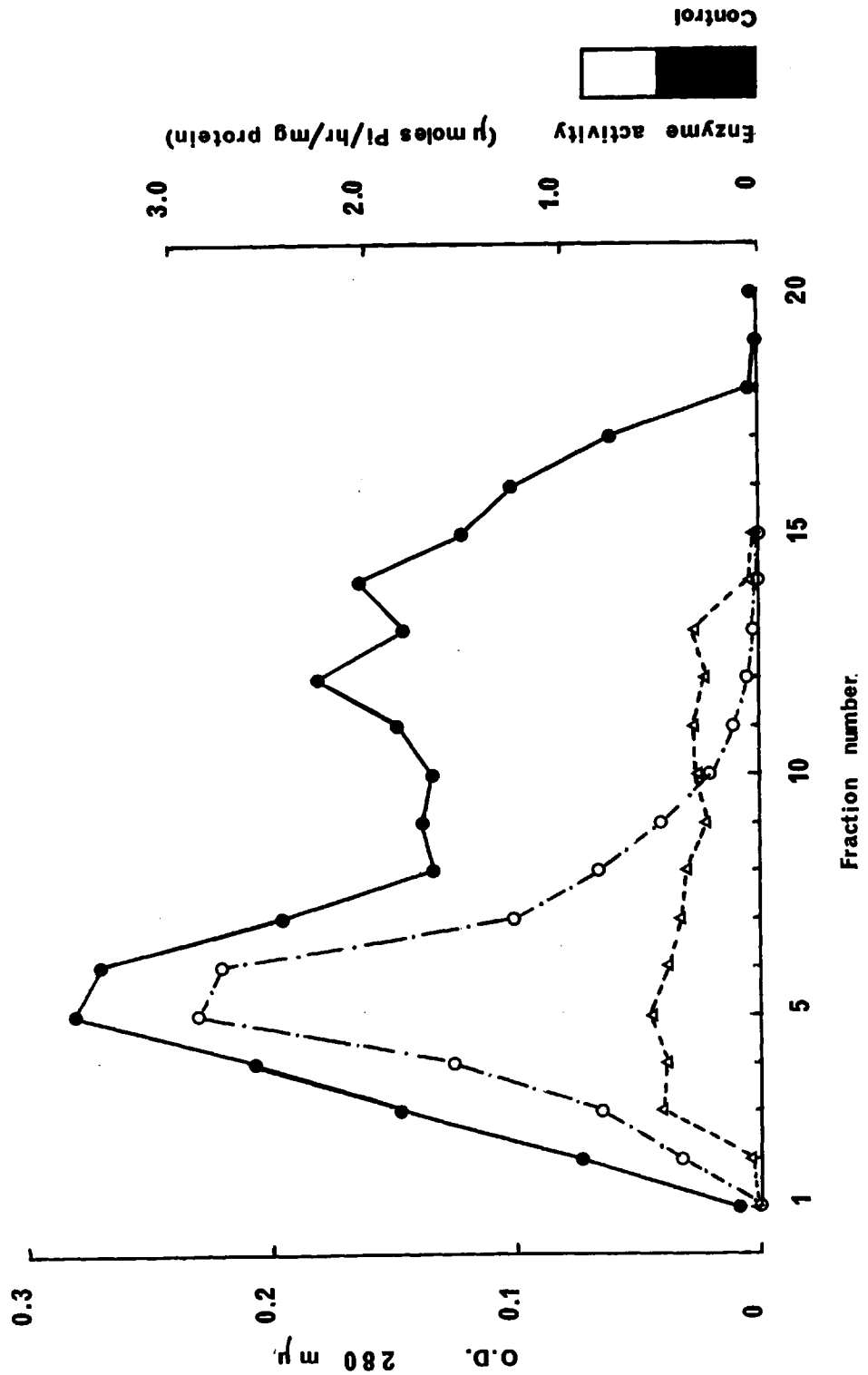


FIG. IV 10 Elution pattern of dog myocardial microsomal fraction from a Sephadex G 100 column. 10 mg of microsomal suspension put on column. 15 mls was collected before the first protein peak was eluted. ●, protein at 280 m μ ; —-O—-, (Na^+, K^+) -ATPase; --Δ-- Mg^{++} -ATPase. Control ■, Mg^{++} -ATPase ; □ (Na^+, K^+) -ATPase activity before column.

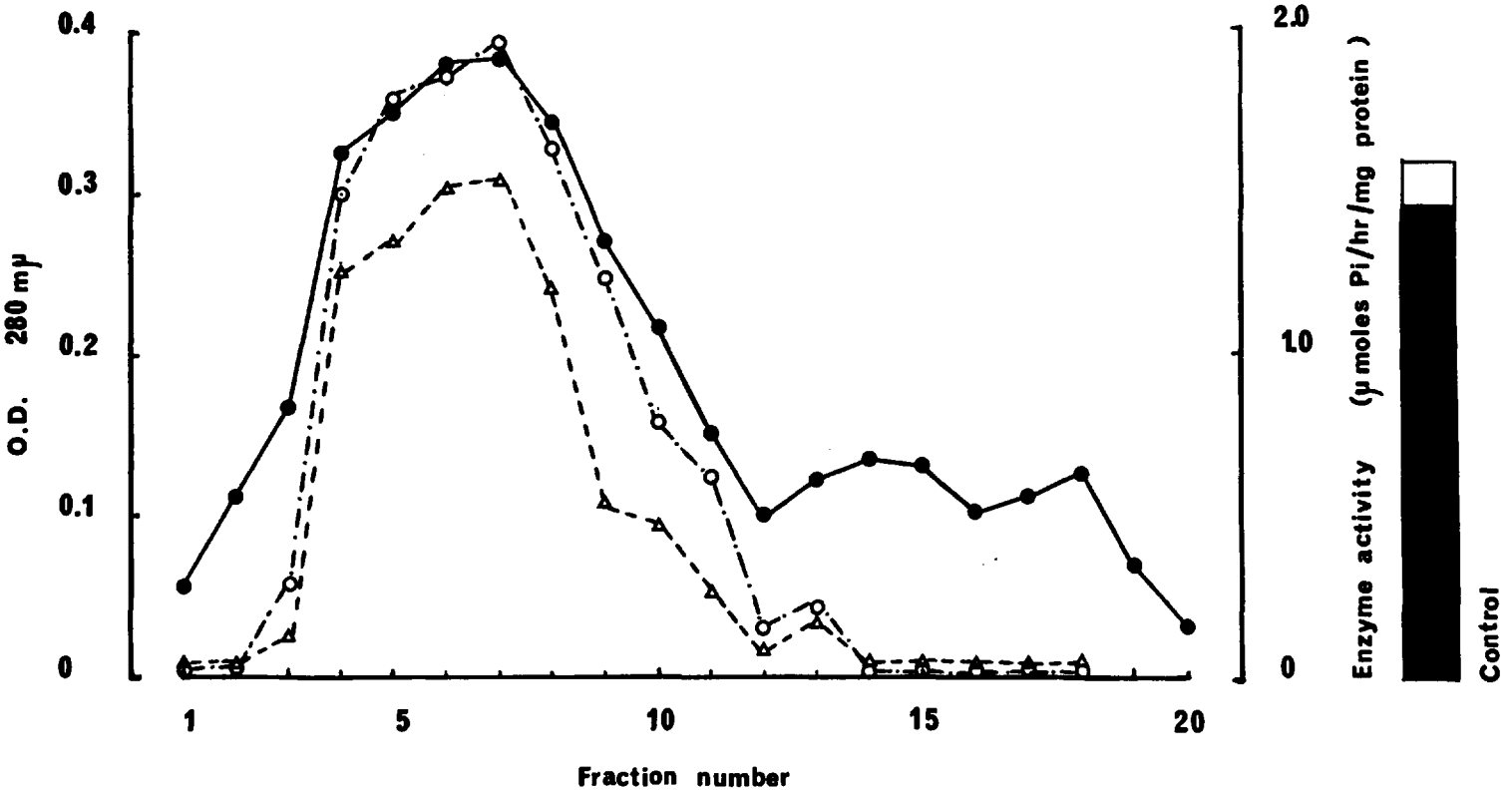


FIG. IV 11 Elution pattern of dog myocardial microsomal fraction from a Sephadex G 150 column. 9.0 mg of microsomal protein was put on the column. 40 mls was collected before the first protein peak was eluted. Symbols as in FIG. IV 10.

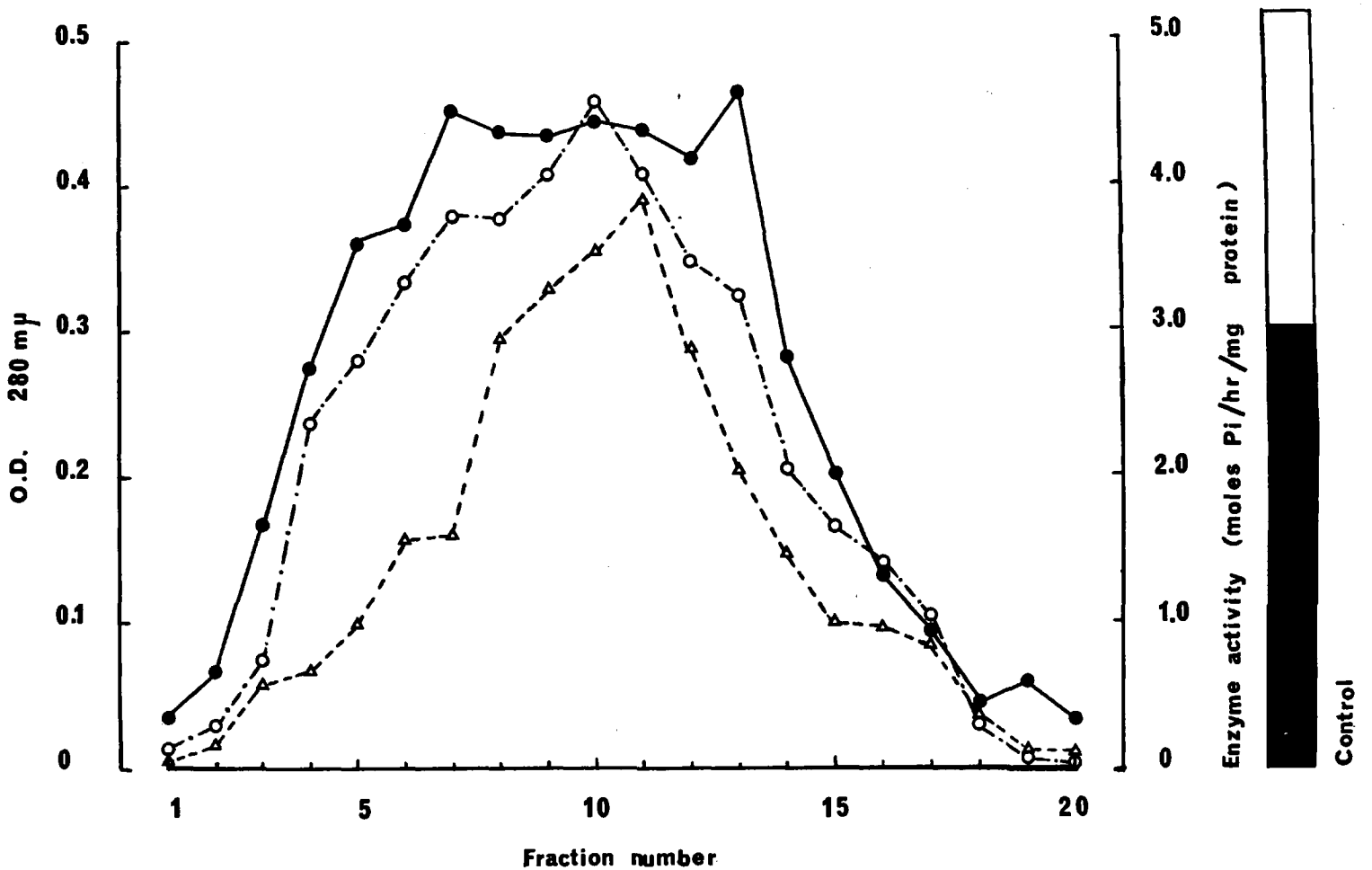


FIG. IV 12 Elution pattern of dog myocardial microsomal fraction from a Sephadex G 200 column. 7.8 mg of microsomal protein was put on the column. 56 mls was collected before the first protein peak was eluted. Symbols as in FIG. IV 10.

microsomal suspensions it was not possible to separate the (Na^+, K^+) - ATPase from the Mg^{++} - ATPase. However, passing the microsomal samples through the columns could activate them to Na^+ and K^+ ion stimulation, possibly by removing an inhibitor protein, mentioned earlier.

Gel electrophoresis of microsomal fractions

The methods of disc and preparative gel electrophoresis have been well described (Smith, 1968) and full details of apparatus and procedure will not be given here. The technique allows separation of small quantities of substances by their size and mobility in an electrical field. The pore size of the gel can be varied depending on the quantity of gel added.

The apparatus consists, essentially, of an acrylamide gel supported in a glass tube situated between two buffer solutions each of which has an electrode. The sample, in sucrose, is applied to the top of the column and buffer is added gently to cover the gels and electrode. Sucrose prevents the samples mixing with the buffer. Current is applied at 1 mA per tube for 10 mins to compact the sample then at 3 - 4 mA per tube for about 90 mins. In "disc" electrophoresis the gel columns are removed from the glass tubes after electrophoresis and stained for protein or enzyme. The procedure is qualitative. In the preparative procedure the protein bands are removed as they separate at the base of the column. As the bands are

eluted by buffer it is passed to a fraction collector via a "Uvicord" or equivalent instrument to measure absorbancy at 260 mμ or 280 mμ (or both) depending on the absorbancy of the sample being eluted. The preparative gel column is larger and the current applied stronger than in disc electrophoresis and a cooling system is therefore, required to remove the heat evolved by the current. The outer glass tube has a water jacket surrounding it and a cold finger on the inside through which either tap water or iced water is circulated.

By separating microsomal proteins after various treatments of deoxycholate it was hoped that differences in the resulting protein patterns may give some indication of the effects of deoxycholate on the microsomal protein constituents. A microsomal fraction was divided into several pottions and treated with various concentrations of deoxycholate. They were kept for 24 hours at -10°C after which they were centrifuged at 105,000 x g for 60 mins. The resulting pellets were resuspended in 20% sucrose solution. Fig. IV 13 shows diagrams of gels after electrophoresis of the various samples. The lowest concentration used (0.05% deoxycholate) has a striking effect on the protein pattern. There is a reduction in the faster moving protein bands in all the samples treated with deoxycholate. With increasing concentrations of deoxycholate there is a gradual reduction in the size and quantity of the slower running protein bands. It seemed likely, therefore, that this method could be used to separate a protein fraction which may contain ATPase activity and

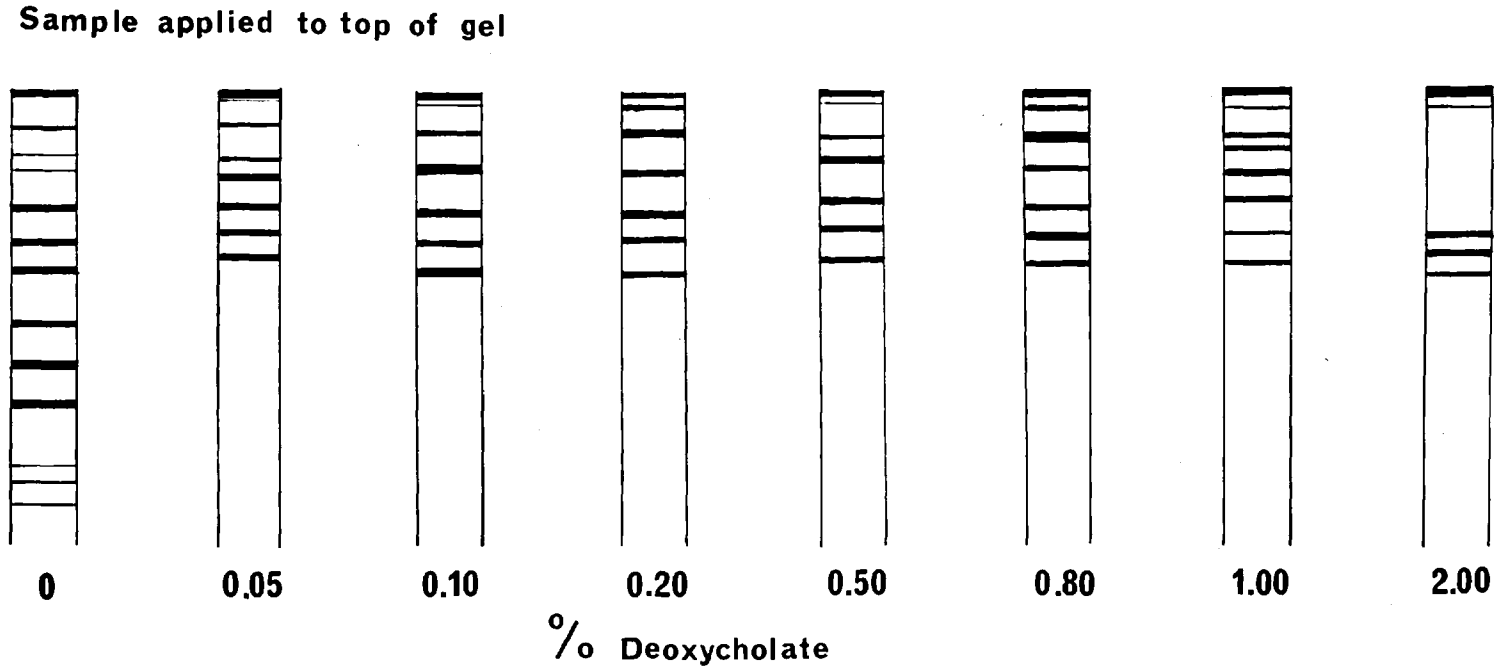


FIG. IV 13 Diagram showing results obtained by disc electrophoresis of human myocardial 'microsomes' treated with various concentrations of deoxycholate. Gel 7%. Samples: 40 µl of a 5 mg/ml protein solution in 25% sucrose. Current; 12 mA for 10 mins, then 28 mA for 90 mins. Stained with naphthlene black and decolorised with acetic acid: methanol: water (20:50:50).

and possibly reduce the non enzymic portion of the microsomal protein. The preparative acrylamide gel technique was used for this purpose but the method met with little success. Using a large pore gel it was difficult to continue electrophoresis to completion, the pores of the gel appeared to clog and electrophoresis stopped. Reducing the protein content applied to the top of the column overcame the clogging problem and resulted in a single protein peak being eluted which was devoid of any ATPase activity. This may be the result of disrupting the membranes to such an extent that membrane integrity is lost or the removal of a protein important in conserving the enzyme activity.

Discussion

As most workers in this field have reported it is very difficult to obtain a 'pure' (Na^+, K^+) - ATPase enzyme free from Mg^{++} - ATPase. By far the most convenient routine procedure has proved to be deoxycholate treatment of microsomal preparations. This method increases the $\frac{(Na^+, K^+) - \text{ATPase}}{Mg^{++} - \text{ATPase}}$ ratio and liberates the activity of the Na^+ plus K^+ ion stimulated ATPase from the membrane fraction. NaI treatment was a longer procedure and yielded no better results than deoxycholate treatment.

Separation on Sephadex gels, sucrose density gradients and glass bead columns removed some non ATPase enzyme protein from the microsomal fraction but the

Mg^{++} - ATPase activity followed closely the (Na^+, K^+) - ATPase activity elution pattern with one notable exception. This was the 60 : 40 : 20% sucrose density gradient centrifugation experiment. A partial separation was achieved with a high (Na^+, K^+) - ATPase activity compared to Mg^{++} - ATPase activity separating in higher fractions (19 to 24) while a high Mg^{++} - ATPase activity compared to (Na^+, K^+)- ATPase activity was found in fractions 15 to 24. However, fractions 15 to 24 all contained both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. It was interesting to note that all these methods caused an unmasking of the Na^+ plus K^+ ion stimulated portion of the ATPase from microsomal preparations. This could be by the removal of a protein inhibitor discussed earlier or by some structural rearrangement which allows the enzyme to react with the substrate ATP. The removal of an inhibitor protein seems the most likely in view of the results obtained by the sucrose density gradient studies. When fractions at the top of the gradient, low in ATPase activity but high in protein, are added to samples with a high (Na^+, K^+) - ATPase activity there is a reduction in the (Na^+, K^+) - ATPase activity.

Suggestions that the Mg^{++} - ATPase activity is derived from mitochondrial sources (Samaha and Gergely, 1966) would seem to be further repudiated by these findings together with the electron microscopy and enzyme marker studies (Chapter II). Mg^{++} - ATPase activity is also present in red cell membranes (Dunham and Glynn, 1961; Tosteson and Hoffman, 1960) which do not have mitochondrial structures associated

with them. This would further support suggestions by Skou (1964) that there is a close relation between these two enzymes and that they may have some interconnected role. Skou (1964) suggests that the Mg^{++} - ATPase may be formed from (Na^{+}, K^{+}) - ATPase and the addition of a soluble factor converts the Mg^{++} - ATPase to respond to Na^{+} and K^{+} ion activation.

Deoxycholate treatment caused a rearrangement of the protein pattern shown by disc electrophoresis. The nature of the proteins affected is not known and difficulties in the preparative procedure have for the present prevented further elucidation of the positioning of the ATPases in the various protein bands.

CHAPTER V

HUMAN MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase

A Mg^{++} - dependent Na^+ plus K^+ ion stimulated, ouabain sensitive, ATPase has been identified in human tissues. Dunham and Glynn (1961) and Post et al (1960) found erythrocyte membranes contained (Na^+, K^+) - ATPase activity. Samaha and Gergeley (1965) reported its presence in human striated muscle and Rawson and Pincus (1968) detected it in human brain homogenates.

This chapter describes the methods used to obtain microsomal (Na^+, K^+) - ATPase from biopsy and post mortem samples of the human heart. Some of its properties are described. Initially it was thought that fresh tissue was required for the study of myocardial (Na^+, K^+) - ATPase. Human myocardial biopsy specimens were obtained during cardiac operations. The biopsy specimens were small and provided only a limited quantity of material for study. When the opportunity to study post mortem myocardium was made available it was considered that this would provide a greater bulk of microsomal material from a single specimen, provided the enzyme could still be extracted from it. This would then allow more accurate comparative kinetic studies to be made.

A study was also made of the distribution of microsomal (Na^+, K^+) - ATPase in the various chambers of post mortem hearts.

Materials

Biopsy specimens were taken during the course of cardiac operations and kept in 0.25 M sucrose solution at 4°C and used on the same day for the preparative procedure. Most of the specimens were atrial appendages weighing about 0.2 to 1 g each.

Post mortem myocardium was obtained from St. Mary's Hospital and transferred to the laboratory surrounded by ice in a large thermos container. Cadavers were stored at + 5°C prior to autopsy. Myocardial specimens not used immediately were stored at - 10°C until required.

Methods

Homogenates from biopsy specimens were prepared by the glass and teflon homogenizer method while homogenates from the post mortem tissue were homogenized by the MSE homogenizer. Centrifugation procedure, assay conditions for the measurement of enzyme activity and protein content are those described in Chapter III. An electron micrograph of human biopsy myocardial microsomal fraction is shown in Fig. II. 11.

Microsomal fractions were suspended in 0.1% deoxycholate and stored at -10°C until required for assay. For the comparative studies of biopsy and post

mortem samples the microsomal suspensions in deoxycholate were assayed after 20 hrs storage at - 10°C.

Results

Both, biopsy and post mortem, microsomal fractions looked very similar under the electron microscope. They were composed of vesicles with no apparent gross mitochondrial contamination, Fig. II 11.

Results from human biopsy specimens

For the preliminary investigations of the properties of human myocardial microsomal (Na^+, K^+) - ATPase only biopsy specimens were available. Fig. V 1 shows a time / activity curve under various assay conditions. In the absence of Mg^{++} ions activity was negligible. In the presence of Mg^{++} ions the activity was a linear response. With Mg^{++}, Na^+ and K^+ ions present there was a further stimulation of Mg^{++} - ATPase activity but the response was not a linear one. In the presence of Mg^{++}, Na^+ and K^+ ions plus 10^{-2} M ouabain the ATPase activity was similar to that found with Mg^{++} ions alone. These results justify the measurement of human myocardial (Na^+, K^+) - ATPase activity by a similar method to that described in Chapter III. One assay mixture contained Mg^{++}, Na^+ and K^+ ions while another assay mixture contained Mg^{++}, Na^+ and K^+ ions plus 10^{-2}M ouabain. (Na^+, K^+) - ATPase activity was calculated as the difference between the two assay mixtures. Assay mixtures were incubated for 45 minutes at 37°C.

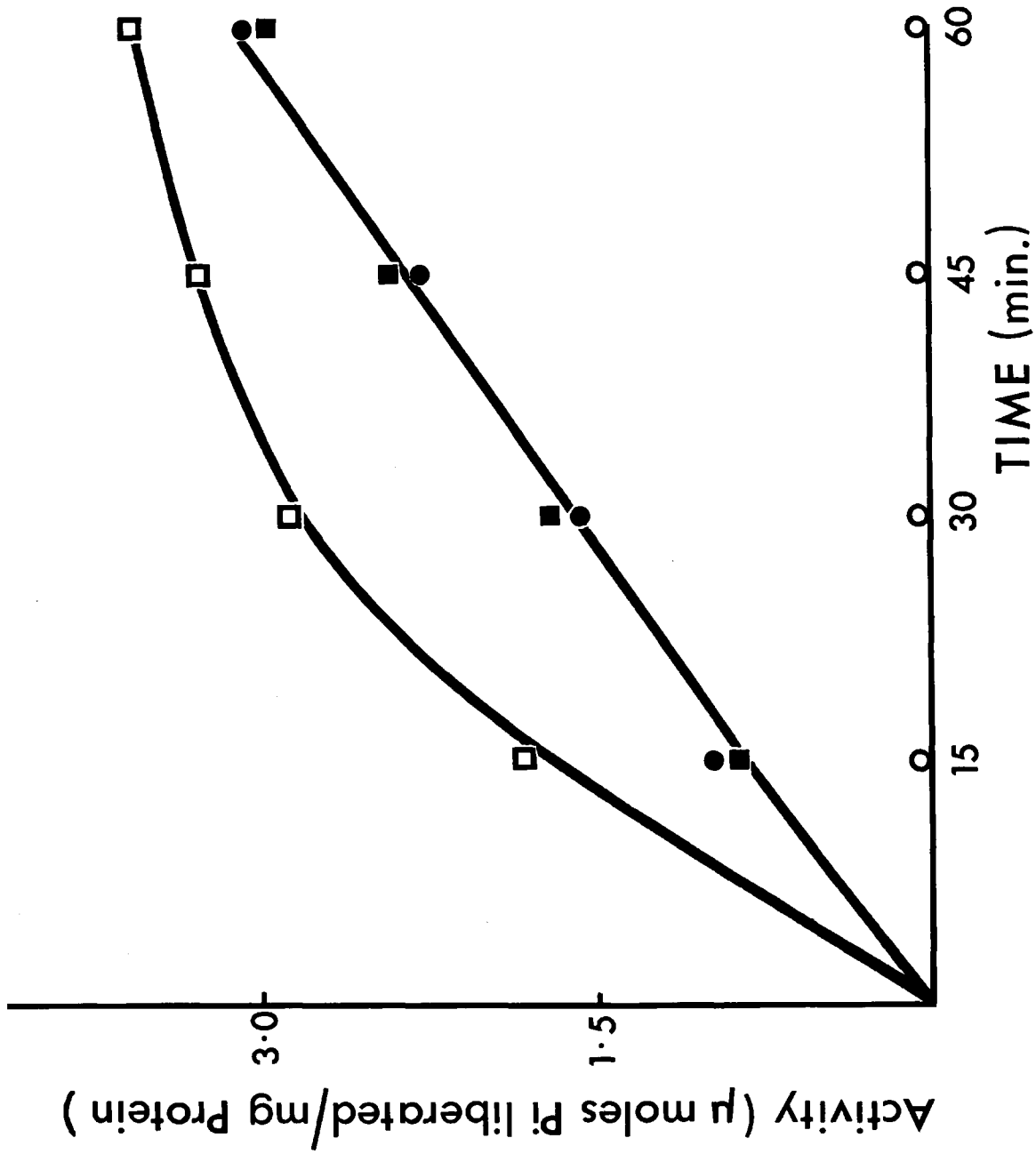


FIG. V I Human biopsy myocardial microsomal ATPase activity as a function of time. □, complete assay system; ●, Na^+ and K^+ ions omitted from assay; ■ complete assay system plus 10^{-3} M ouabain; ○, Mg^{++} ions omitted from assay.

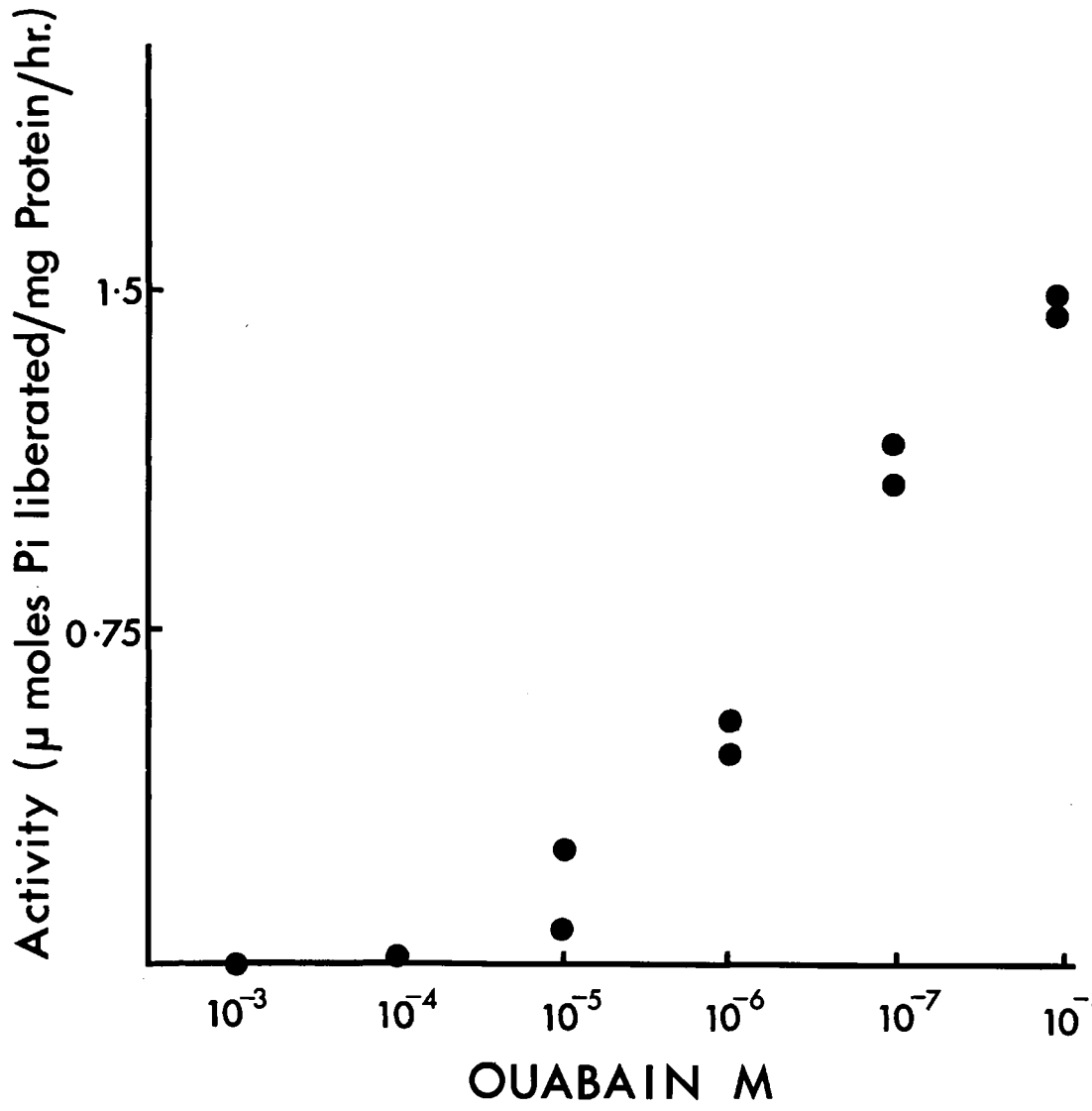
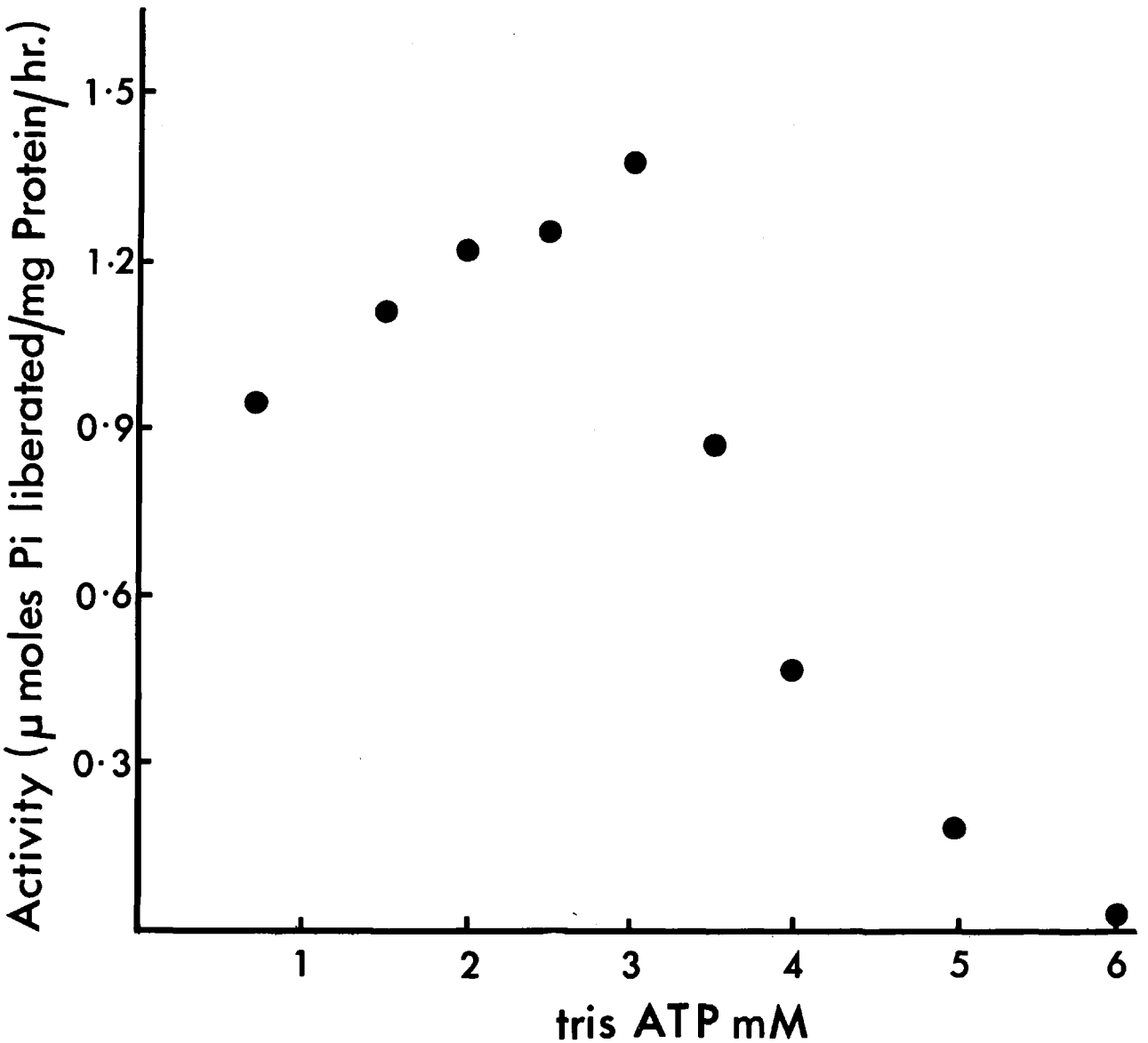


FIG. V 2 The effect of varying concentrations of ouabain on human biopsy myocardial microsomal (Na^+, K^+) -ATPase activity.

FIG. V 3 The effect of varying concentrations of ATP on human biopsy myocardial (Na^+, K^+)-ATPase



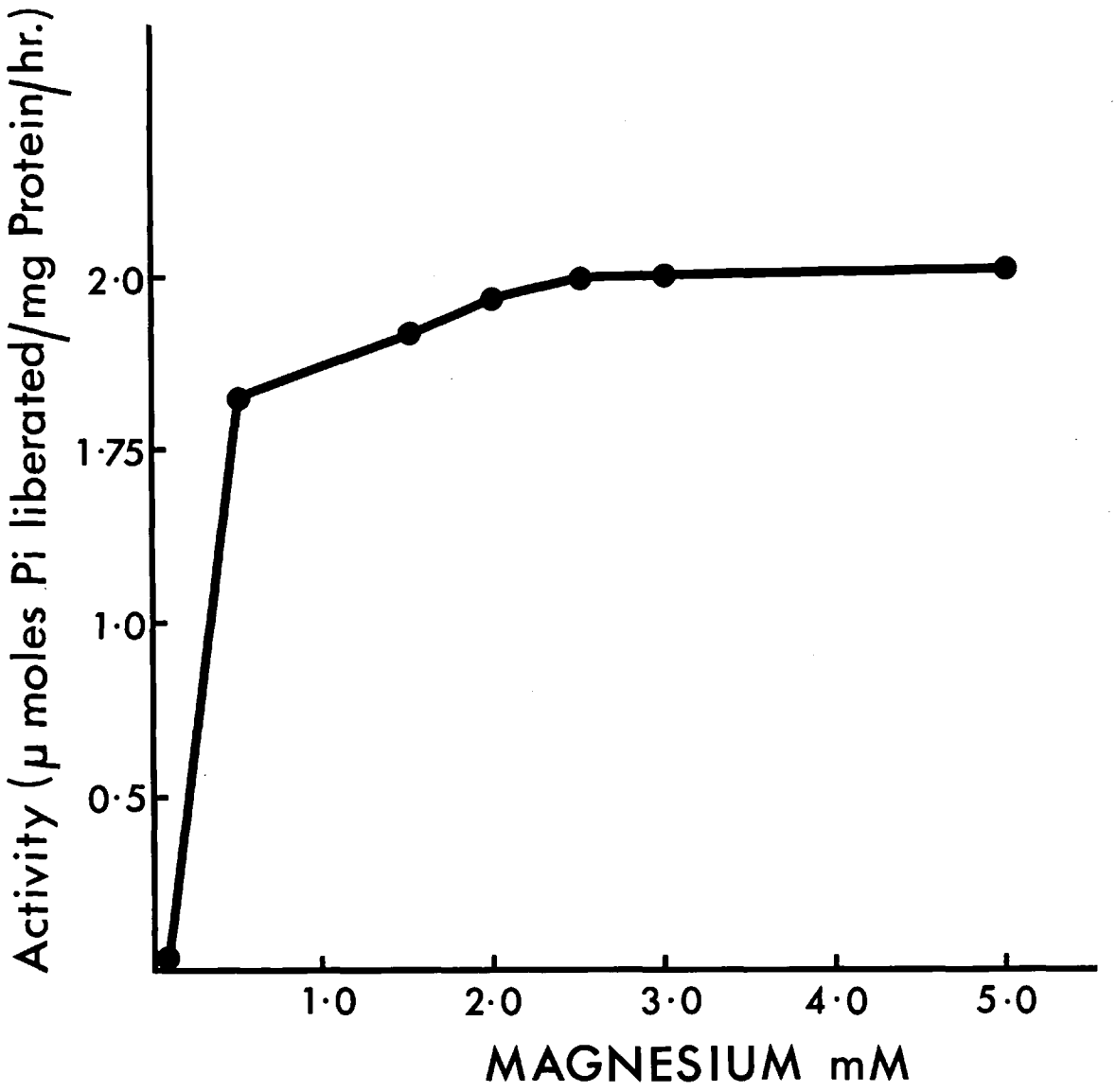


FIG. V 4 The effect of varying concentrations of Mg^{++} ions on human biopsy myocardial microsomal (Na^{+}, K^{+}) -ATPase.

FIG. V 5 The influence of pH on human biopsy myocardial microsomal, (Na^+, K^+)-ATPase activity.

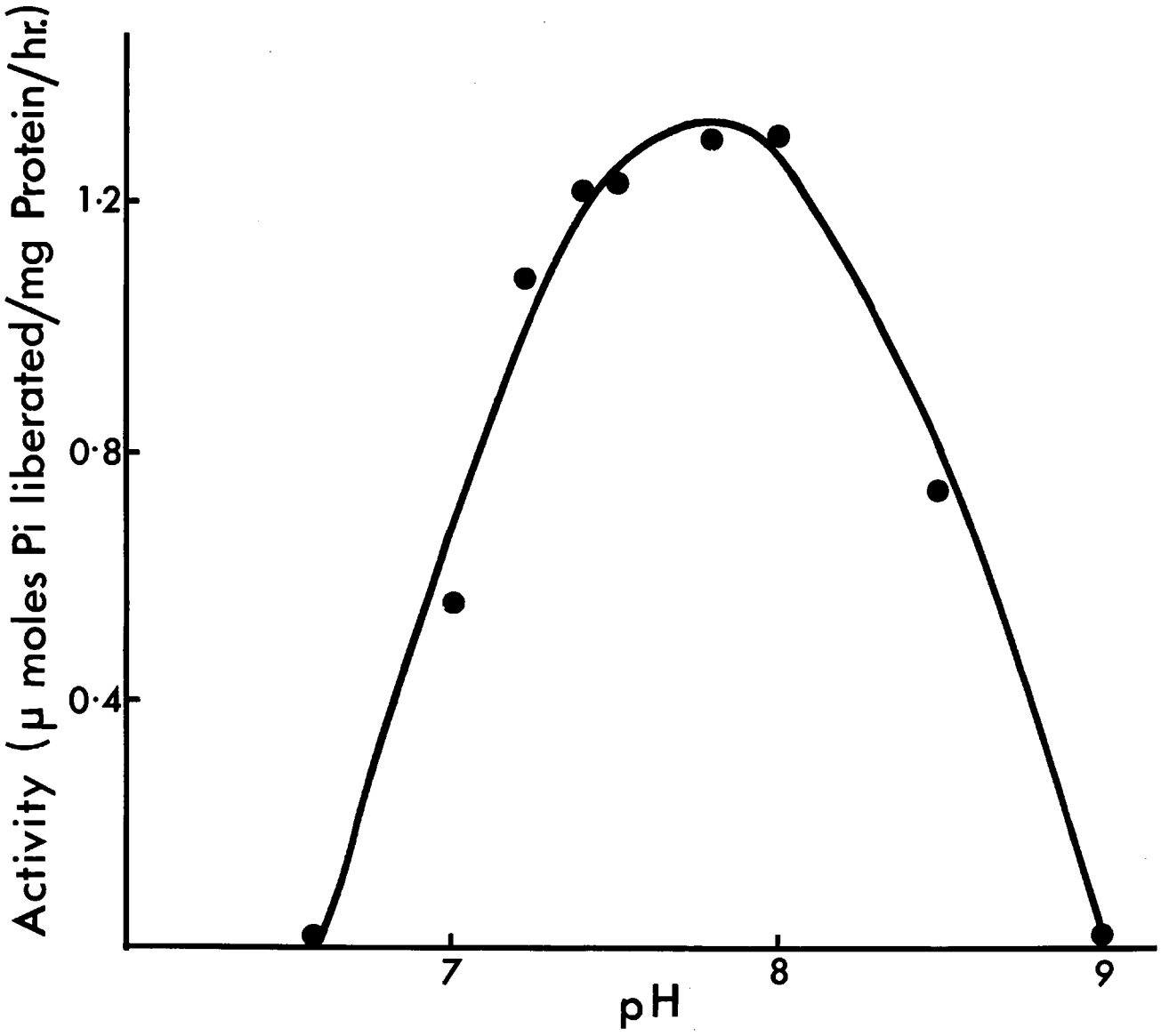
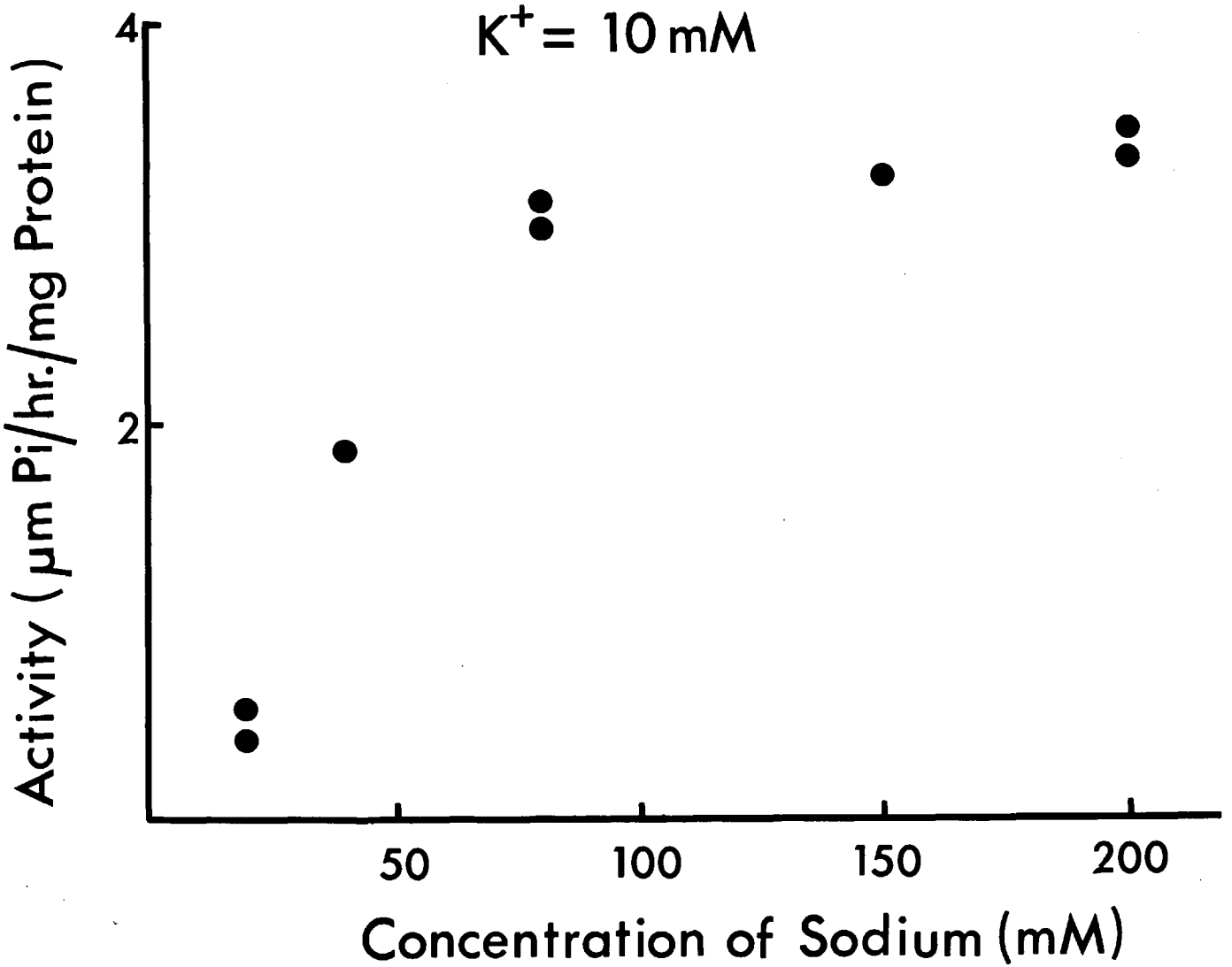


FIG. V 6 The effect of varying sodium ion concentrations on human biopsy myocardial microsomal, $(Na,^{+} K^{+})$ -ATPase.



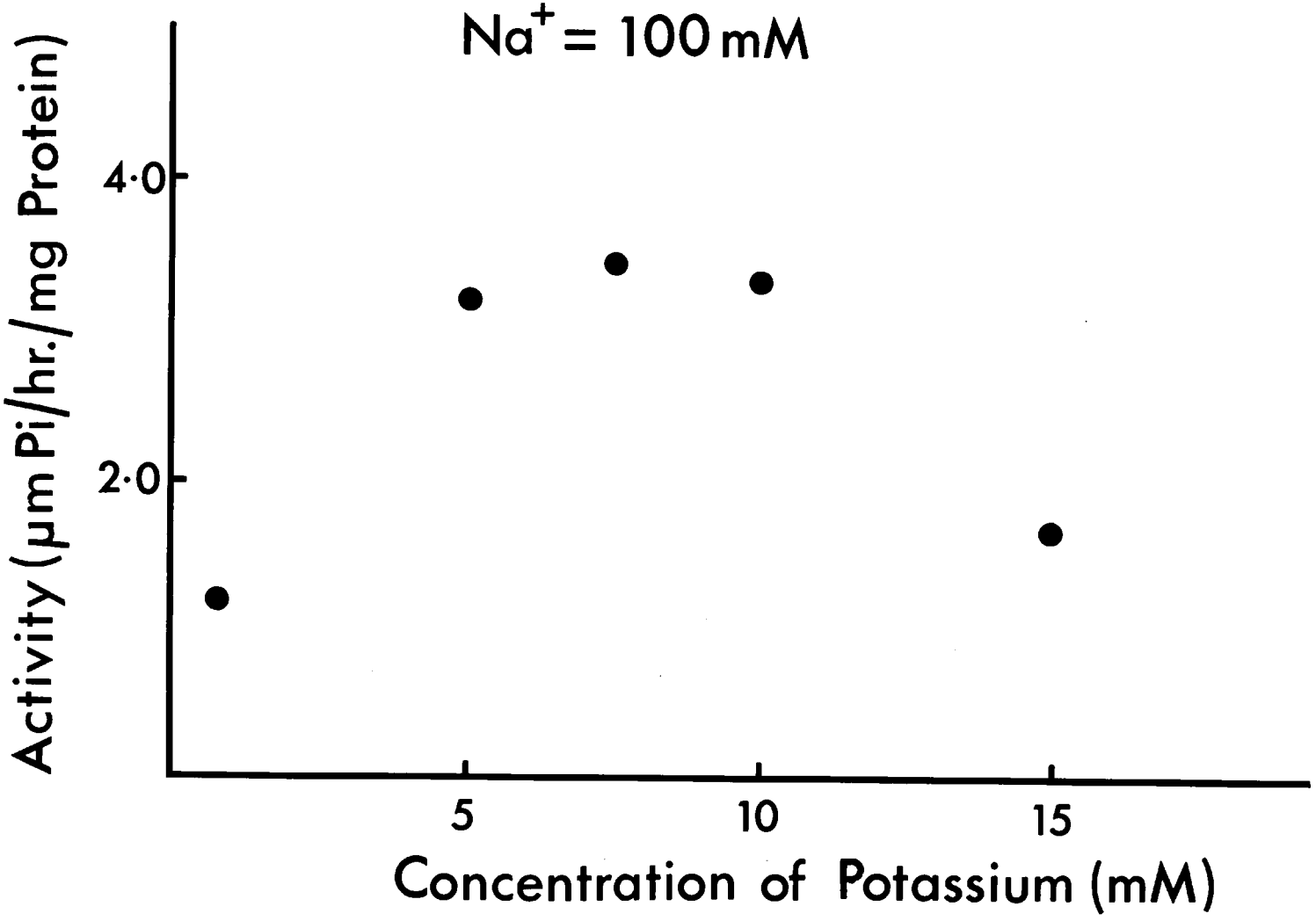


FIG. V 7 The effects of potassium ions on human biopsy myocardial microsomal (Na^+, K^+) -ATPase activity.

Fig. V 2 shows the effects of adding varying concentrations of ouabain. The KI for ouabain was $10^{-6.4}$ M. Maximal inhibition occurred at 10^{-4} M ouabain.

Fig. V 3 shows that the optimal ATP concentration was 3 mM above which there was inhibition by ATP. A 2 mM substrate concentration was chosen for routine assay mixtures. The activity was only slightly less than 3 mM and avoided the risk of falling on the steep, inhibitory, portion of the curve above 3 mM ATP substrate concentrations.

The Mg^{++} ion optimum concentration was 3 mM (Fig. V 4). Mg^{++} ions in concentrations up to 8 mM did not cause any inhibition of (Na^+, K^+) - ATPase activity.

The pH optimum for (Na^+, K^+) - ATPase activity was 7.8 (Fig. V 5). The pH of the assay solution was measured after the incubation period to check that the pH of the reaction mixtures had not altered. At a pH below 6.5 and above 9.0 the enzyme was inactivated.

Fig. V 6 shows the effects of adding increasing concentrations of Na^+ ions while maintaining the K^+ ion concentration at 10 mM. Na^+ ions were not inhibitory up to a concentration of 200 mM. The most rapid increase in activity was up to the addition of 75 mM sodium after which the curve flattens.

Fig. V 7 shows the effects of increasing K^+ ion concentration while maintaining the Na^+ ion concentration at 100 mM. There was a peak of optimal K^+ ion

TABLE V 1

SUBSTRATE SPECIFICITY OF HUMAN BIOPSY CARDIAC MICROSOMAL (Na^+, K^+) - ATPase TOWARDS SOME NUCLEOTIDES

SUBSTRATE	μ moles Pi liberated / mg protein / hr.
GUANOSINE TRIPHOSPHATE	0.2
INOSINE TRIPHOSPHATE	0.1
CYTIDINE TRIPHOSPHATE	0.2
ADENOSINE DIPHOSPHATE	0.4
ADENOSINE TRIPHOSPHATE	2.9

TABLE V 2

ATPase ACTIVITY OF HUMAN BIOPSY SPECIMENS

No. of Samples	Mg^{++} - ATPase		(Na^+, K^+) - ATPase		$\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
8	3.0	2.5	2.9	0.8	1.5	0.9

TABLE V 3

EFFECTS OF 0.1% DEOXYCHOLATE TREATMENT ON MICROSOMAL FRACTIONS OF HUMAN POST MORTEM TISSUE AND BIOPSY TISSUE

	+ DEOXYCHOLATE		- DEOXYCHOLATE	
	(Na^+, K^+) - ATPase	Mg^{++} - ATPase	(Na^+, K^+) - ATPase	Mg^{++} - ATPase
Biopsy Specimens	3.8 2.2	3.2 3.0	0.1 0.3	2.6 3.1

	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Post Mortem Tissue (8 specimens)	5.3	2.7	3.8	1.6	4.7	3.2	3.1	1.2

concentration between 5 and 10 mM above which K^+ ions became inhibitory. Table V 1 shows the substrate specificity. ADP could be used as a substrate but the activity was considerably lower than with ATP. Cytidine triphosphate showed a little activity above that found for guanosine triphosphate and inosine triphosphate.

In general the biopsy specimens had a high Mg^{++} - ATPase activity and a low $\frac{(Na^+, K^+) - \text{ATPase}}{Mg^{++} - \text{ATPase}}$ ratio, Table V 2. The activity of neither enzyme was greatly altered after storage at -10°C for up to 1 month. Both, (Na^+, K^+) - ATPase and Mg^{++} - ATPase showed a slight decrease in activity after 1 month's storage at -10°C.

Results from human post mortem specimens

Table V 3 shows the results of a series of experiments to determine the effects of 0.1% deoxycholate on post mortem myocardial microsomal fractions. There was a varying response to deoxycholate treatment in post mortem preparations. Many of the samples were indifferent to pretreatment by deoxycholate already having a high (Na^+, K^+) - ATPase activity before deoxycholate was added. This was unlike the biopsy specimens where pretreatment was essential to release (or unmask) (Na^+, K^+)- activity.

TABLE V 4

SUCCINIC DEHYDROGENASE ACTIVITY OF HUMAN POST MORTEM HOMOGENATE AND MICROSOMAL FRACTION

Details of assay are given in Chapter II

SAMPLE	No.	Succinic dehydrogenase activity O.D. change / 0.1 mg protein	S.D.
Homogenate	3	0.60	0.01
Microsomal suspension in 0.25 M sucrose	3	0.11	0.01

Succinic dehydrogenase activity

Succinic dehydrogenase activity was measured in post mortem myocardial homogenates and microsomal fractions. Table V 4 shows the results of these assays. The succinic dehydrogenase activity found in microsomal fractions would account for less than 2% of the activity found in the homogenate.

Ion requirements

Addition of gradually increasing concentrations of Na^+ ions showed an optimum at 75 to 100 mM after which there was a gradual decrease in activity up to 200 mM (Fig. V 8). The potassium ion requirements are shown in Fig. V 9. The sodium ion concentration was kept at 100 mM. Optimal potassium ion concentration was 10 mM above which they became inhibitory. Fig. V 10 shows that the optimal magnesium ion concentration, in the presence of 2 mM ATP, was 3 mM. Magnesium ions were not inhibitory above 3 mM.

pH Optimum

Fig. V 11 shows that the optimal pH for (Na^+, K^+) - ATPase was 7.7. The optimal pH for Mg^{++} - ATPase activity was 8.9.

ATP substrate concentration

The optimal ATP substrate concentration was 3 mM. Above 3 mM ATP rapidly

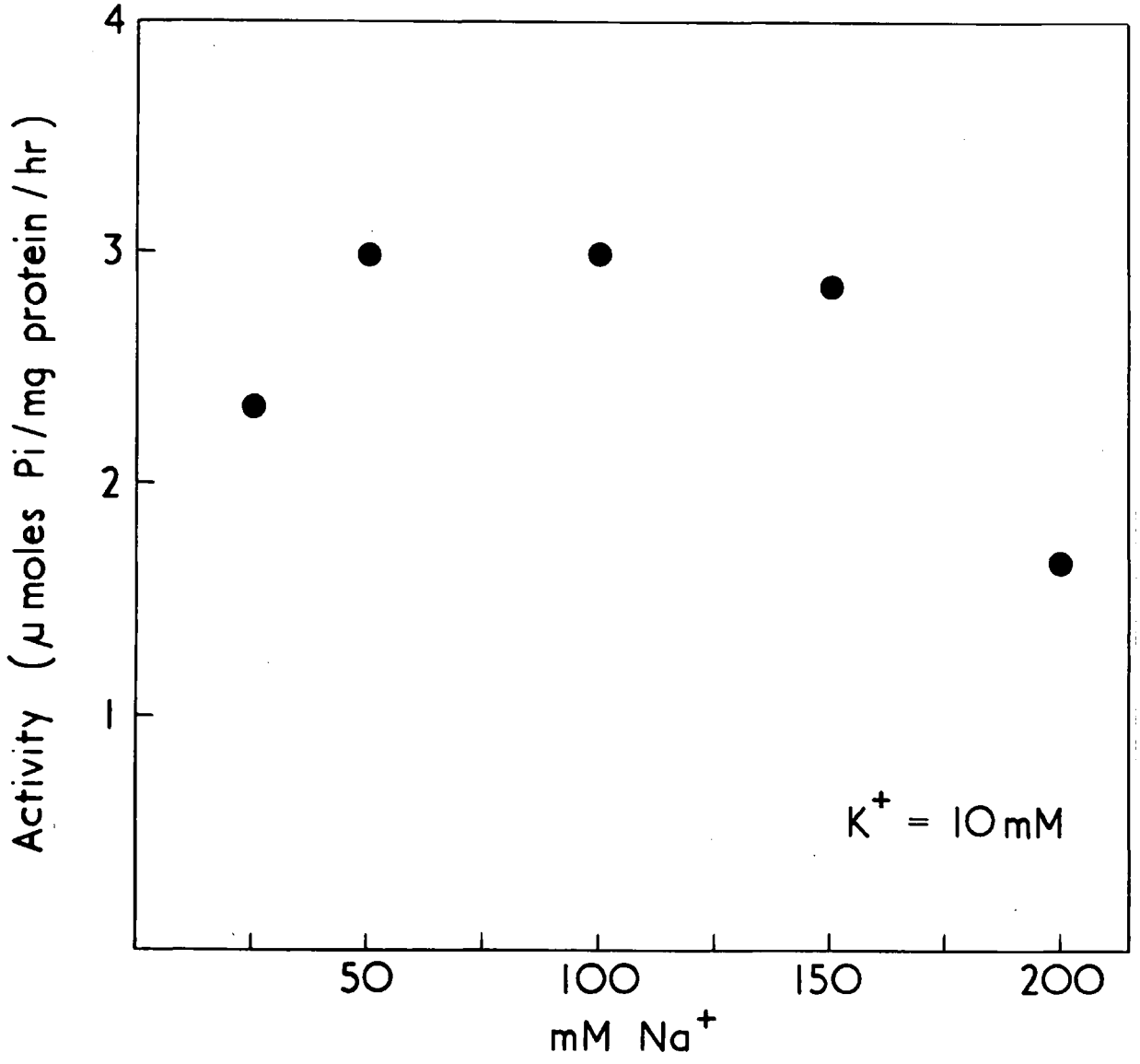


FIG. V 8 The effects of various concentrations of sodium ions on human post mortem myocardial (Na^+, K^+) -ATPase. The potassium ion concentration was kept at 10 m M.

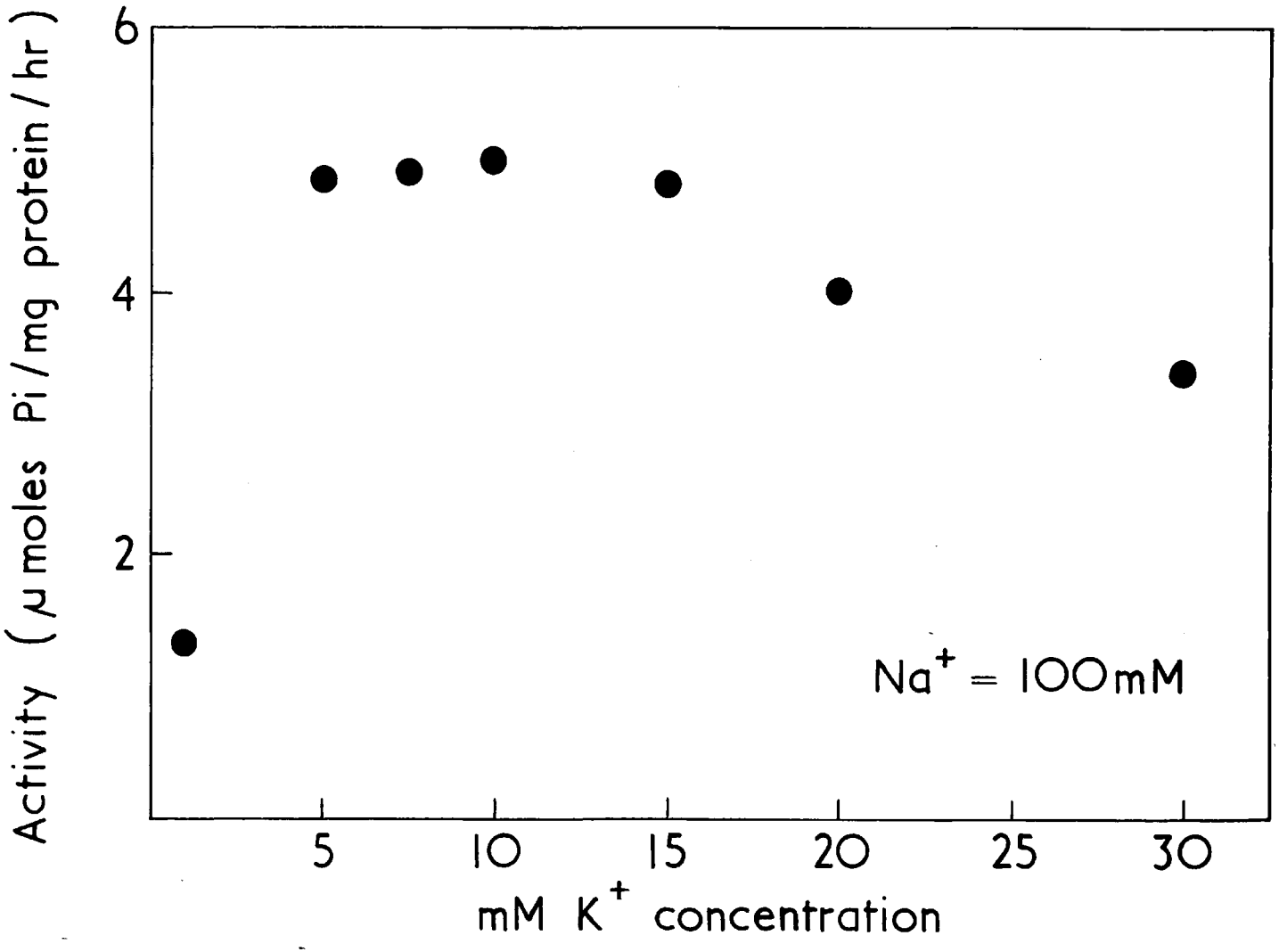


FIG. V 9 The effects of various concentrations of potassium ions on human post mortem myocardial $\overline{(Na\ ,\ K)}$-ATPase activity. The sodium ion concentration was held at 100 mM.

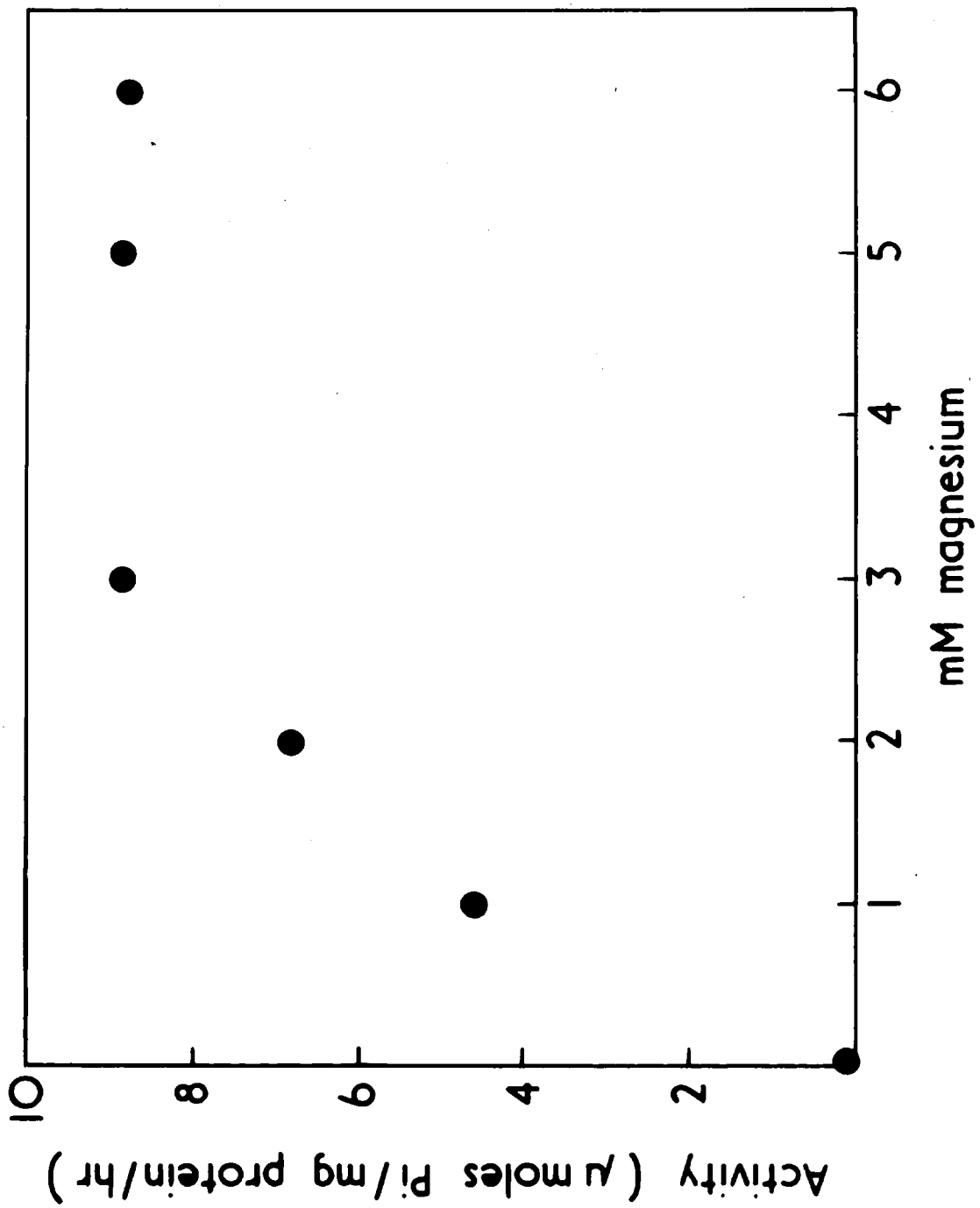


FIG. V 10 The effects of magnesium ions of human post mortem myocardial $\overline{(Na^+, K^+)}$ -ATPase activity.

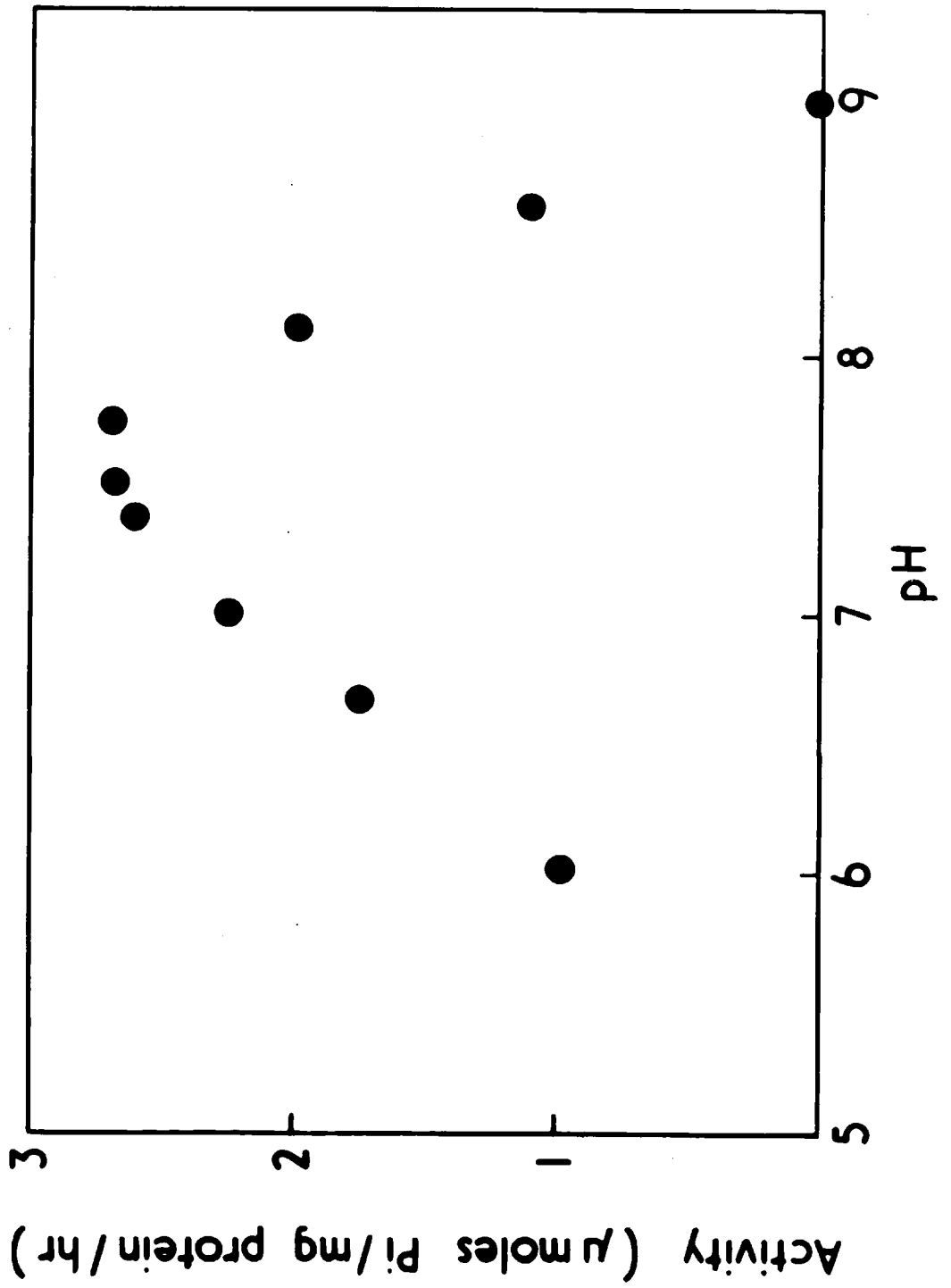


FIG. V II The effects of pH on human post mortem myocardial, $\overline{(Na^+, K^+)}$ -ATPase activity.

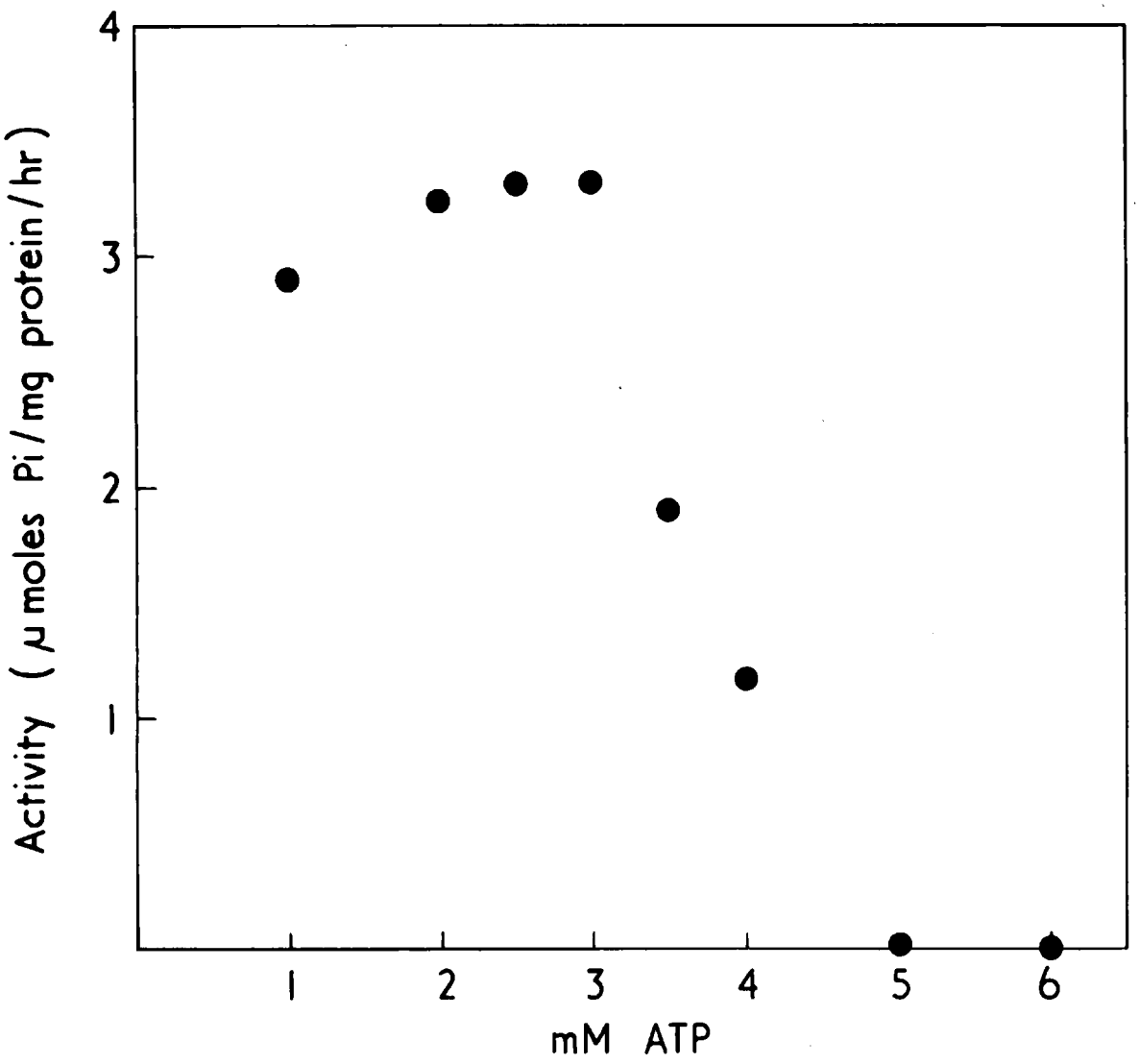


FIG. V 12 The effects of substate ATP on human post mortem myocardial $\overline{(Na^+, K^+)}$ -ATPase activity.

TABLE V 5

SUBSTRATE SPECIFICITY OF HUMAN POST MORTEM MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase

SUBSTRATE	μ mole Pi liberated / mg protein / hr.
GUANOSINE TRIPHOSPHATE	0.2
INOSINE TRIPHOSPHATE	0.2
CYTIDINE TRIPHOSPHATE	1.9
ADENOSINE DIPHOSPHATE	2.1
ADENOSINE TRIPHOSPHATE	5.0

became inhibitory (Fig. V 12).

Substrate specificity

The substrate specificity towards various nucleotides is shown in Table V·5. Negligible activity was found with GTP and ITP. CTP and ADP were more effective substrates but not so effective as ATP.

Time / Activity relationship

Fig. V 13, shows the activity plotted against time under various assay conditions. In the absence of magnesium ions there was negligible ATPase activity. Maximal activity was found in the presence of Mg^{++}, Na^{+} and K^{+} ions. In the absence of sodium and potassium ions the activity was lightly higher than that found in a assay system containing sodium ions, potassiumions and 10^{-3} M ouabain.

An ATP regenerating system was not added to the routine assays as the reactions were stopped after 30 minutes at 37°C up till which time the time / activity relationship was linear.

Sensitivity to ouabain

Fig. V. 14 shows the response of (Na^{+}, K^{+}) - ATPase to varying concentrations of ouabain. Half maximal inhibition was $10^{-7.5}$ M ouabain while complete inhibition occurred at 10^{-6}M.

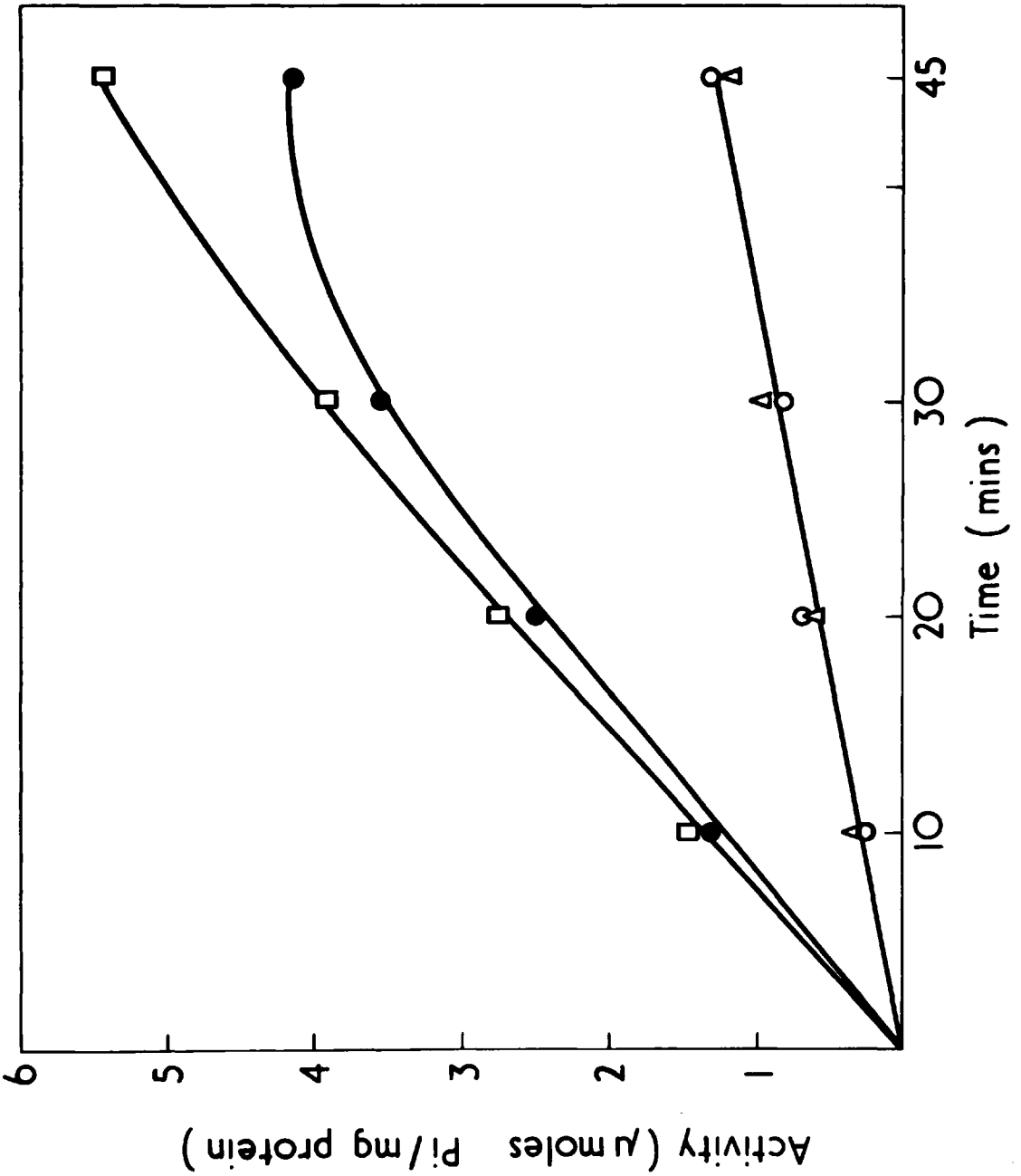


FIG. V 13 The time/activity relationship of human post mortem myocardial $\overline{(Na^+, K^+)}$-ATPase under various assay conditions. ●, complete assay system; □, assay system plus an ATP regenerating system; O, assay system plus 10^{-3} M ouabain; Δ, assay without Na^+ and K^+ ions added.

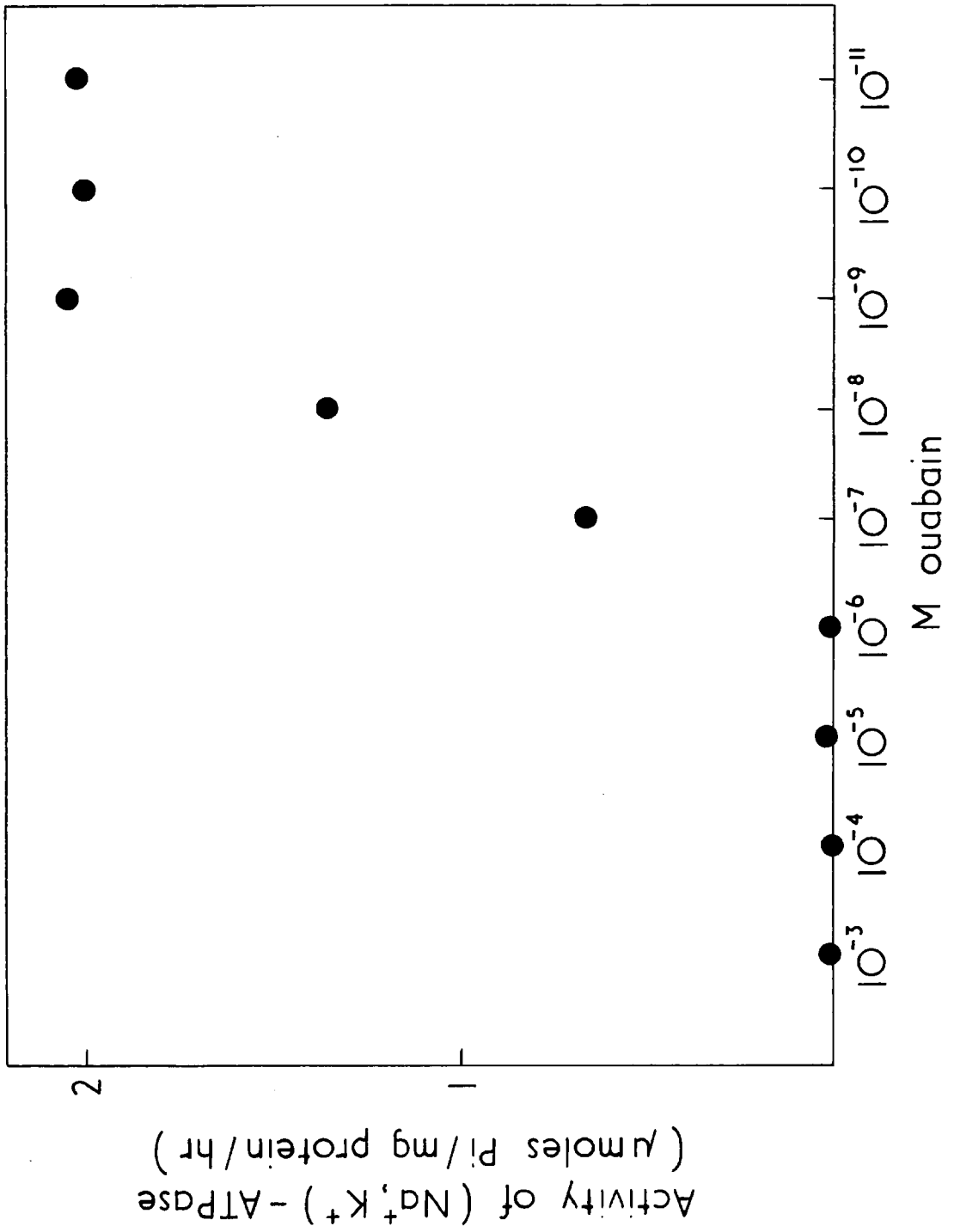


FIG. V 14 The effects of various concentrations of ouabain on human post mortem myocardial (Na^+, K^+) -ATPase activity.

TABLE V 6

THE EFFECTS OF AZIDE ON HUMAN POST MORTEM MYOCARDIAL ATPase ACTIVITY

CONCENTRATION mM				ATPase ACTIVITY		
Na^+	K^+	AZIDE	OUABAIN			
100	10	-	-	3.3	3.5	4.2
100	10	-	1	0.7	0.6	1.2
100	10	5	-	2.5	2.6	3.6
100	10	5	1	0.8	0.3	1.0
-	-	-	-	1.0	-	1.7
-	-	-	1	0.8	-	1.1
-	-	5	-	0.5	-	1.4
-	-	5	1	0.6	-	1.0

TABLE V 7

COMPARISON OF BIOPSY AND AUTOPSY MYOCARDIAL MICROSOMAL ATPases

(μ moles Pi liberated / mg protein / hr)

	No. of SAMPLES	Mg^{++} - ATPase		(Na^+, K^+) - ATPase		$\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
BIOPSY SPECIMENS	8	3.0	2.5	2.9	0.8	1.5	0.9
AUTOPSY SPECIMENS	25	2.1	1.4	5.4*	2.6	3.6	2.7
NON CARDIAC CASES	16	1.8	1.7	5.0	2.5	3.9	2.6
CARDIAC CASES	9	2.7	0.9	6.0	2.6	3.1	2.9

* Difference between biopsy and autopsy (Na^+, K^+) - ATPase significant $P = < 0.01$

Effects of 5 mM azide

The influence of 5 mM azide on ATPase activity under various assay conditions is shown in Table V 6. Azide had an inhibitory effect on both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities.

Table V 7 shows a summary of the (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities from human biopsy and autopsy specimens. There was a higher activity of (Na^+, K^+) - ATPase in the autopsy specimens compared to biopsy specimens. There was also an increasein the $\frac{(Na^+, K^+) - \text{ATPase}}{Mg^{++} - \text{ATPase}}$ ratio. The difference between post mortem cardiac and non-cardiac cases was not significant for either (Na^+, K^+) - ATPase or Mg^{++} - ATPase activities. There was a significantly higher (Na^+, K^+) - ATPase activity ($P < 0.01$) in post mortem samples compared to biopsy samples. There was no significant difference in Mg^{++} - ATPase activity between post mortem and biopsy samples ($P = 0.50$).

Tables V 8, V 9 and V 10 show a list of the individual cases used in these studies from post mortem non-cardiac, cardiac and biopsy tissues respectively.

The effects of high and low Mg^{++} - ATPase activity, present in microsomal preparations, on kinetic studies of (Na^+, K^+) - ATPase are shown in Fig. V 15 and V 16. Both graphs are plotted according to the method described by Lineweaver and Burk (1934). Fig. V 15 shows the effects of a high Mg^{++} - ATPase activity. The

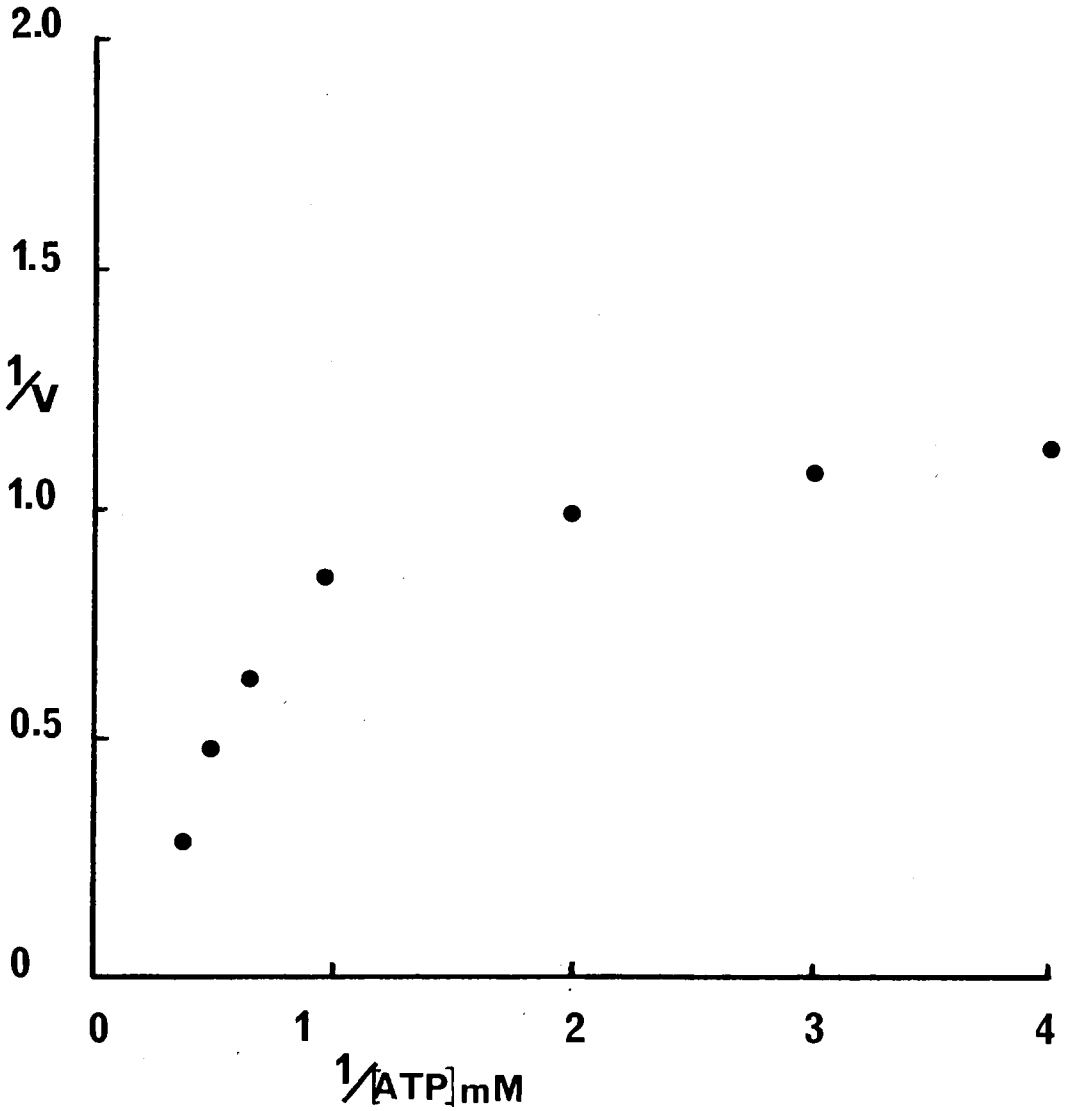


FIG. V 15 Lineweaver Burk plot of human myocardial microsomal, (Na^+, K^+)-ATPase containing a high Mg^{++}-ATPase activity.

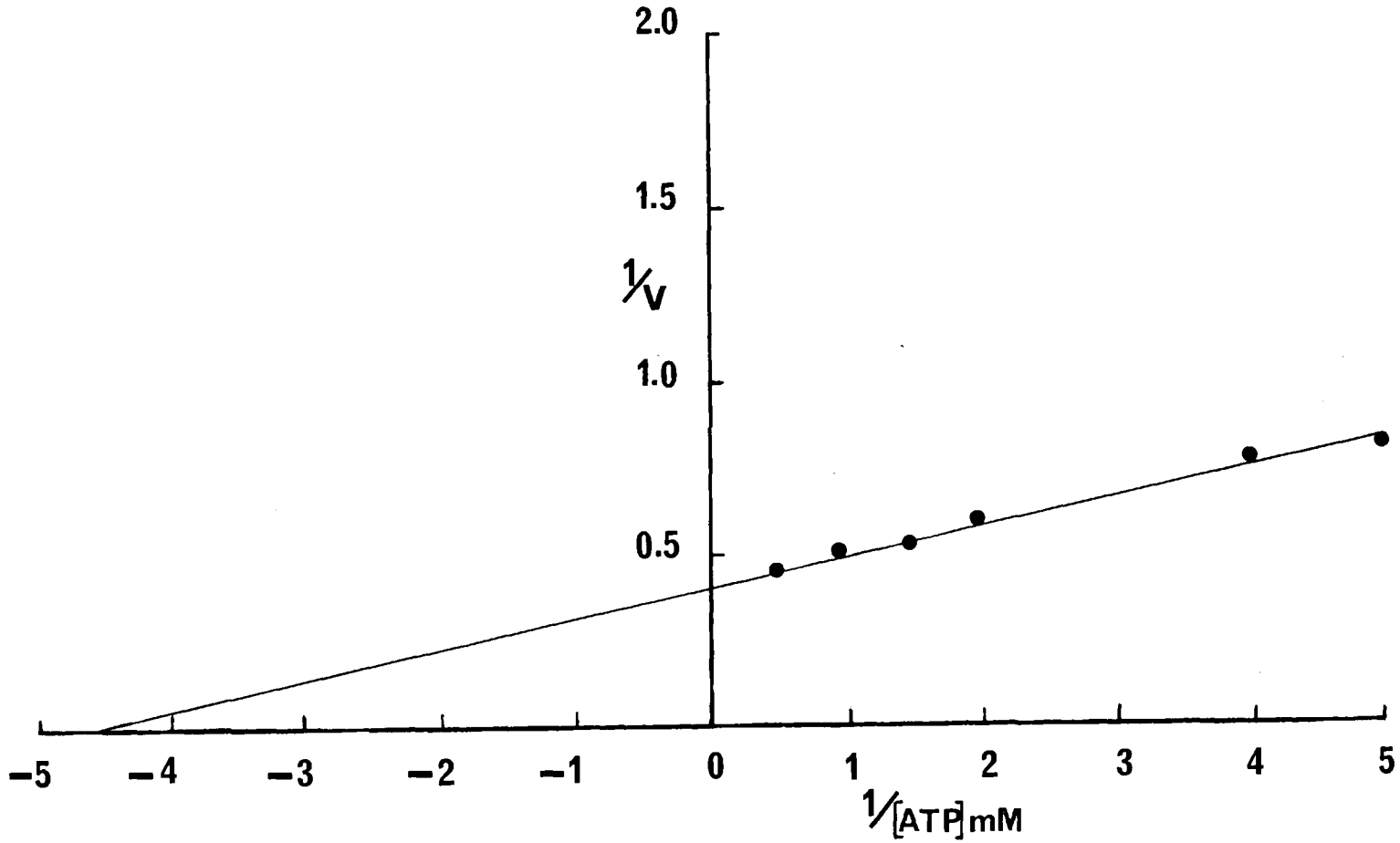


FIG. V 16 Human myocardial (Na^+, K^+) -ATPase activity plotted according to Lineweaver and Burk. The enzyme preparation contained very little Mg^{++} -ATPase activity.

TABLE V 8

HUMAN MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase FROM POST MORTEM TISSUE. NON CARDIAC CASES

Sample	Cause of death	Time after death before autopsy	VENTRICLE	
			Mg^{++}-ATPase	(Na^+,K^+)-ATPase
M.S.	chronic renal failure	48 hrs.	1.0	7.3
E.C.	cerebrovascular accident	24 hrs.	1.0	3.1
P.W.	chronic bronchitis	48 hrs.	1.3	4.4
W.Z.	carcinoma of kidney	72 hrs.	0.4	3.2
A.C.	broncho pneumonia	48 hrs.	0.6	5.5
-	dissecting aneurysm	24 hrs.	1.3	4.9
V.L.	carcinoma of ovary	48 hrs.	0.0	3.6
-	melanoma	48 hrs.	1.3	5.3
T.F.	bacterial meningitis	24 hrs.	1.0	2.3
W.G.	acute aneurysm	24 hrs.	2.4	4.8
G.L.	cerebrovascular accident	24 hrs.	1.8	9.6
R.H.	abdominal aneurysm	24 hrs.	2.0	1.1
E.F.	aortic aneurysm	24 hrs.	7.5	7.3
E.A.	pulmonary fibrosis	24 hrs.	3.3	0.8
C.M.	carcinoma of bronchus	24 hrs.	1.5	7.0
R.B.	carcinoma of oesophagus	24 hrs.	3.2	9.4

TABLE V 9

HUMAN MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase FROM POST MORTEM TISSUE. CARDIAC CASES

Sample	Cause of death	Time after death before autopsy	VENTRICLE	
			Mg^{++}-ATPase	(Na^+,K^+)-ATPase
H.B.	myocardial infarct	24 hrs.	3.6	5.2
E.H.	" "	24 hrs.	1.2	7.1
.B.	" "	24 hrs.	4.4	6.2
.W.	" "	24 hrs.	2.2	2.3
O.L.	coronary thrombosis	24 hrs.	2.9	4.7
W.D.	" "	8 hrs.	1.2	12.1
R.A.	" "	24 hrs.	3.4	7.1
E.S.	congestive cardiac and renal failure	24 hrs.	2.4	6.6
F.K.	myocardial fibrosis	24 hrs.	2.8	2.8

TABLE V 10

HUMAN MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase FROM BIOPSY SPECIMENS REMOVED DURING CARDIAC SURGERY

SAMPLE		Mg^{++} - ATPase	(Na^+, K^+) - ATPase
E.J.	right atrial appendage	3.2	3.8
V.J.	" " "	3.5	2.7
D.S.	left " "	4.9	1.1
S.L.	right " "	8.7	4.2
-	" " "	0.0	3.1
-	" " "	1.6	3.1
-	" " "	1.1	1.9
-	" " "	1.2	3.2

TABLE V 11

THE DISTRIBUTION OF (Na^+, K^+) - ATPase AND Mg^{++} - ATPase IN HUMAN POST MORTEM MYOCARDIUM

	No. of Samples	(Na^+,K^+)-ATPase		Mg^{++}-ATPase		$\frac{(Na^+,K^+)\text{-ATPase}}{Mg^{++}\text{-ATPase}}$	
		Mean	S.D.	Mean	S.D.	Mean	S.D.
LEFT ATRIUM	9	3.0	1.5	2.0	1.0	2.0	1.0
RIGHT ATRIUM	9	3.6	1.7	2.2	1.4	2.0	.1.2
LEFT VENTRICLE	9	3.9	1.6	1.7	1.3	3.6	2.2
RIGHT VENTRICLE	9	3.1	1.4	1.9	1.2	3.1	2.1

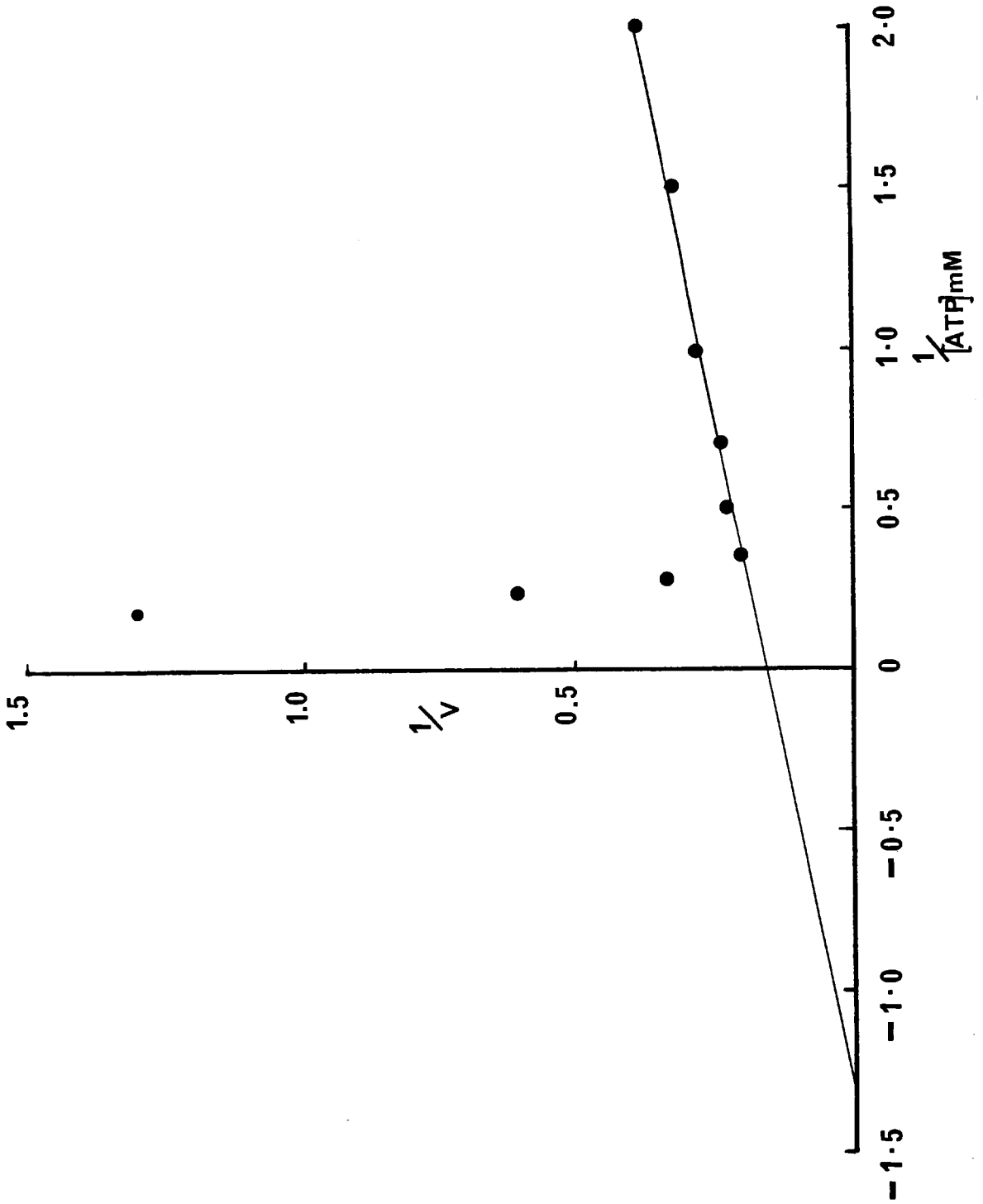


FIG. V 17 Human myocardial (Na^+, K^+) -ATPase activity plotted according to Lineweaver and Burk. The graph shows the effect of excess substrate.

graph is characteristic of one obtained when two enzymes are acting on a single substrate (Dixon and Webb, 1966). Fig. V 16 shows a similar plot using an enzyme very low in Mg^{++} - ATPase activity and a $\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$ ratio of 4.8. This type of curve is characteristic of a single enzyme reacting with its substrate.

Fig. V 17 shows the effects of excess ATP substrate on (Na^+, K^+) - ATPase activity when plotted by the Lineweaver and Burk method. ATP concentrations higher than 2.5 mM caused a rapid fall in (Na^+, K^+) - ATPase activity.

Distribution of (Na^+, K^+) - ATPase in the various chambers of human post mortem myocardial samples

Table V 11 shows the results from a series of experiments to compare the activities of (Na^+, K^+) - ATPase and Mg^{++} - ATPase in the various chambers of human post mortem hearts. There was no statistically significant difference between the four areas of myocardium studied.

Discussion

The presence of (Na^+, K^+) - ATPase has been demonstrated in human myocardial biopsy and post mortem tissues. Some of the properties of the enzyme have been described. It would appear that the enzyme has very similar properties to

(Na^+, K^+) - ATPase isolated from other tissues and even other animal species. A pH optimum of 7.8 was found for human biopsy myocardium compared to 7.7 for post mortem myocardium. This is of the same order as that found for human skeletal muscle, pH 7.5 to 8 (Samaha and Gergely, 1965) and rat myocardium, pH 7.8 (Tashima et al, 1966). For comparison the pH optimum for calf heart is 7.2 (Matsui and Schwartz, 1966) and rabbit ventricle is 7.5 (Auditore and Murray, 1962) which are lower than that found for human myocardium.

The biopsy and post mortem human myocardial preparation had very similar requirements for ions and substrates. Oreexception was that sodium ions were inhibitory above 100 mM in post mortem tissues but were not inhibitory up to 200 mM in biopsy specimens. The substrate specificity was similar in both post mortem and biopsy specimens except that CTP and ADP were more effective substrates in post mortem compared to biopsy preparations. Mg^{++} ion and substrate requirements are similar to those reported for other tissues (Auditore and Murray, 1962; Lee and Yu, 1963 and Matsui and Schwartz, 1966). The sensitivity to ouabain was higher in the post mortem tissue. The Ki for ouabain was $10^{-7.5}$ M in post mortem and $10^{-6.4}$ M in biopsy myocardium. The maximal inhibition was reduced from 10^{-4} in biopsy tissue to 10^{-6} in post mortem specimens.

It is appreciated that neither the post mortem nor biopsy specimens can be considered 'normal' but the materials have provided a clear demonstration that the

enzyme can be located in human myocardial microsomal fractions. Very little is known of the action of drugs on (Na^+, K^+) - ATPase in general and even less of their action on human myocardial (Na^+, K^+) - ATPase. The presence of (Na^+, K^+) - ATPase in human myocardial specimens presented the opportunity to study some of the in vitro effects of some cardio-active drugs and compounds on this enzyme system.

(Na^+, K^+) - ATPase activity could be measured in many of the human post mortem specimens without the necessity to pretreat the microsomal suspensions with deoxycholate. This would indicate that autolysis may have been responsible for some modification to the membrane allowing the enzyme to react with the substrate. An alternative may be the decay of a labile inhibitor substance present in the myocardium or the release of an activator of (Na^+, K^+) - ATPase. That keeping tissue at + 5° C for some time before preparation of microsomal fractions could lead to an activation or 'unmasking' of the (Na^+, K^+) - ATPase has been further demonstrated with dog heart (Chapter VI).

The inhibitory effects of azide on post mortem myocardial ATPases were not specific. Samaha and Gergely (1965) found that azide specifically inhibited human skeletal muscle Mg^{++} - ATPase and Schwartz and Laseter (1964 a) found a similar response in guinea pig myocardium. However, similar studies on the hearts of rabbits (Brown, 1966) and calves (Matsui and Schwartz, 1966) and on the guinea

pig brain (Schwartz and Laseter, 1964 a) have failed to show any such inhibition. The effects of azide in the present study may be due to the ability of azide to combine with phosphate acceptors (Siekevitz et al, 1958).

The presence of a high Mg^{++} activity in the samples interfered with kinetic studies of (Na^+, K^+) - ATPase. Only samples having a low Mg^{++} - ATPase activity ($\frac{(Na^+, K^+) - \text{ATPase}}{Mg^{++} - \text{ATPase}}$ 74) were used in kinetic studies. The substrate concentration did not exceed 2.5 mM in the Kinetic studies as excess substrate strongly inhibits (Na^+, K^+) - ATPase activity.

The enzyme has a highly specific requirement for ATP as substrate. ADP is inhibitory in the presence of ATP and this may explain the curve obtained in the time / activity plot of biopsy microsomal fraction high in Mg^{++} - ATPase activity. ADP inhibition could be partly removed by an ATP regenerating system. The enzyme had an absolute requirement for Mg^{++} ions. Experiments to determine whether Na^+ and K^+ ions could be replaced were not performed but in other myocardial tisues it has been found that Na^+ ion replacement is very difficult and only Na^+ ions will cause a maximal stimulation of enzyme activity. K^+ ions are more easily replaceable and with NH_4^+ ions the stimulation is even higher than with K^+ ions (Skou, 1964).

The distribution of the enzyme, from the limited study made, showed no

significant difference in activity between any of the heart chambers.

CHAPTER VI

ANIMAL (Na^+, K^+) - ATPase

During this study the experiments were performed with dog, rabbit, sheep and rat myocardium according to the availability of the specimens. Rats have been used for all the in vivo studies. Therefore a preliminary investigation of rat myocardium was made to determine optimum assay conditions to be used for routine in vivo work. A study of the distribution of (Na^+, K^+) - ATPase in the atrium, left and right ventricles of the rat was also made as no previous work has been reported on this.

Materials

Dog myocardium was obtained from The Royal Vetinary College and transported to the laboratory surrounded by ice in a large thermos container. The dogs were mongrels killed by an overdose of phenobarbital given intraperitoneally.

New Zealand White rabbits were obtained from The London Hospital and killed in the laboratory by a sharp blow to the head.

Sheep myocardium was obtained from The London Hospital and transported to the laboratory in the same way as dog myocardium.

Sprague-Dawley albino rats were used in all the experiments. The rats were killed by a sharp blow to the head.

Methods

Dog and Sheep myocardium were homogenized with a MSE homogenizer as described in Chapter II. Rabbit and rat myocardium were homogenized using a glass and teflon homogenizer as detailed in Chapter II.

The microsomal fraction was obtained by the shortened centrifugation scheme described in Chapter II.

Results

Storage experiments with dog myocardium

The (Na^+, K^+) - ATPase assay conditions for the dog myocardium were those described in Chapter II with the addition of an ATP regenerating system consisting of 1 mg of PEP and 1 μ g of PK per 1 ml assay volume. Incubation time was 45 mins at 37°C.

The left ventricle of a single heart was divided into 8 portions. One portion was homogenized and a microsomal fraction prepared and the remainder stored at + 5°C for various time intervals up to 5 days before homogenization and microsomal preparation. Fig. VI 1 shows the results of the (Na^+, K^+) - ATPase and Mg^{++} - ATPase assays on the various samples suspended in 0.1% deoxycholate and assayed after 2 hours at + 5°C. There was a gradual increase in the activity of (Na^+, K^+) - ATPase up to 3 days while the Mg^{++} - ATPase fell slowly after

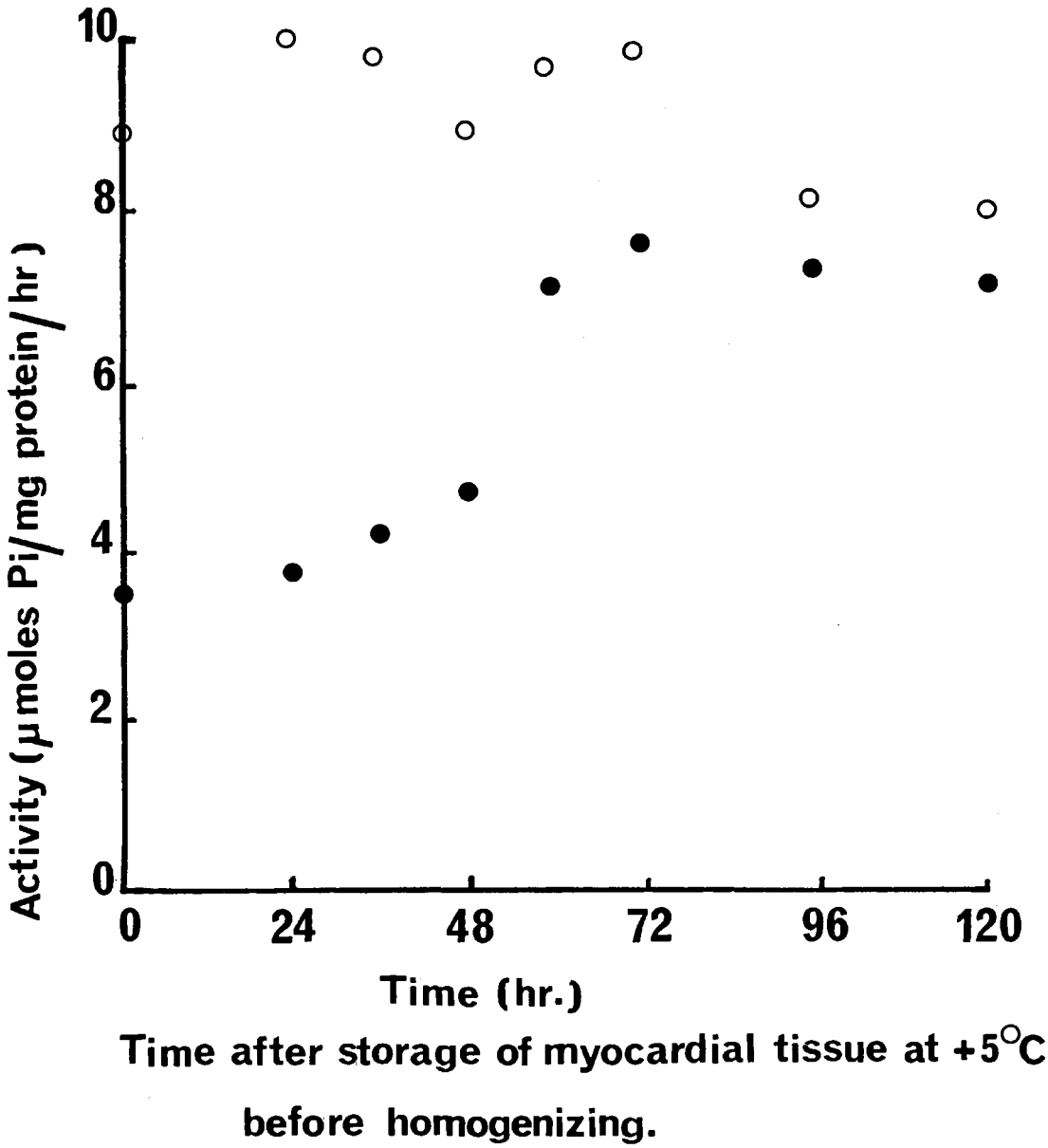
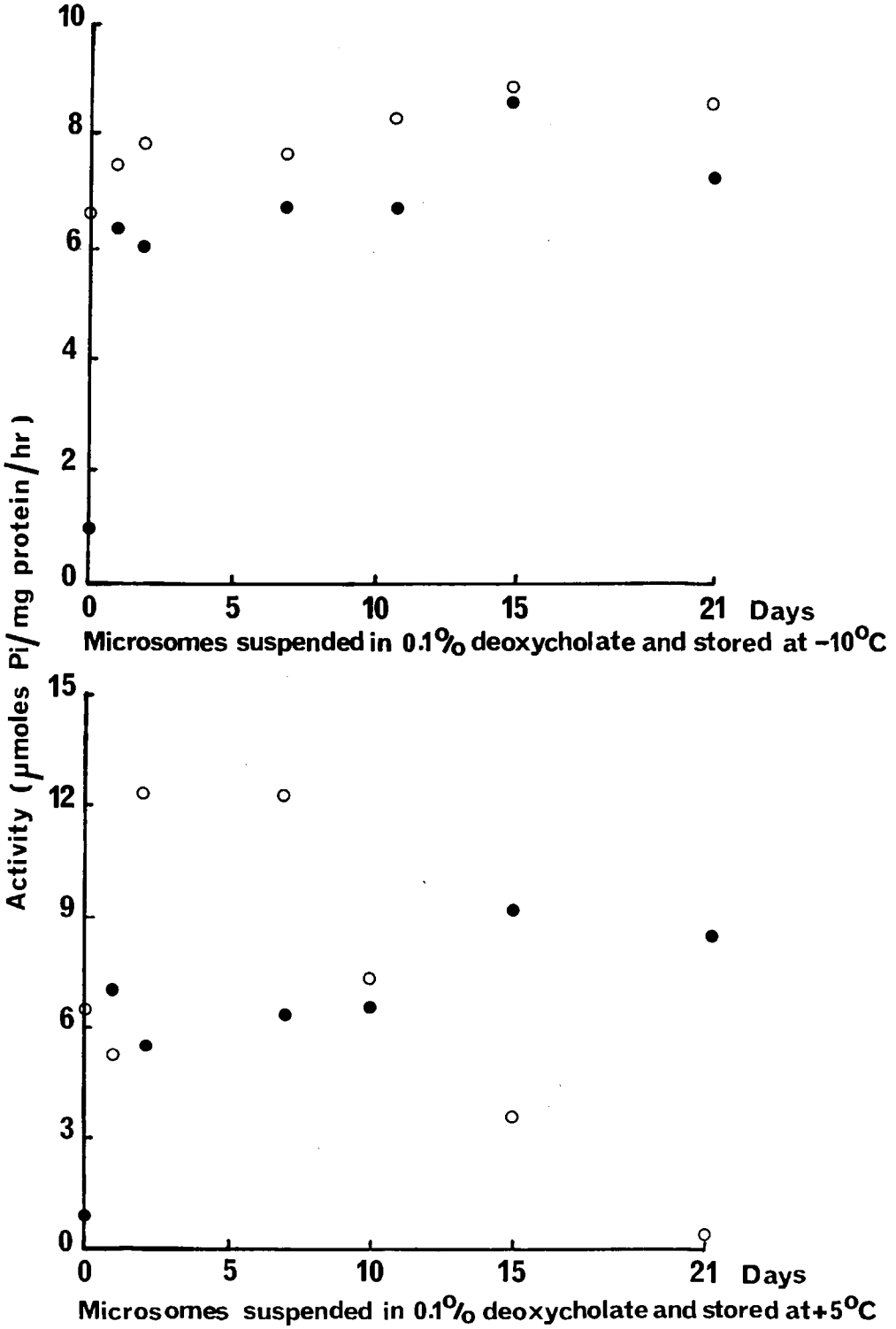


FIG. VI I The effects of storing dog myocardial tissue at + 5°C on microsomal ATPase activity. ●, (Na^+, K^+) -ATPase; Mg^{++} -ATPase



Storage experiment with dog myocardial microsomal fraction suspension.
FIG. VI 2 Upper diagram
FIG. VI 3 Lower diagram
●, (Na^+, K^+) -ATPase activity; O, Mg^{++} -ATPase activity.

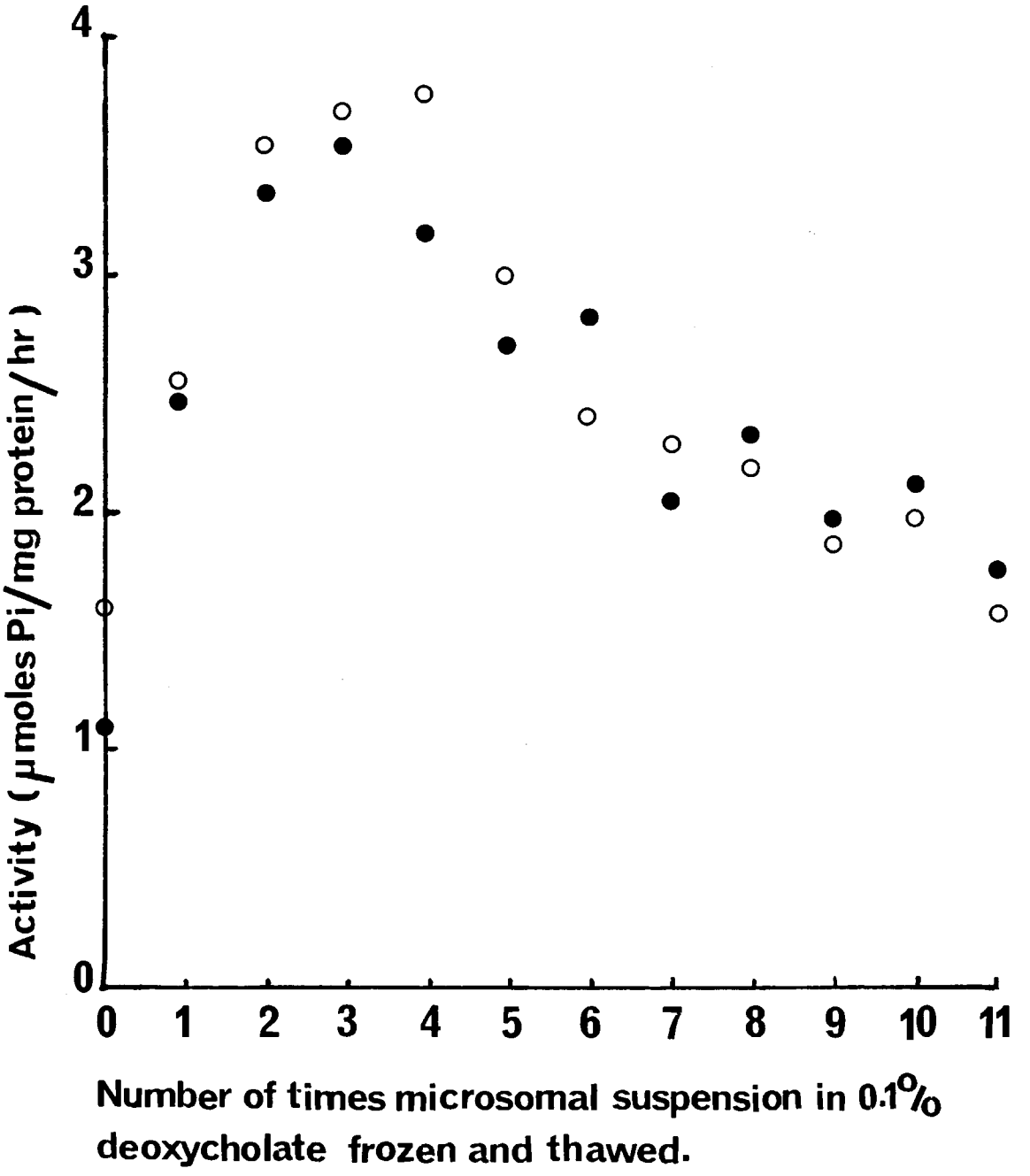


FIG. VI 4 The effects of freezing and thawing on dog myocardial microsomal ATPase activity. ●, (Na^+, K^+) -ATPase activity; ○ Mg^{++} -ATPase activity.

storage. After 5 days storage at + 5°C the (Na^+, K^+) - ATPase activity was higher than in the freshly prepared myocardium.

A large quantity of microsomal fraction was prepared from a single dog ventricle and suspended in 0.1% deoxycholate to give a protein concentration of 0.8 mg / ml. The microsomal suspension was divided into a series of tubes one half of which were stored at - 10°C whilst the remainder were stored at + 5°C for various time intervals. Figs. VI2 and VI 3 show the results of the (Na^+, K^+) - ATPase and the Mg^{++} - ATPase assays of these samples. There was an initial increase in (Na^+, K^+) - ATPase activity after storage at - 10°C for 24 hours. After 24 hours and up to 21 days storage at - 10°C there was little change in (Na^+, K^+) - ATPase or Mg^{++} - ATPase activities. Storage at + 5°C showed a drastic decrease in Mg^{++} - ATPase activity after 8 days. After 21 days there was negligible Mg^{++} - ATPase activity. Throughout storage at + 5°C there was little change in (Na^+, K^+) - ATPase activity after the initial increase at 24 hours.

A dog myocardial microsomal fraction was suspended in 0.1% deoxycholate to give a 0.8 mg / ml protein content. The sample was frozen and thawed eleven times. The activity of Mg^{++} - ATPase and (Na^+, K^+) - ATPase was followed and results are shown in Figure VI 4. Freezing and thawing the sample twice led to an increase in the activity of both the (Na^+, K^+) - ATPase and the Mg^{++} - ATPase activity. After the third freezing and thawing there was a gradual fall in the

TABLE VI I

THE EFFECTS OF FREEZE DRYING ON DOG MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase

CONTROL MICROSOMES	(Na^+, K^+) - ATPase	Mg^{++} - ATPase	$\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$
Suspended in 0.1% deoxycholate and kept at - 15°C for 1 week	0.74	1.42	0.52
Freeze-dried preparation after 1 week at - 10°C	1.39	1.34	1.04
Freeze-dried preparation after 1 year at - 10°C	4.10	5.80	0.71

activity of both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. The Mg^{++} - ATPase activity was slightly above that of the (Na^+, K^+) - ATPase at the beginning of the experiment but after five freezings and thawings the activity of the Mg^{++} - ATPase was slightly below that of the (Na^+, K^+) - ATPase activity.

Seven microsomal pellets were prepared from a single dog ventricle homogenate. One was suspended in 0.1% deoxycholate to give 0.8 mg / ml protein content and kept for 24 hours at - 10°C before assaying (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. The remaining microsomal pellets were stored at - 10°C for various time intervals before suspending in 0.1% deoxycholate, as described for the first pellet. Fig. VI 5 shows the results of storing the microsomal pellets for up to 45 days at - 10°C. There was an increase in (Na^+, K^+) - ATPase activity after 2 to 4 days storage but then it returned to the initial value at the eleventh day. From the eleventh day up to 45 days storage there was little change in enzyme activity.

From a microsomal suspension in 0.1% deoxycholate containing 0.8 mg / ml protein content a sample was removed and the remainder freeze-dried. The powder was divided into two portions both of which were stored at - 10°C. After one week one sample was resuspended with distilled water and assayed for ATPase activity. The remaining sample was kept for 1 year before resuspending with distilled water and assaying for ATPase activity. The results are shown in Table VI 1. (Na^+, K^+) - ATPase activity increased after freeze-drying and storage for 1 week,

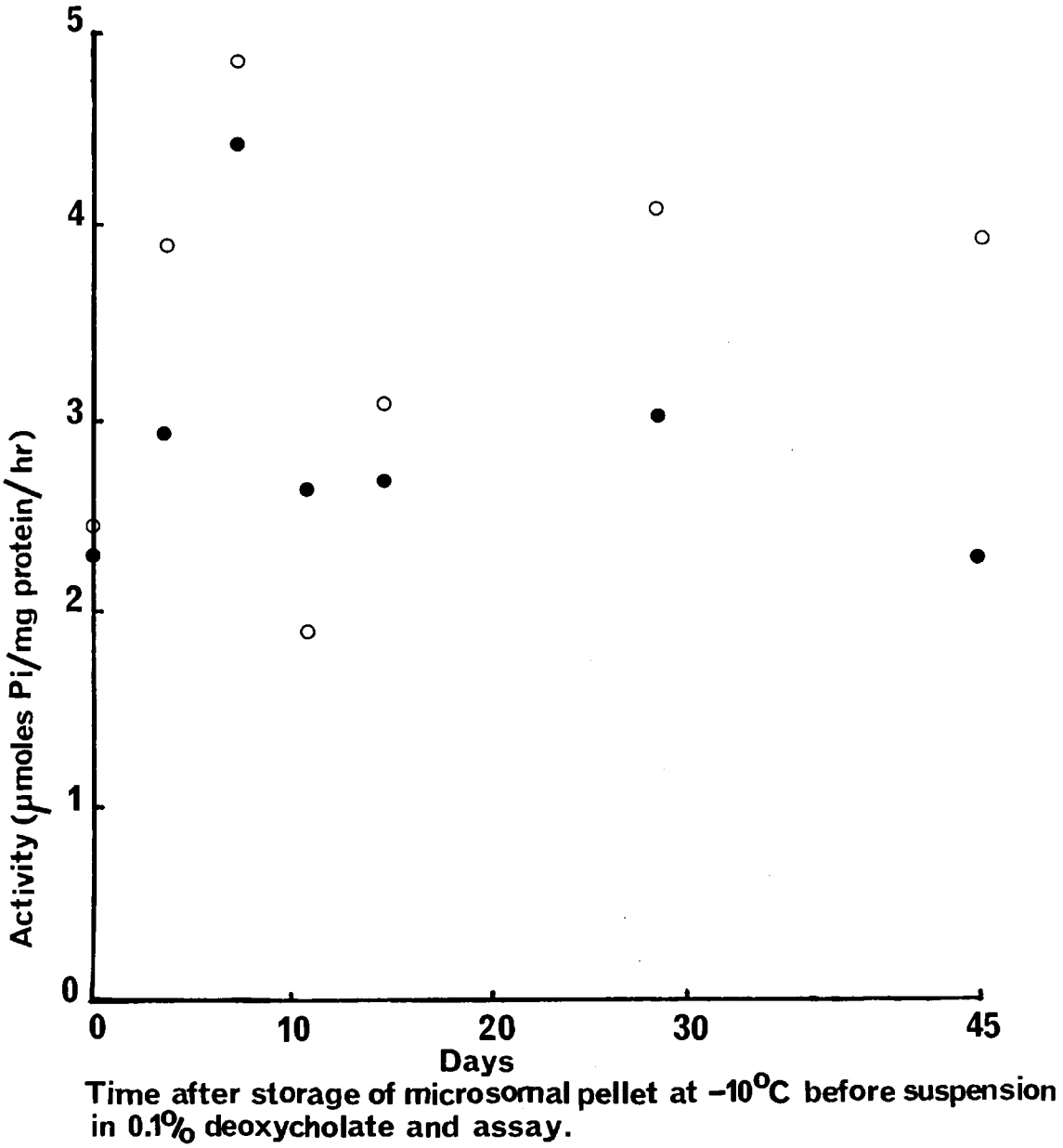


FIG. VI 5 Storage experiment with dog myocardial microsomal fraction. ●, (Na^+, K^+) -ATPase activity; ○, Mg^{++} -ATPase activity.

TABLE VI 2

SUMMARY TABLE OF RABBIT MYOCARDIAL MICROSOMAL ATPases

RABBIT MYOCARDIAL MICROSOMAL ATPase ACTIVITIES

3 month old animals

SAMPLE	No.	(Na^+, K^+) - ATPase Mean	S.D.	Mg^{++} - ATPase Mean	S.D.
Atrium	6	0.4	0.2	0.7	0.4
Left ventricle	6	0.4	0.2	0.8	0.4
Right ventricle	6	0.6	0.3	0.7	0.4

4 day old animals

SAMPLE	No.	(Na^+, K^+) - ATPase Mean	Mg^{++} - ATPase Mean
Atrium	2	0.1	0.3
Left ventricle	2	0.2	0.2
Right ventricle	2	0.2	0.2

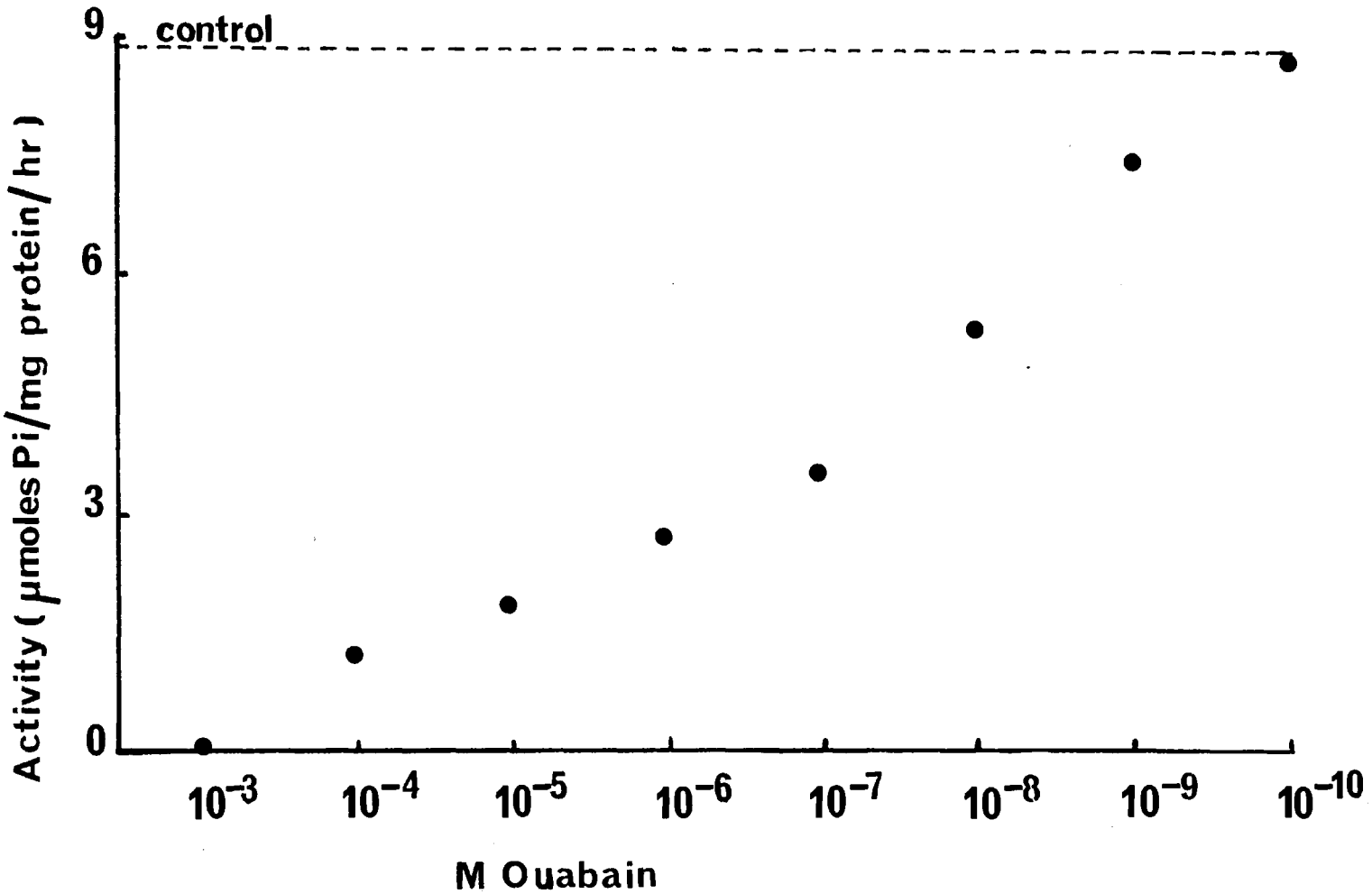


FIG. VI 6 The effects of ouabain on sheep myocardial microsomal (Na^+, K^+) -ATPase activity.

and the activity ratio ($\frac{(Na^+, K^+) - ATPase}{Mg^{++} - ATPase}$) was doubled. After storage for 1 year there was an increase in both Mg^{++} - ATPase and (Na^+, K^+) - ATPase activity. The activity ratio was slightly reduced after 1 year's storage at - 10°C.

Myocardial microsomal ATPase from Sheep

Only two sheep hearts were available for this study. (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity could be found in the atria and ventricle of each sample. The mean (Na^+, K^+) - ATPase activity found in the atria was 3.9 and the Mg^{++} - ATPase was 3.7. The mean (Na^+, K^+) - ATPase found in the ventricle was 8.7 and the Mg^{++} - ATPase was 6.3. Sensitivity to ouabain is shown in Fig. VI 6. The Ki for ouabain was $10^{-7.5}$M.

Myocardial microsomal ATPase from Rabbit

The (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities from the rabbit myocardium were extremely low compared to all the other species studied. Table VI 2 summarizes the results from 3 month old and 4 day old rabbits. The highest activity was 0.6 μ moles Pi liberated / mg protein / hour from the right ventricle of the 4 month old animals. The assays were repeated 2 weeks later and similar results obtained.

As the results from rabbit myocardium were of very low activity no further

experiments were continued with rabbit myocardium. There was insufficient data for a statistical analysis of these results.

Rat myocardial microsomal ATPase

Fig. VI 7 shows the effects of adding increasing quantities of potassium ions while maintaining the sodium ion concentration at 100 mM. The optimum potassium ion concentration in 10 mM above which potassium ions were inhibitory. Fig. VI 8 shows the effects of increasing sodium ion concentration while maintaining the potassium ion concentration constant at 10 mM. The optimum sodium ion concentration was 75 mM to 100 mM. At concentrations of 150 to 200 mM sodium ions had a slight inhibitory effect.

The optimal pH for rat myocardial microsomal (Na^+, K^+) - ATPase was found to be 7.4.

A linear time / activity relationship up to 45 mins was obtained using rat myocardial microsomal suspension (Fig. VI 9). The assay mixture did not contain an ATP regenerating system.

In a series of 13 experiments ouabain at 10^{-3} M concentration had the same effect as the omission of sodium and potassium ions from the assay mixture. The mean value for (Na^+, K^+) - ATPase in the presence of Mg^{++}, Na^+ and K^+ ions was 3.77 and the addition of ouabain caused the value to fall to 2.33 while that obtained by the omission of sodium and potassium ions was 2.51.

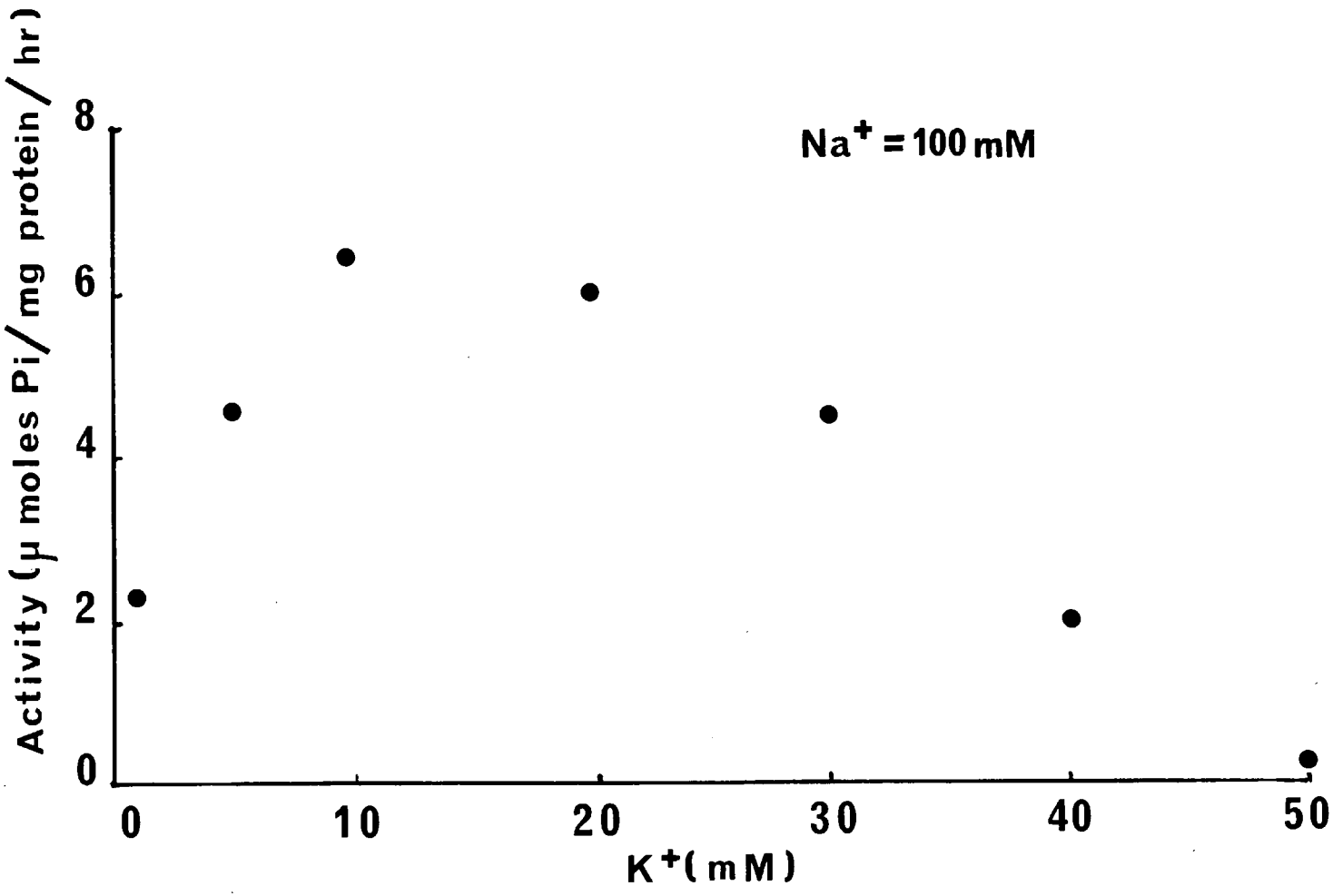


FIG. VI 7 The effects of potassium ions on rat myocardial microsomal (Na^+, K^+) -ATPase activity. The sodium ion concentration was held at 100 mM.

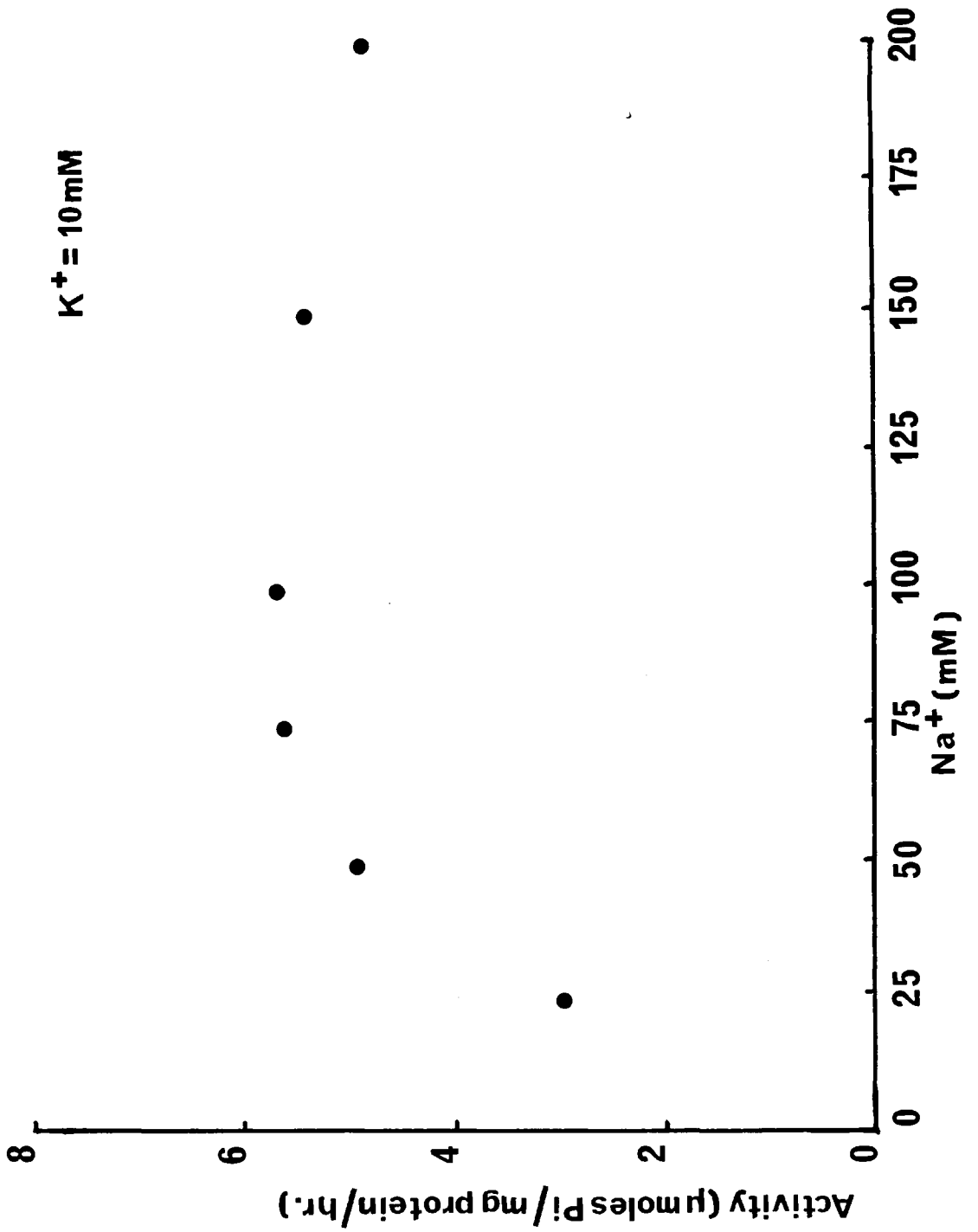


FIG. VI 8 The effects of various concentrations of sodium ions on rat myocardial microsomal (Na^+, K^+) -ATPase. The potassium ion concentration was held at 10 mM.

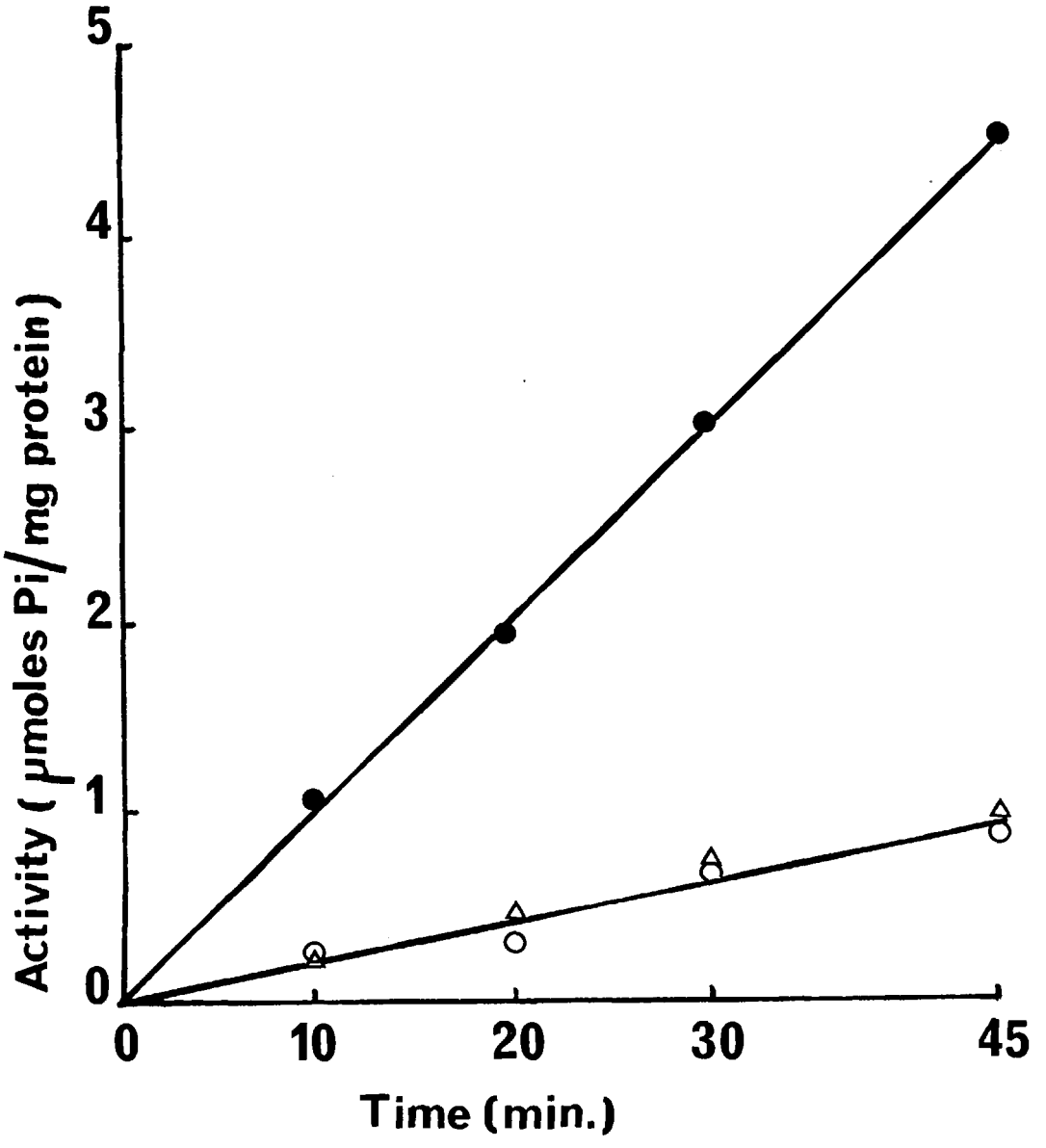


FIG. VI 9 Time/activity relationship of rat myocardial microsomal ATPase. ●, complete assay mixture; △, Na^+ and K^+ omitted from assay mixture; ○, complete assay mixture plus 10^{-3} M ouabain.

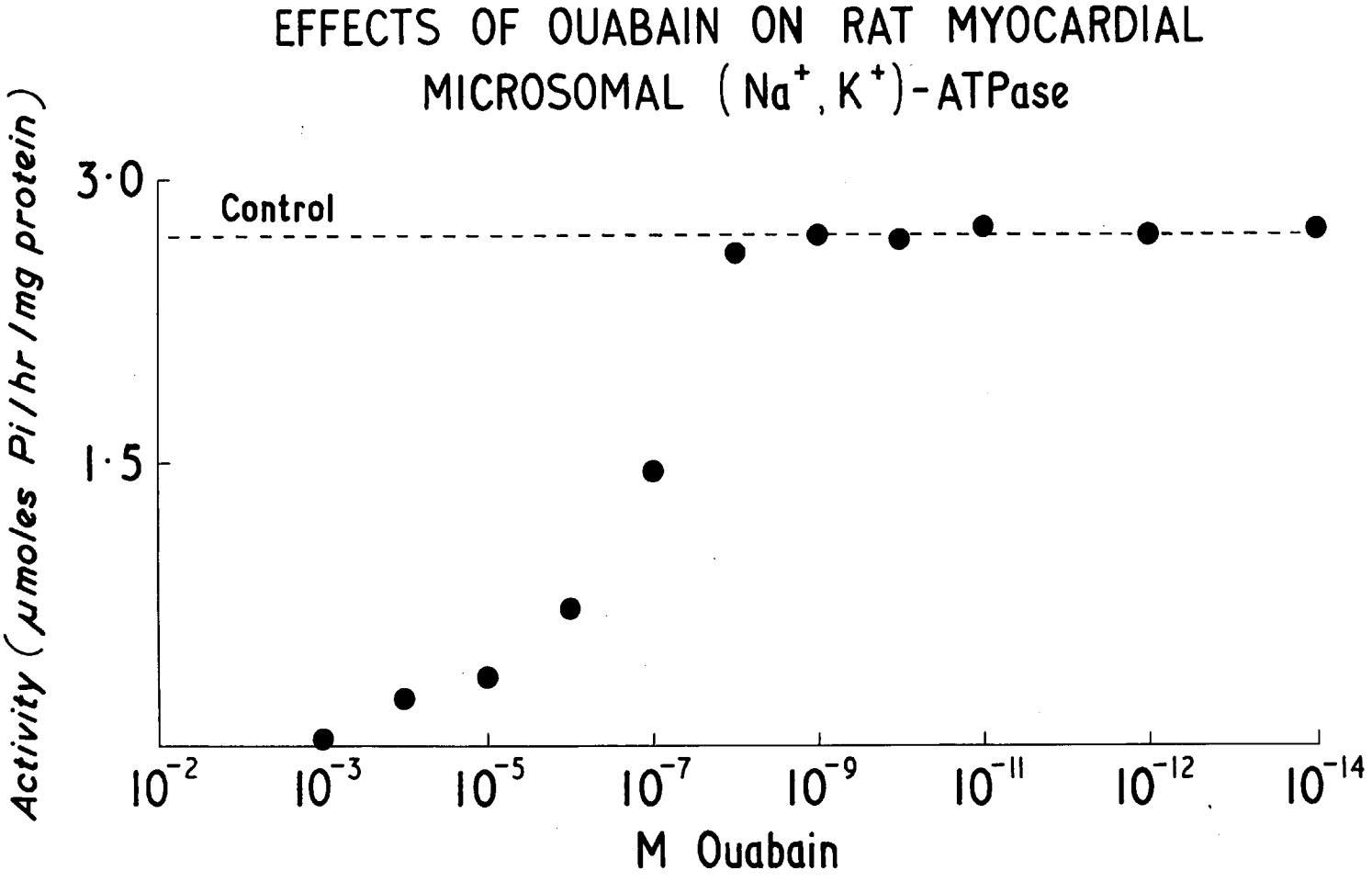


FIG. VI 10 The effects of ouabain on rat myocardial microsomal (Na^+, K^+) -ATPase activity.

The Ki for the effect of ouabain on rat (Na^+, K^+) - ATPase was $10^{-6.5}$ M. At no concentration down to 10^{-14} M was ouabain stimulatory, Fig. VI 10.

Storage of rat myocardial microsomal suspensions

Extensive studies were not performed on this aspect of rat myocardial microsomal ATPase.

A microsomal preparation was divided into two samples. One was suspended in sucrose versene tris solution while the other sample was suspended in sucrose versene tris solution plus 0.1% deoxycholate. Both suspensions had a protein concentration of 0.8 mg / ml. The deoxycholate treated suspension was divided into several tubes. One was assayed for ATPase activity immediately and the remainder stored at -10°C for various time intervals up to one month. The untreated suspension was assayed for ATPase activity immediately after suspension and after storage for 20 hours at -10°C.

	Suspension without deoxycholate		Suspension plus 0.1% deoxycholate	
	(Na^+,K^+)-ATPase	Mg^{++}-ATPase	(Na^+,K^+)-ATPase	Mg^{++}-ATPase
Initial activity	0.5	6.3	0.5	5.6
Storage at -10°C for 20hrs	0.3	6.1	3.0	6.0
" " " " 2 days			3.2	6.7
" " " " 7 days			2.9	5.9
" " " " 14 days			3.1	6.0
" " " " 28 days			2.9	5.8

(Results are the mean values of three experiments)

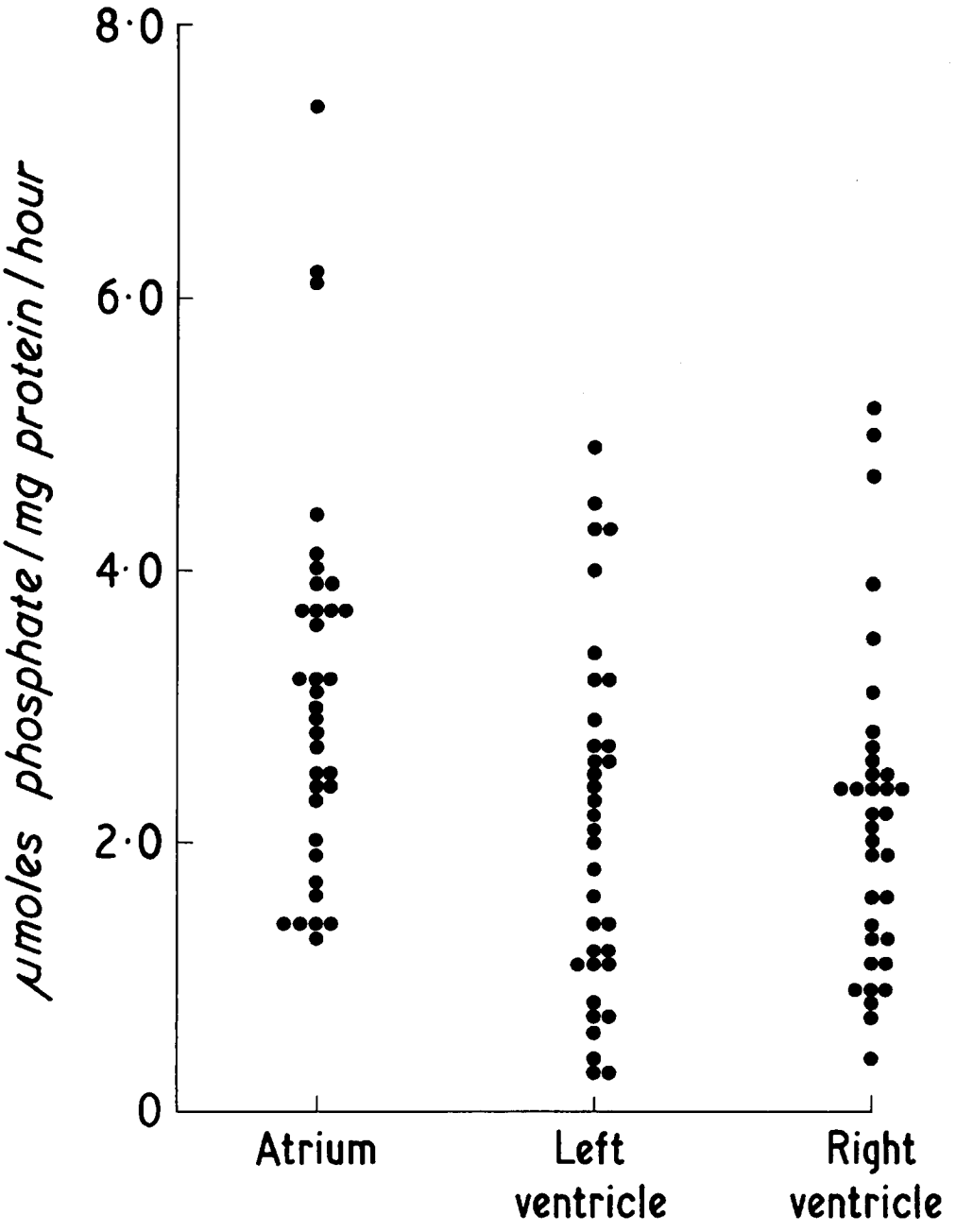


FIG. VI II The distribution of (Na^+, K^+) -ATPase activity in rat heart.

After the initial rapid increase in (Na^+, K^+) - ATPase activity after storage at -10°C for 20 hours there was little change in either (Na^+, K^+) - ATPase or Mg^{++} - ATPase activity for up to 1 month.

Distribution of (Na^+, K^+) - ATPase activity in the rat myocardium

The 2 atria from a single rat heart were pooled to give an atrial sample of sufficient weight to enable a microsomal fraction to be prepared. The ventricle was divided into left and right chambers. The interventricular septum was included in the left ventricular sample.

Fig. IV 11 shows the results of a series of experiments on 50 control animals to determine the (Na^+, K^+) - ATPase enzyme distribution. The difference in the (Na^+, K^+) - ATPase activity between left and right ventricle was not significant but the difference between atria and ventricles, analysed by a paired 't' test, was unlikely to be due to chance ($P < 0.01$). There was no significant difference between atria, left and right ventricle with regard to Mg^{++} - ATPase activity.

Effects of body weight on rat myocardial microsomal (Na^+, K^+) - ATPase and Mg^{++} - ATPase

Groups of control rats with body weights in the range 50 g to 300 g were examined for distribution of myocardial (Na^+, K^+) - ATPase and Mg^{++} - ATPase activity. No statistically significant differences were found in (Na^+, K^+) - ATPase

or Mg^{++} - ATPase levels in the atria, or ventricle.

		ATRIUM				VENTRICLE			
No. of animals	body weight range	(Na^+, K^+)-ATPase Mean	S.D.	Mg^{++}-ATPase Mean	S.D.	(Na^+, K^+)-ATPase Mean	S.D.	Mg^{++}-ATPase Mean	S.D.
32	45-55g	5.4	2.3	5.1	2.0	4.8	1.7	6.8	1.8
(tissues from 3 animals pooled to make 1 sample)									
7	150-165g	5.7	1.9	5.3	2.0	5.4	1.6	5.4	1.4
8	290-315g	7.4	2.6	7.3	1.4	6.8	1.6	6.8	1.2

Discussion

It appears that myocardial microsomal (Na^+, K^+) - ATPase has similar properties in all the animal species studied. The enzyme in fact would also appear to have very similar characteristics from all the various tissues studied (Skou, 1962).

Storage of dog myocardium at + 5°C led to a gradual increase in (Na^+, K^+) - ATPase and a fall in Mg^{++} - ATPase activity after 5 days. This would further support the suggestion made in Chapter V that autolysis has some effect on the membrane either unmasking enzyme activity or causing the decay of an inhibitor. Such a phenomenon has not been previously reported. Storage of microsomal

preparations at -10°C has no marked effect on either (Na^+, K^+) - ATPase or Mg^{++} - ATPase activity. Schwartz (1962) found that storage of cardiac preparations at -5°C caused a decrease in (Na^+, K^+) - ATPase but this was small compared to the Mg^{++} - ATPase which decreased at a faster rate. This effect is comparable to the storage of dog heart microsomal suspension at + 5°C for up to 3 weeks. During this time there was little effect on (Na^+, K^+) - ATPase but the Mg^{++} - ATPase activity almost disappeared. Auditore and Murray (1962) using rabbit ventricle also found that there was little change in the Na^+ plus K^+ stimulated ATPase but a very rapid decrease in Mg^{++} - ATPase when stored at 0°C for 10 days.

Freezing and thawing of microsomal suspensions led to an initial increase in (Na^+, K^+) - ATPase activity but the activity fell rapidly after about three freezings and thawings. The initial rise may possibly be due to disruption of the membranes which would expose more enzyme or alternatively lead to a destruction of the labile cardiac inhibitor protein. Repeated freezing and thawing however destroys the (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities possibly by further destruction of the membrane.

Freeze-drying the microsomal preparation did not destroy the ATPase activity. Indeed, after 1 year's storage of a freeze-dried preparation both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities had increased four fold.

It was apparent from these preliminary studies that conditions of storage were an important aspect of any proposed quantitative work or comparative studies. It was decided that in all such experiments microsomal suspensions would be kept at -10°C for 20 hours before assay. Activity of the ATPases reached a peak after this treatment and did not change during the next month. The specimens would be thawed for assay once only and stored in ice immediately prior to assay.

There have not been reported any studies on sheep myocardium microsomal (Na^+, K^+) - ATPase. It was, therefore, interesting to find that the (Na^+, K^+) - ATPase activity was high compared to rat and dog. Tosteson and Hoffman (1960) found that sheep of two distinct genetic types, one having red cells with a high potassium content and the other a low potassium content, had differing (Na^+, K^+) - ATPase activities in the red cells membranes. It was not possible, unfortunately, to check the genetic type from which the myocardium was obtained but the high activity may indicate the high intracellular K^+ type. The sensitivity to ouabain was higher than that found for human biopsy myocardium. The Ki value for ouabain was $10^{-7.5}$M.

Rabbit myocardial microsomal (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities were extremely low compared to all the other species investigated. Auditore and Murray (1962) reported high activities for both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities from rabbit ventricle although they do not specify the variety of rabbit used.

The reagents and preparative procedure were checked using a portion of dog myocardium and found to be satisfactory. It is possible that the New Zealand white rabbits chosen for the experiment have a lower myocardial microsomal ATPase level than other strains.

The pH optimum for rat myocardial (Na^+, K^+) - ATPase was 7.4. Schwartz (1962) found pH 7.0 to be optimal while Tashima et al. (1966) report 7.8. Ion requirements found in this study were 100 mM for sodium ions and 10 mM potassium ions. This is similar to that reported by Schwartz and Tashima et al. Low concentrations of ouabain have been reported by some authors to stimulate (Na^+, K^+) - ATPase activity (Repke, 1963 a; Brown, 1966 and Palmer et al., 1966) although the stimulation has not always been reproducible (Repke, 1963 a). In these experiments at no concentration, down to 10^{-14} M, did ouabain activate (Na^+, K^+) - ATPase activity. The Ki value for ouabain was $10^{-6.5}$ M in rat myocardium.

The measurement of (Na^+, K^+) - ATPase activity used in these studies is, strictly, a measure of a ouabain sensitive ATPase. However, in the assay system used both dog and rat preparations gave the same (Na^+, K^+) - ATPase activity value whether Mg^{++} - ATPase was measured by the omission of Na^+ and K^+ ions or whether Na^+ and K^+ ions were present plus 10^{-3}M ouabain. This was also found in the experiment on human myocardium, Chapter V.

A higher atrial activity compared to ventricular activity in rat heart would imply that there is a difference in the active transport of sodium and potassium ions in these regions. It, therefore, seems likely that the differences in distribution may be associated with differences in intracellular sodium and potassium levels and consequently the resting transmembrane potential. However, the resting trans-membrane potential of the atrial cell does not differ, significantly, from that of the ventricle (Hoffman et al., 1960). In the frog, turtle (Mazel and Holland, 1958) and ox (Davis et al., 1952) the intra-cellular concentration of sodium has been found to be higher in the atria than in the ventricles, while the intracellular concentration of potassium is higher in the ventricle than in the atria. In the dog (Hoffman and Cranefield, 1960 and Hoffman and Suckling, 1952) and turtle (Sano et al., 1956) the resting transmembrane potential has not, however, been found to be notably different between ventricular and atrial cells. The results of such admittedly few and diverse observations might imply that the cell membrane was more permeable and active transport of ions more active in the atria than in the ventricles. If, as seems probable (Skou, 1964), active transport of ions depends on the activity of microsomal (Na^{+}, K^{+}) - ATPase, such conclusions are consistent with the present observations.

The Mg^{++} - ATPase distribution was not significantly different in any of the chambers. There was a higher activity in atria compared to ventricles but it

was not statistically significant.

No significant difference in (Na^+, K^+) - ATPase activity was observed in rats of different body weight ranges from 50 g to 300 g. This would imply that with increasing age and deposition of new membranes there is an equal increase in the synthesis of (Na^+, K^+) - ATPase enzyme protein laid down in the cell membrane.

CHAPTER VII

THE EFFECTS OF FEEDING NaCl, KCl and DIGITALIS ON RAT MYOCARDIAL AND RENAL MICROSOMAL ATPases

Electrolytes play an important role in myocardial excitability, on respiratory function of mitochondria and on the contractile mechanisms (Leonard and Hadju, 1962). Sodium and potassium ions in addition to magnesium ions leads to a stimulation of microsomal ATPase activity in vitro. It was, therefore, of interest to investigate the effect of feeding sodium or potassium salts on myocardial (Na^+, K^+) - ATPase.

Disturbances in renal function accompany congestive heart failure. The renal defect is usually a failure to excrete sodium and secondarily, water. Although the failure to excrete sodium is in some part due to reduced renal blood flow and glomerular filtration rate, it is also related to other factors including elevated aldosterone levels. It was considered pertinent to investigate renal (Na^+, K^+) - ATPase activity as well as myocardial (Na^+, K^+) - ATPase in the NaCl, KCl and digitalis feeding studies.

Although much work has been reported on the in vitro effects of digitalis on (Na^+, K^+) - ATPase activity it has not been studied in vivo. The in vivo effects of digitalis treatment on rat myocardial and renal (Na^+, K^+) - ATPase have therefore been investigated. The rat was chosen because it was the only species available in the sufficiently large numbers required for in vivo studies of this nature. Unfortunately,

the rat is much less susceptible to digitalis than many other species. Therefore large doses were given, about twenty times the normal human therapeutic dose.

Materials and Methods

Animals

The animals used in these and subsequent studies reported in this thesis were Sprague-Dawley male albino rats supplied and kept at The London Hospital Medical College. The rats were kept on a MRC diet 41 B and tap water was given ad libitum throughout the experiment. The rats in the feeding studies reported in this chapter had a body weight in the range 140 to 150 g at the start of the experiment.

Dosing

The rats were dosed with a solution of the compounds or, in the case of digitalis, a fine suspension in water, by gastric intubation using a size 0 needle with a bulbous end.

The rats were killed by a sharp blow to the head. The heart and kidneys were quickly dissected. The heart was divided into atria, left ventricle and right ventricle. The interventricular septum was included in the left ventricle sample. After blotting the samples free of blood on a filter paper they were weighed. The tissues were then placed in glass bottles surrounded by ice in a large thermos container for transport to the laboratory.

TABLE VII 1 THE EFFECTS OF FEEDING WITH DIGOXIN ON CARDIAC AND RENAL MICROSOMAL ATPase ACTIVITY

(Activity expressed as μ moles Pi / mg protein / hour)

Site	Weeks of treatment	Number of observations	(Na^+, K^+) - ATPase		Mg^{++} - ATPase	
			Mean	Standard Deviation	Mean	Standard Deviation
ATRIUM	0	15	2.3	1.0	5.5	1.9
	1	8	5.3*	1.1	9.9*	2.1
	2	10	3.2	1.6	8.9*	2.5
	3	7	2.3	1.0	7.1	1.0
	4	8	2.1	0.9	6.3	2.4
RIGHT VENTRICLE	0	21	2.1	0.7	4.5	2.0
	1	8	3.9*	1.7	8.9*	1.5
	2	10	2.8	1.0	4.5	1.5
	3	7	2.5	0.8	3.0	1.5
	4	8	2.3	1.0	4.9	1.3
LEFT VENTRICLE	0	21	1.8	0.7	5.5	2.4
	1	8	1.8	0.6	9.7*	1.9
	2	10	2.1	0.5	5.4	1.2
	3	7	1.7	0.4	5.0	1.6
	4	8	2.1	1.3	5.0	1.5
KIDNEY	0	7	5.2	2.1	6.5	1.8
	1	7	0.9*	0.4	9.3	2.1
	2	10	0.8*	0.4	6.1	0.7
	3	7	0.6*	0.3	6.3	1.0
	4	7	0.5*	0.8	5.6	0.5

* $P < 0.01$ (compared to week 0)

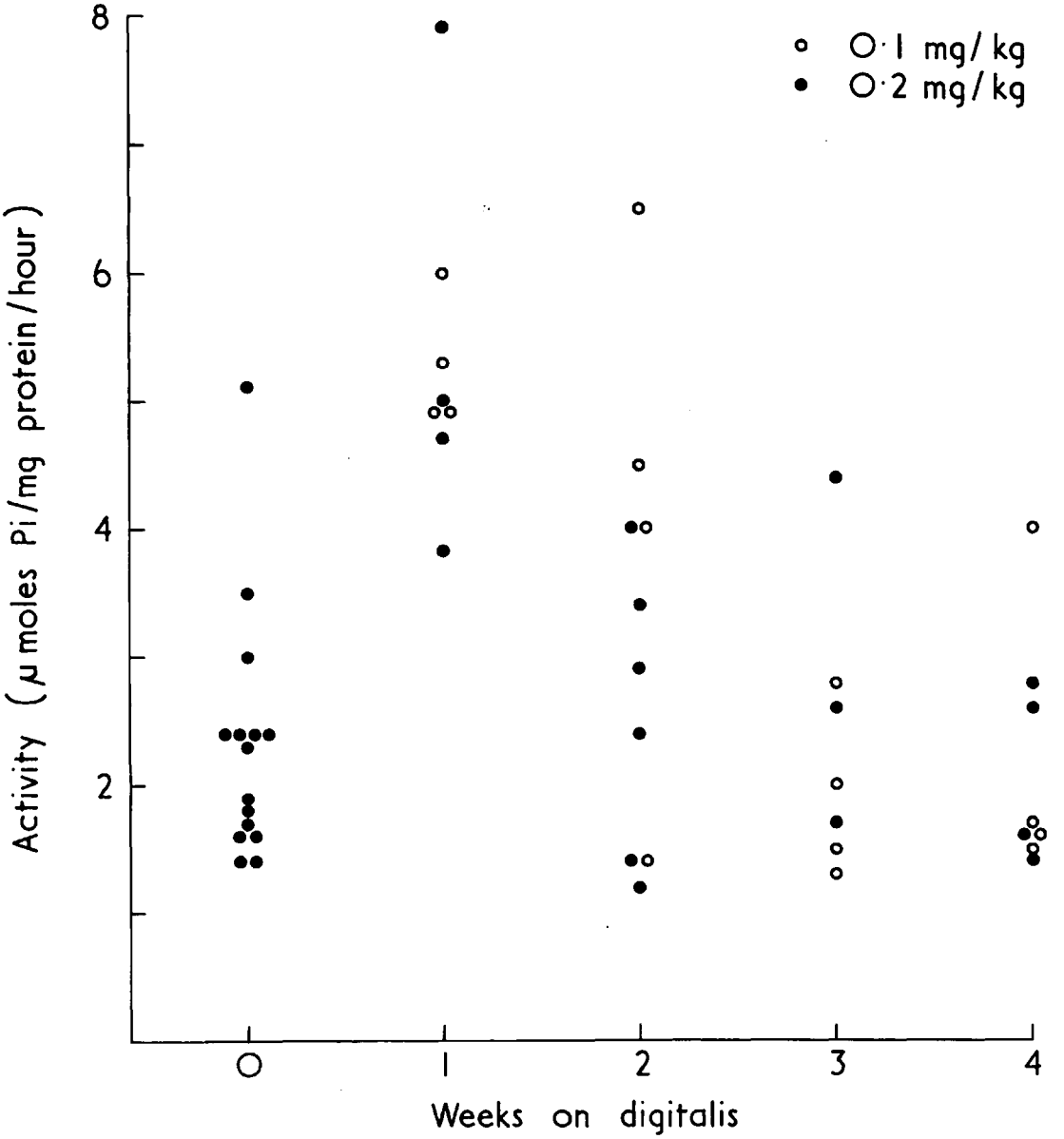


FIG. VII 1 The effects of digitalis feeding on microsomal (Na^+, K^+) - ATPase activity in rat atrium

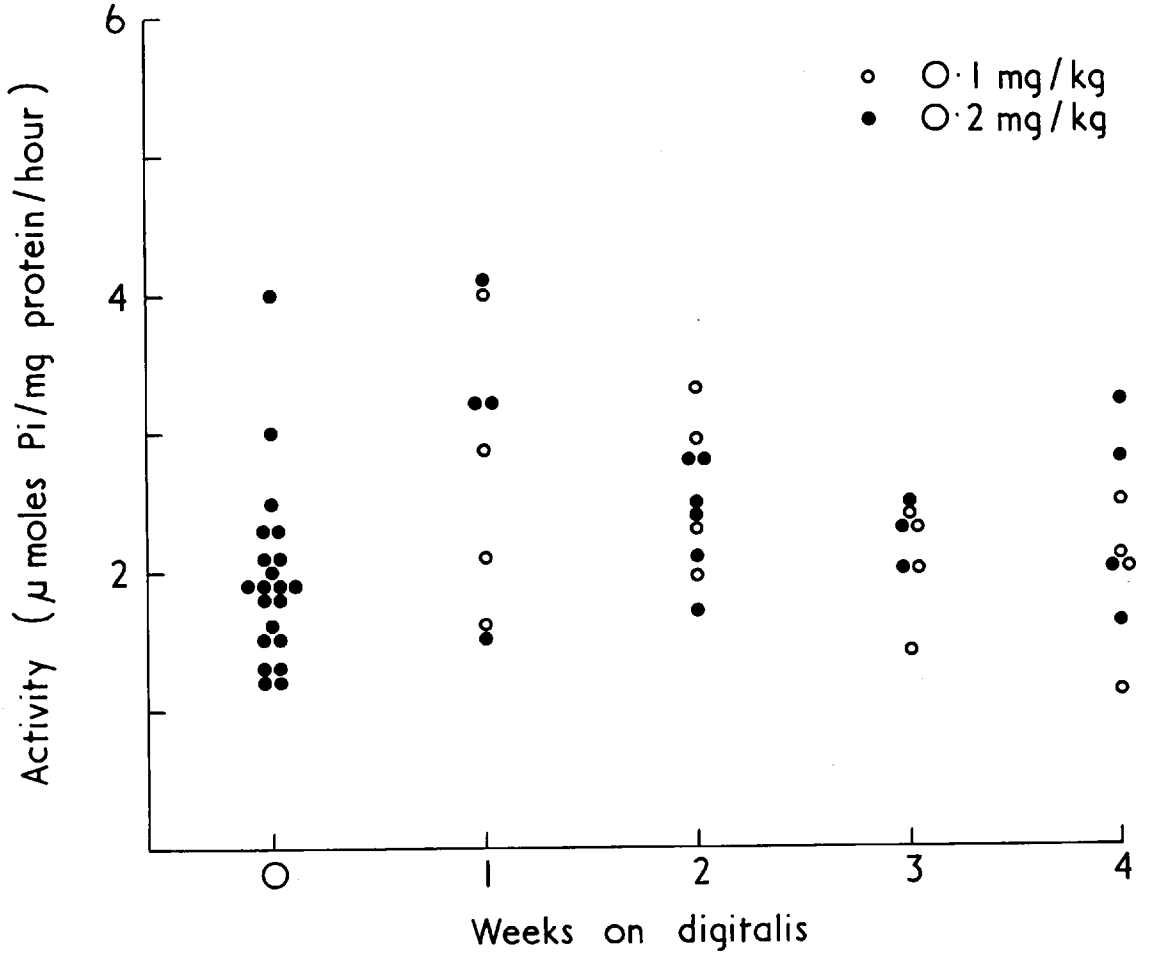


FIG. VII 2 The effects of digitalis feeding on microsomal (Na^+, K^+) - ATPase activity in rat ventricle.

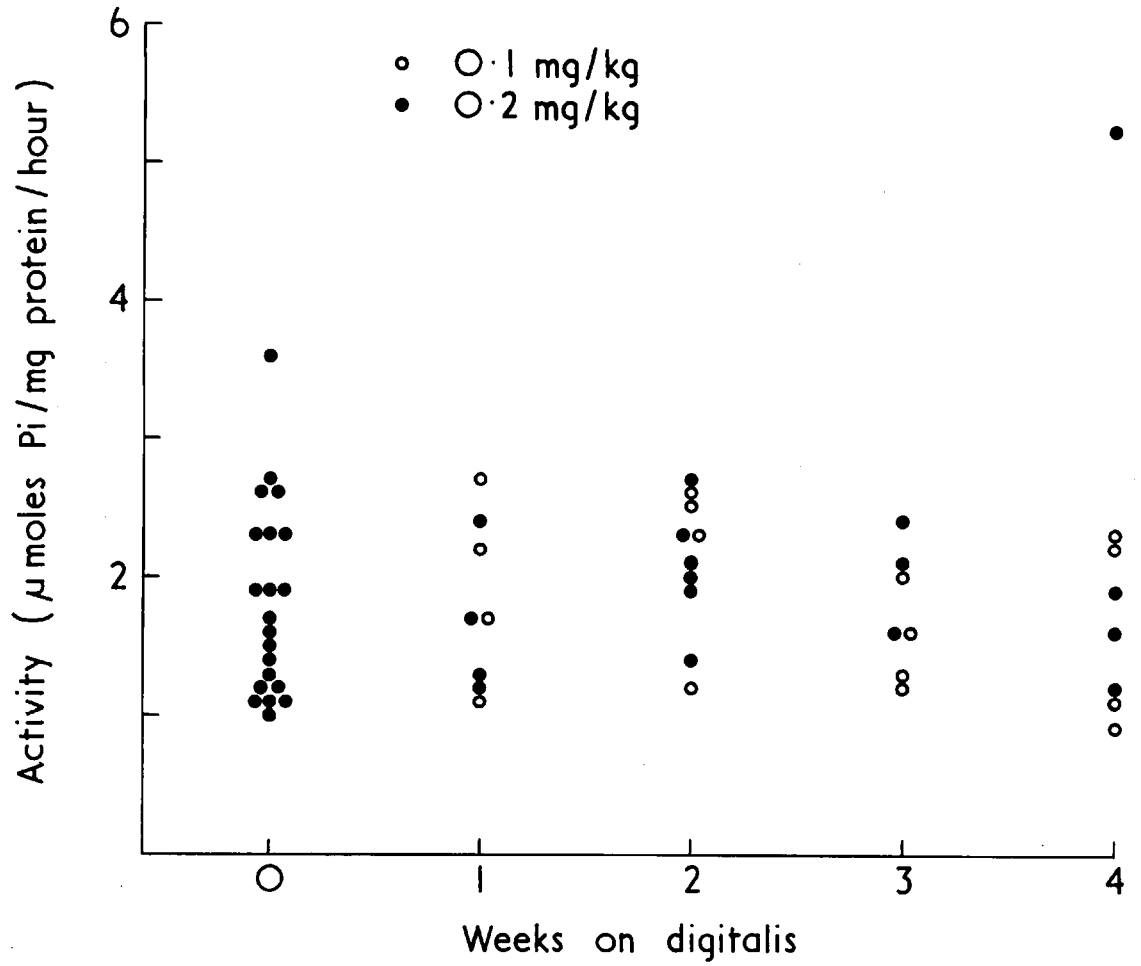


FIG. VII 3 The effects of digitalis feeding on microsomal (Na^+, K^+) - ATPase activity in rat left ventricle

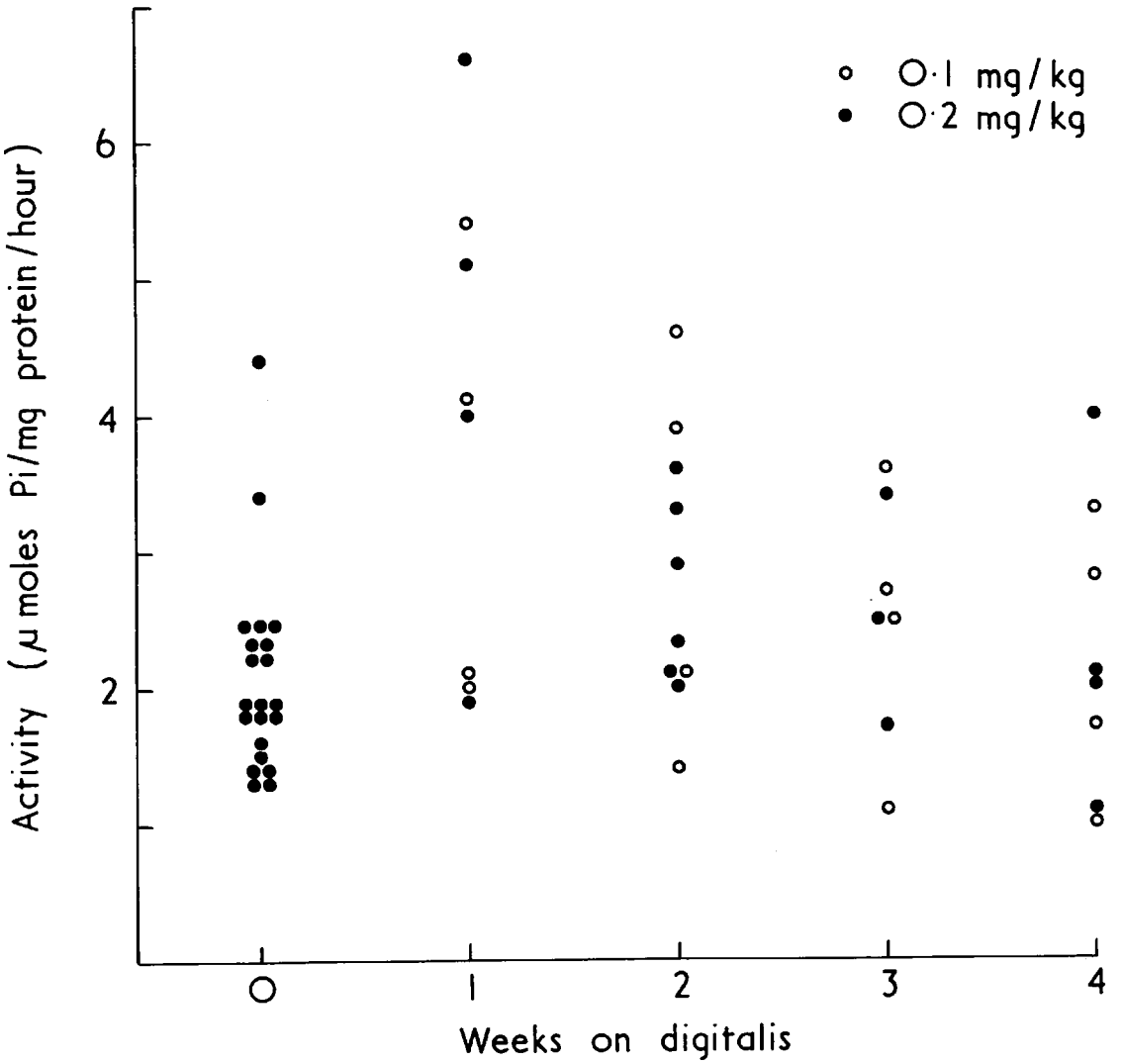


FIG. VII 4 The effects of digitalis feeding on microsomal (Na^+, K^+) - ATPase in rat right ventricle.

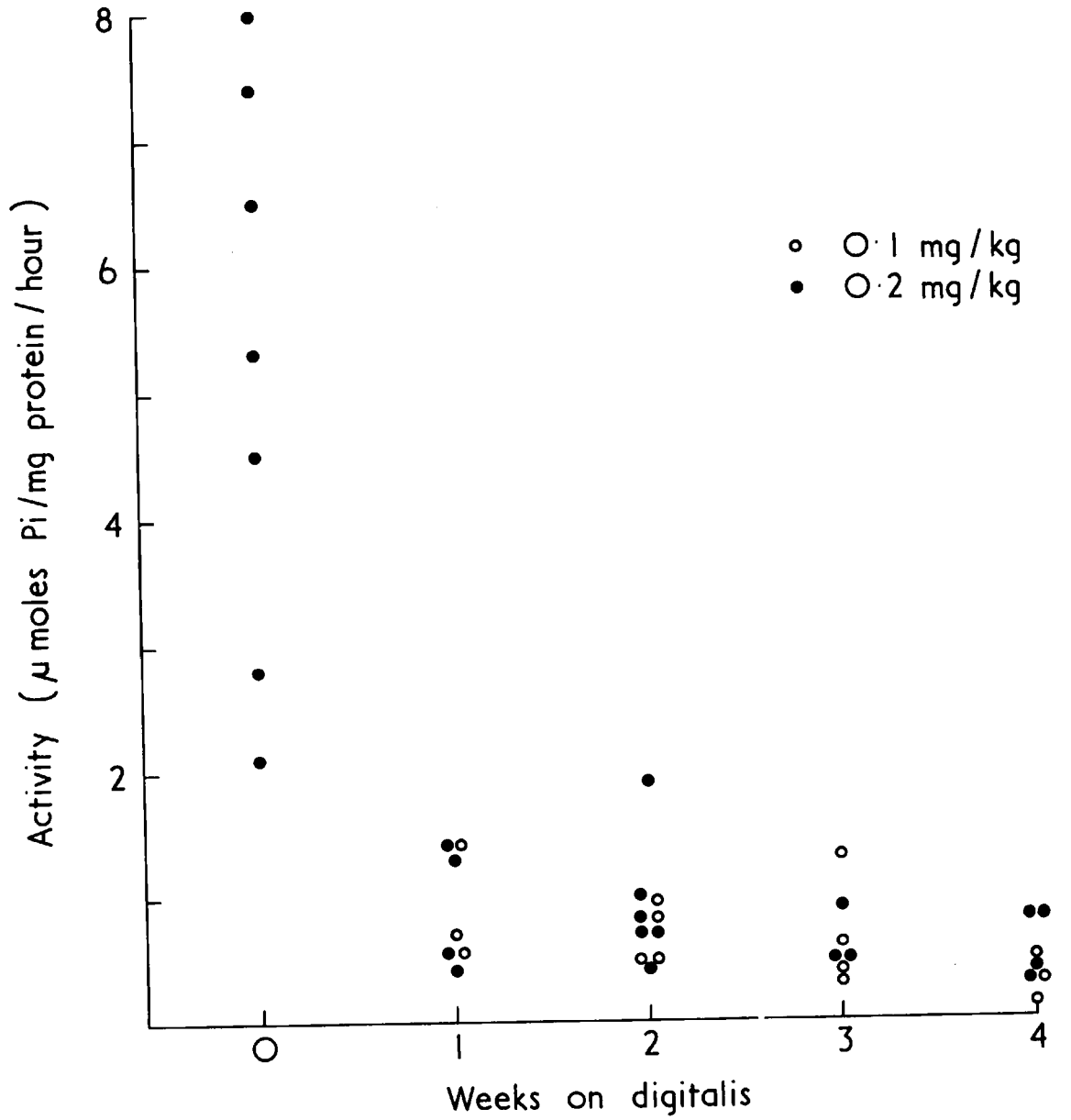


FIG. VII 5 The effects of digitalis feeding on microsomal (Na^+, K^+) - ATPase activity in rat kidney

Preparation of samples and assay

Because of the large differences in enzyme activity observed under various keeping and preparative conditions great care was taken to standardize all the procedures.

The animals were killed at 9.30 a.m. each morning and the tissues processed the same day using the same homogenizer and centrifugation conditions throughout. The microsomal samples were suspended in sucrose, versene tris solution containing 0.1% deoxycholate to give a final protein concentration of 0.8 mg / ml. The suspensions were then stored in the refrigerator at - 10°C. The assays of (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities were carried out the following afternoon, 20 hours after storage of the microsomal suspensions.

Results

Digitalis feeding experiment

Two dose levels were used, 0.2 mg digoxin / kg body weight / day and 0.1 mg digoxin / kg body weight / day. Groups of rats were killed at intervals of one, two, three and four weeks after commencing treatment with digoxin. The results of the ATPase activities are shown in table VII 1. Figs VII 1, 2, 3, 4 and 5 show the (Na^+, K^+) - ATPase activities plotted for each of the tissue samples. Since there was no difference in the effects of the two dose levels of digoxin the results

TABLE VII 2

THE EFFECTS OF SODIUM AND POTASSIUM FEEDING ON ATRIAL VENTRICULAR AND RENAL MICROSOMAL ATPase IN THE RAT (Activity expressed as μ moles Pi/mg protein/hour)

		(Na^+, K^+) - ATPase		Mg^{++} - ATPase	
	Number of Observations	Mean	Standard Deviation	Mean	Standard Deviation
CONTROL					
Atrium	15	2.3	1.0	3.5	1.5
Ventricle	21	2.0	0.6	3.2	0.8
Kidney	7	5.2	2.1	10.1	4.5
POTASSIUM FEEDING					
Atrium	10	4.3*	1.7	4.3	3.2
Ventricle	11	3.3*	1.3	4.7*	1 7
Kidney	11	5.1	1.9	7.8	1.3
SODIUM FEEDING					
Atrium	10	2.0	0.5	2.6	0.6
Ventricle	12	2.1	0.4	3.8	1.3
Kidney	12	6.7	2.2	10.4	3.6

* $P < 0.01$

of both test groups have been pooled. The increase in (Na^+, K^+) - ATPase activity in the atrium from 2.3 to 5.3 μ moles Pi / mg protein / hr and in the right ventricle from 2.1 to 3.9 μ moles Pi / mg protein / hr after the first week of treatment is statistically significant. The decrease in renal (Na^+, K^+) - ATPase activity from 5.2 to 0.9 μ moles Pi / mg protein / hr after the first week and the decreased activity during the subsequent weeks was statistically significant.

There was a significant increase in Mg^{++} - ATPase activity in atrial samples after one week slowly decreasing during the remainder of the experiment. The Mg^{++} - ATPase activity after two weeks was still significantly higher than the control group. In both the left and right ventricle there was a significantly higher Mg^{++} - ATPase activity after the first week of treatment. The remaining weeks were not significantly different from the control group. Mg^{++} - ATPase activity in the kidney was not significantly altered throughout the experiment.

Sodium and Potassium Chloride Feeding

One group of rats was treated with 3 g KCl / Kg body weight / day for two weeks and another group treated with 3 g NaCl / Kg body weight / day for two weeks. The results of both KCl and NaCl treatments on myocardial and renal (Na^+, K^+) - ATPase and Mg^{++} - ATPase are tabulated in Table VII 2.

After KCl feeding the (Na^+, K^+) - ATPase activity was significantly increased

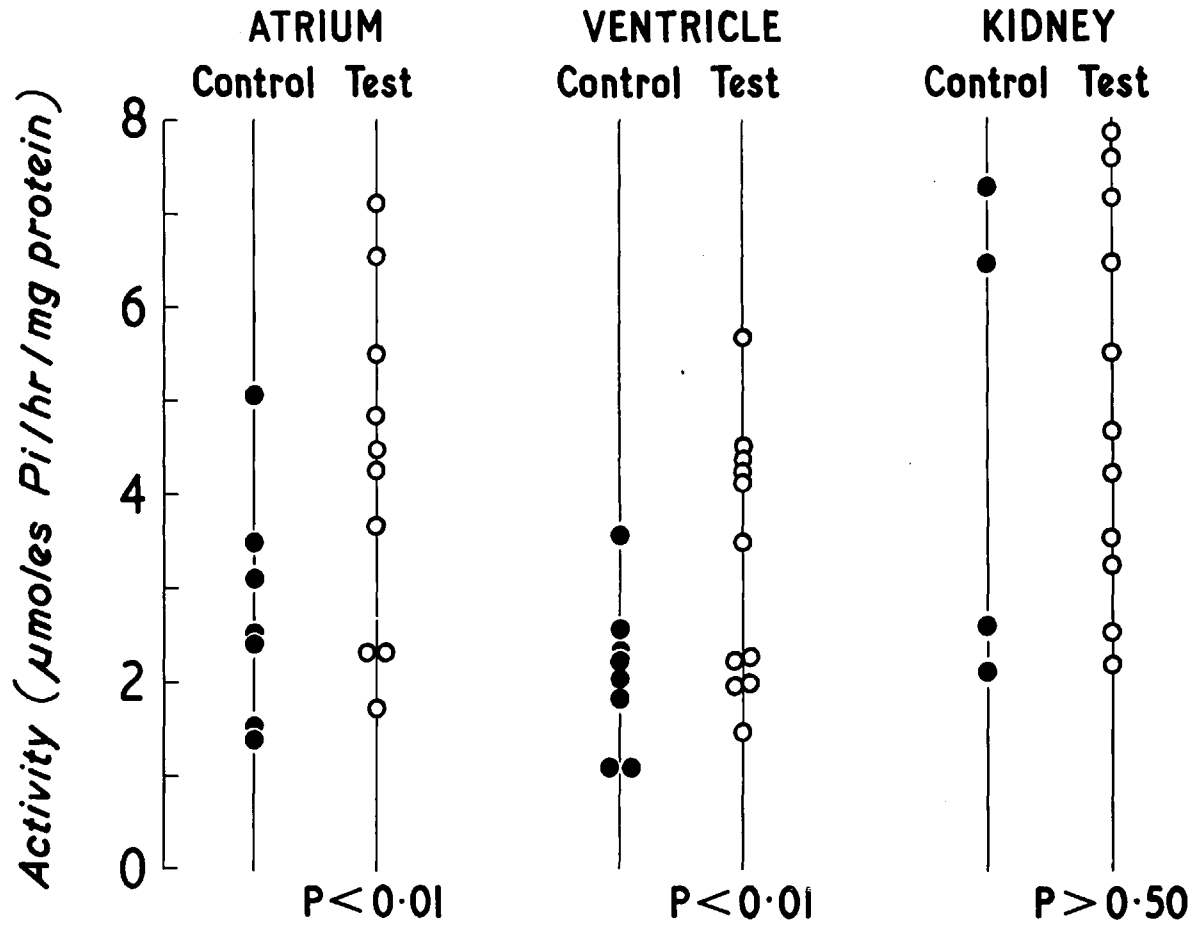


FIG. VII 6 The effects of feeding rats potassium chloride (3 g/Kg body-weight/day for 2 weeks).

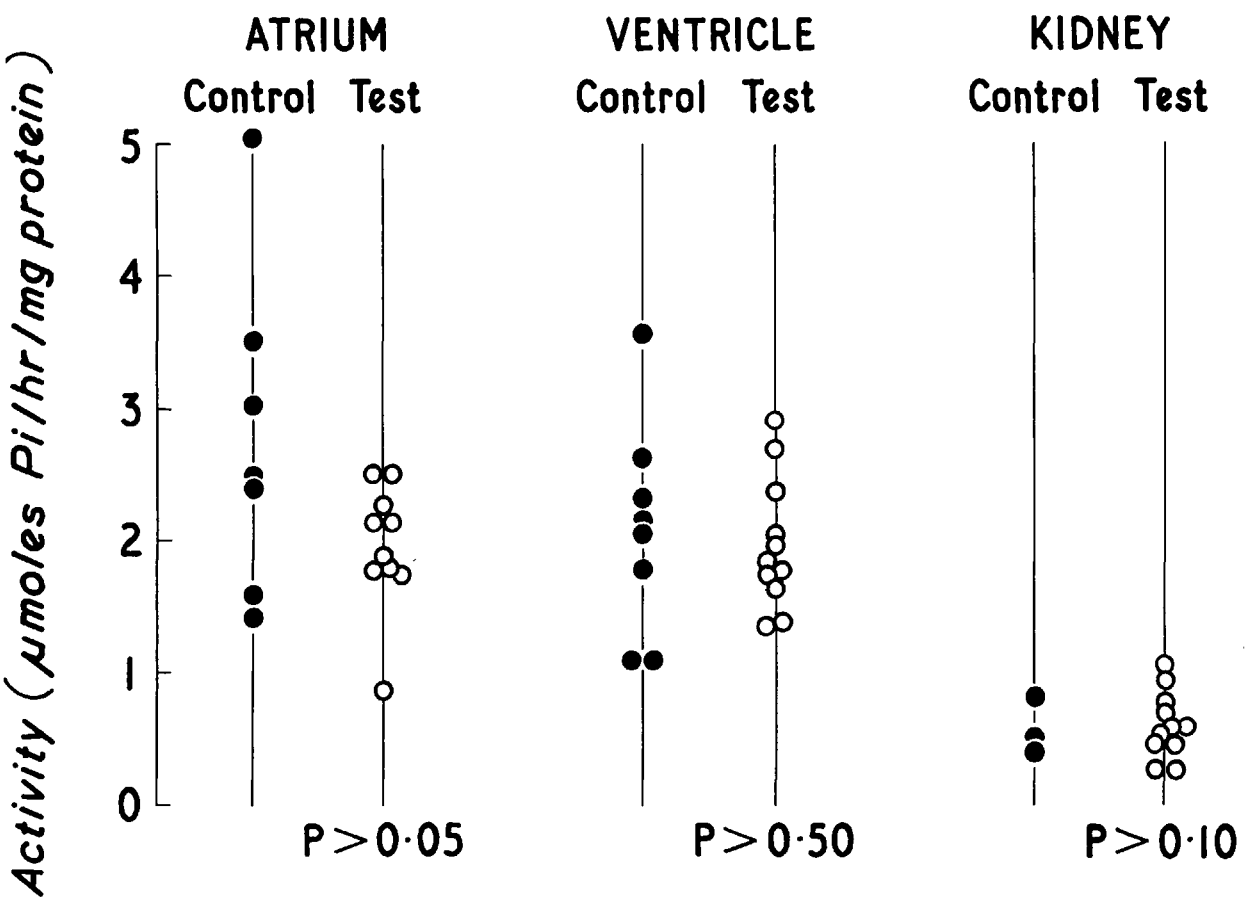


FIG. VII 7 The effects of feeding rats sodium chloride (3 g/Kg body-weight/day for 2 weeks).

in the atrium and ventricle but not in the kidney, Fig. VII 6.

Feeding sodium chloride did not significantly alter the (Na^+, K^+) - ATPase activity of the myocardium or renal tissues. However, a trend towards a decreased activity is noted, Fig. VII 7.

The only significant change in Mg^{++} - ATPase activity was found in the ventricles of rats treated with potassium chloride. There was a significant increase in Mg^{++} - ATPase activity in these tissues.

Discussion

The transient stimulating effect of digoxin on atrial and right ventricular (Na^+, K^+) - ATPase activity after one week was surprising in view of the inhibitory action digoxin has on (Na^+, K^+) - ATPase in vitro. There have been several reports that therapeutic concentrations of ouabain stimulate (Na^+, K^+) - ATPase activity in vitro (Brown, 1966; Repke, 1963 a and Palmer et al., 1966). These results have not always been consistent (Repke, 1963 b). In the present study ouabain failed to stimulate (Na^+, K^+) - ATPase activity in rat or human myocardial microsomal preparations, Chapter V page 147 and Chapter V page 100. It is possible that the transient stimulation observed in these experiments represents the therapeutic action of digitalis, while the continued administration of relatively high doses caused a toxic effect which reversed the stimulatory properties. The

inhibitory action of digitalis glycoside on (Na^+, K^+) - ATPase has been used as the basis for a hypothesis regarding its therapeutic action (Lee, 1967). However, a stimulatory effect would seem to be more directly in keeping with the therapeutic action produced by digitalis.

The decrease in renal (Na^+, K^+) - ATPase after feeding digoxin may be due to glycoside bound to the microsomal membranes. However, this would not be supported by the myocardial (Na^+, K^+) - ATPase results which show an activation of enzyme activity after one week. Digoxin has been reported to have a diuretic action which is independent of increased renal blood flow. Cardiac glycosides appear to reduce renal tubular reabsorption of sodium in the kidney (Farher et al., 1951; Hayman et al., 1956; Schatzman et al., 1958; Wilde and Howard, 1960; Stickler and Kessler, 1961; Vogel et al., 1963 and White and Rolf, 1963), which could be related to the inhibition of the renal (Na^+, K^+) - ATPase activity. Another possibility may be that digitalis glycosides effect the rate of synthesis of (Na^+, K^+) - ATPase enzyme protein secondary to the inhibitory action of the glycoside. Renal ion transport is a complex process each region of the kidney having its own special properties.

The relationship, if any, between (Na^+, K^+) - ATPase and Mg^{++} - ATPase is obscure and, as discussed earlier, may possibly involve two distinct enzyme systems. There has also been the suggestion that Mg^{++} - ATPase is derived from

(Na^+, K^+) - ATPase (Skou, 1964).

Both (Na^+, K^+) - ATPase and Mg^{++} - ATPase activities in atria and right ventricle were increased after 1 week of digoxin treatment. The Mg^{++} - ATPase activity only was increased in the left ventricle after 1 week of digoxin treatment. In contrast the Mg^{++} - ATPase activity of kidney was not affected by digoxin treatment while (Na^+, K^+) - ATPase activity was significantly reduced.

Potassium salts are used in the treatment of arrythmias in man and the electrophysiological action at the cellular level has been studied (Surawig, 1965). It is expected that feeding potassium in large doses would lead to an increased serum potassium level. The potassium activated site of (Na^+, K^+) - ATPase enzyme system is orientated to the outside of the membrane and would therefore be stimulated by an increased extracellular potassium. An increased (Na^+, K^+) - ATPase activity after potassium feeding would be expected because of stimulation by extracellular potassium at the potassium activated site of the (Na^+, K^+) - ATPase enzyme. The increase in (Na^+, K^+) - ATPase activity would suggest that there had been an increase in the synthesis of (Na^+, K^+) - ATPase enzyme protein in response to such a prolonged stimulus. Feeding sodium chloride caused a trend towards decreased (Na^+, K^+) - ATPase activity but this was not statistically significant.

The significance of the increased Mg^{++} - ATPase activity in the ventricle

of rats fed with potassium is obscure.

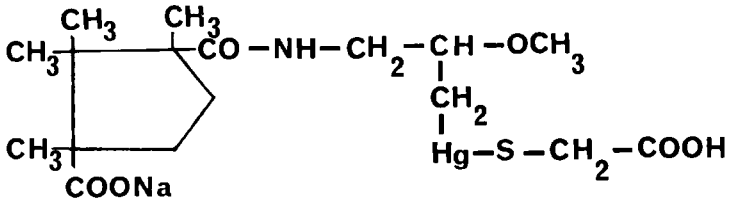
CHAPTER VIII

DIURETICS — IN VIVO AND IN VITRO

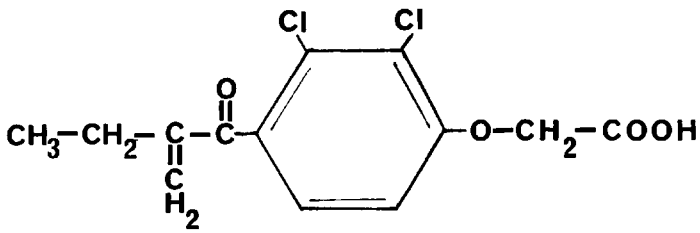
Diuretics are used clinically in the treatment of congestive heart failure. They decrease sodium reabsorption in the proximal tubular region of the kidney. This leads to an increase in the excretion of sodium which is accompanied by an excretion of water and hence diuresis. The renal effects of diuretics are the most notable. For example a 5% change in sodium pumping activity in muscle is barely detectable but a similar change in renal sodium reabsorption has profound physiological effects (Ruch and Patton, 1965). It has been found that in patients with congestive cardiac failure there is a decrease in the intracellular concentration of potassium ions and an increase in the intracellular concentration of sodium ions throughout muscle tissue (Flear, 1966) and probably most other tissues.

It was therefore of interest to study the effects of a range of diurectis on myocardial and renal (Na^+, K^+) - ATPase both in vivo using rats and in vitro using rat and human myocardial microsomal preparations. The diuretics studied were: - mercaptomerin, ethacrynic acid, chlorothiazide, hydrochlorothiazide and frusemide. Fig. VIII 1, shows the structural formulae of these compounds.

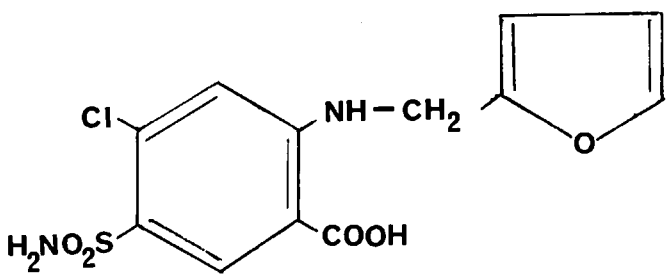
Hook and Williams, (1965) reported an inhibition of potassium ion stimulated renal (Na^+, K^+) - ATPase activity in rats after a single dose of ethacrynic acid or frusemide. Ethacrynic acid also inhibits renal (Na^+, K^+) - ATPase in vitro



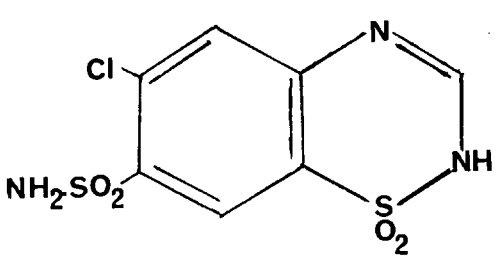
MERCAPTOMERIN



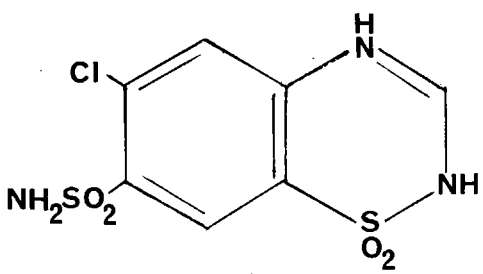
ETHACRYNIC ACID



FRUSEMIDE



CHLOROTHIAZIDE



HYDROCHLOROTHIAZIDE

FIG. VIII I The structural formulae of the diuretics studied.

(Duggan and Noll, 1965). It is interesting to note however that ethacrynic acid is not a diuretic in rats (Beyer et al., 1965) but frusemide does have a diuretic action on rats (Timmerman, 1964).

Jones et al., (1965) found that mercurial diuretics caused a decrease in renal (Na^+, K^+) - ATPase activity. In vitro studies with mercurial diuretics have been reported by a number of authors (Landon and Norris, 1963; Jones et al., 1965 and Taylor, 1963) who found inhibition of renal microsomal (Na^+, K^+) - ATPase. Pitts (1959) suggests that mercurial diuretics function by depressing ion transport by forming inactive mercaptide complexes with sulphydryl enzymes which supply energy to drive the reabsorptive machinery. Thiol groups are an essential structural part of the (Na^+, K^+) - ATPase enzyme system (Skou and Hilberg, 1965) which would support such a mechanism. Cysteine has been reported to be required for full ATPase enzyme activity (Caffery et al., 1956).

Nechay et al., (1967) have questioned the relevance of the in vitro inhibition of (Na^+, K^+) - ATPase by diuretics. They found that at maximal diuresis due to ethacrynic acid, 1000 to 2000 times less drug was bound to renal microsomes than the binding required for 50% inhibition in vitro. While it may be true that in vivo local concentrations of drugs at the cell membrane are relatively high it is doubtful whether they reach the high concentrations found to inhibit (Na^+, K^+) - ATPase in vitro.

Materials and Methods

Animals

The strain of rats, keeping and dosing methods used in these experiments are described in the previous chapter.

In vivo studies

Male rats weighing between 150 - 250 g at the start of the experiment were used. The doses of diuretics given to the various groups of rats were: -

mercaptomerin	1.25 mg / 100 g body weight / day,
ethacrynic acid	2.0 mg / " " " " " " ,
hydrochlorothiazide	3.0 mg / " " " " " " ,
frusemide	1.0 mg / " " " " " " .

Preparations of microsomal fractions from rat and human tissues have also been described previously in Chapter II.

Assay conditions

In all the experiments, with the exception of the in vivo studies of frusemide, the assay system did not contain an ATP regenerating system but reactions were stopped after 30 minutes' incubation at 37°C. In the in vivo experiment with frusemide an ATP regenerating system was used and the incubation time extended to 45 minutes. Separate controls were used in this experiment.

TABLE VIII 1

THE EFFECTS OF FEEDING RATS WITH FRUSEMIDE ON CARDIAC AND RENAL MICROSOMAL ATPase ACTIVITY EXPRESSED AS μ MOLES Pi / mg Protein / hour

		(Na^+, K^+) - ATPase		Mg^{++} - ATPase	
	Number of Observations	Mean	Standard Deviation	Mean	Standard Deviation
CONTROL					
Atrium	11	6.6	2.5	6.6	1.7
Ventricle	11	5.0	2.5	6.0	1.7
FRUSEMIDE (1 mg/100 g rat/day)					
Atrium	12	6.3	2.1	6.9	2.5
Ventricle	12	3.8	1.7	5.6	1.5

TABLE VIII 2

THE EFFECTS OF FEEDING RATS WITH MERCAPTOMERIN, ETHACRYNIC ACID AND HYDROCHLOROTHIAZIDE ON CARDIAC AND RENAL MICROSOMAL ATPase

Activity expressed as μ moles Pi / mg Protein / hr. (Assay system included PEP / PK)

	Number of Observations	(Na^+, K^+) - ATPase		Mg^{++} - ATPase	
		Mean	Standard Deviation	Mean	Standard Deviation
CONTROL					
Atrium	20	7.1	4.0	8.6	4.5
Ventricle	20	5.7	2.0	9.6	6.6
Kidney	20	24.3	5.4	16.4	5.4
MERCAPTOMERIN (1.25 mg/100 g rat/day)					
Atrium	19	10.3	8.5	10.0	6.0
Ventricle	19	7.3	2.9	8.9	2.9
Kidney	19	22.1	4.5	13.0	2.3
ETHACRYNIC ACID (2 mg/100 g rat/day)					
Atrium	14	9.1	5.1	13.1	7.6
Ventricle	15	5.1	2.2	16.8*	7.1
Kidney	15	30.8	6.0	22.5*	4.6
HYDROCHLOROTHIAZIDE (3 mg/100 g rat/day)					
Atrium	15	9.0	3.0	8.6	3.8
Ventricle	15	6.6	2.5	9.3	2.3
Kidney	15	24.8	5.6	14.7	2.0

*$P < 0.01$ compared to controls

Results

In vivo effects of diuretics on myocardial and renal Mg^{++} - ATPase and (Na^+, K^+) - ATPase activity

Tables VIII 1 and VIII 2 summarize the results of these experiments. There was no significant difference between the control and diuretic treated animals in either the myocardial or renal (Na^+, K^+) - ATPase activity. Rats treated with ethacrynic acid had a significantly increased Mg^{++} - ATPase activity in the ventricle and kidney compared to the control animals. The Mg^{++} - ATPase activity was not significantly altered in the other groups.

In vitro studies

Neither the pH of the reaction mixtures nor the inorganic phosphate assay procedure was affected by any of the diuretics studied.

Rat myocardial (Na^+, K^+) - ATPase

All the diuretics inhibited the (Na^+, K^+) - ATPase activity. Figure VIII 2 shows the effects of adding various concentrations of ethacrynic acid and is typical of the type of curve obtained for the other diuretics. The Ki values together with the concentration of the diuretics at the start and at complete inhibition are summarized in Table VIII 3. Mercaptomerin was the most powerful inhibitor followed by ethacrynic acid. Frusemide and the thiazides inhibited

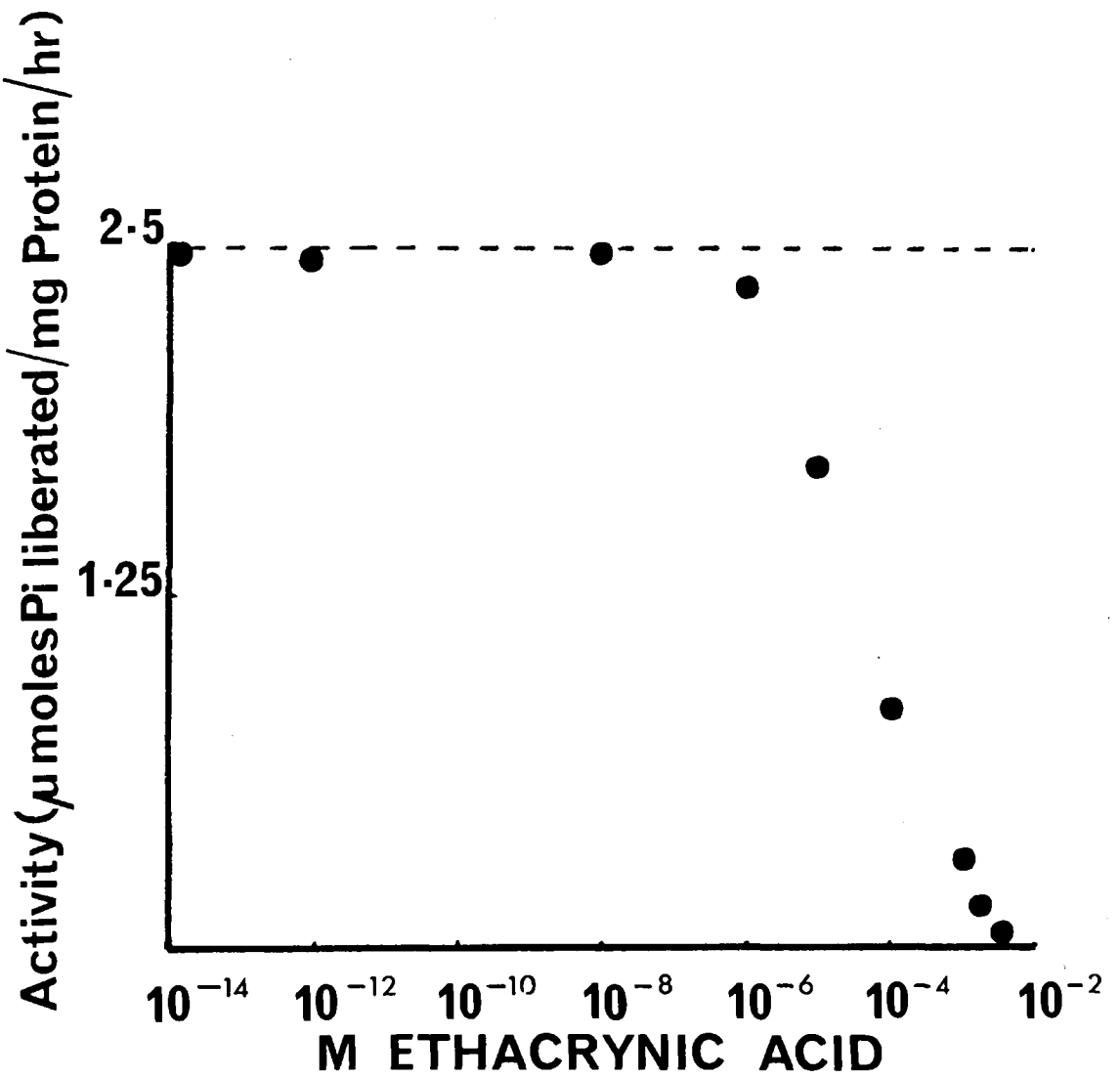


FIG. VIII 2 The effects of various concentrations of ethacrynic acid on rat myocardial microsomal (Na^+, K^+) -ATPase activity. The broken line represents the control activity.

TABLE VIII 3

INHIBITORY EFFECTS OF VARIOUS DIURETICS ON CARDIAC MICROSOMAL (Na^+, K^+) - ATPase ACTIVITY IN VITRO (Molar concentrations)

	RAT PREPARATION			HUMAN PREPARATION		
	Start of inhibition	Ki	Complete inhibition	Start of inhibition	Ki	Complete inhibition
Mercaptomerin	10^{-6}	2.5×10^{-5}	10^{-2}	10^{-9}	5.0×10^{-6}	10^{-2}
Ethacrynic acid	10^{-6}	5.0×10^{-4}	10^{-2}	10^{-6}	1.5×10^{-4}	10^{-2}
Chlorothiazide	10^{-3}	1.0×10^{-3}	10^{-2}	10^{-3}	4.5×10^{-3}	10^{-2}
Hydrochlorothiazide	10^{-3}	2.0×10^{-3}	10^{-2}	10^{-3}	5.0×10^{-3}	10^{-2}
Frusemide	10^{-3}	7.0×10^{-3}	10^{-2}	10^{-4}	1.0×10^{-3}	10^{-2}

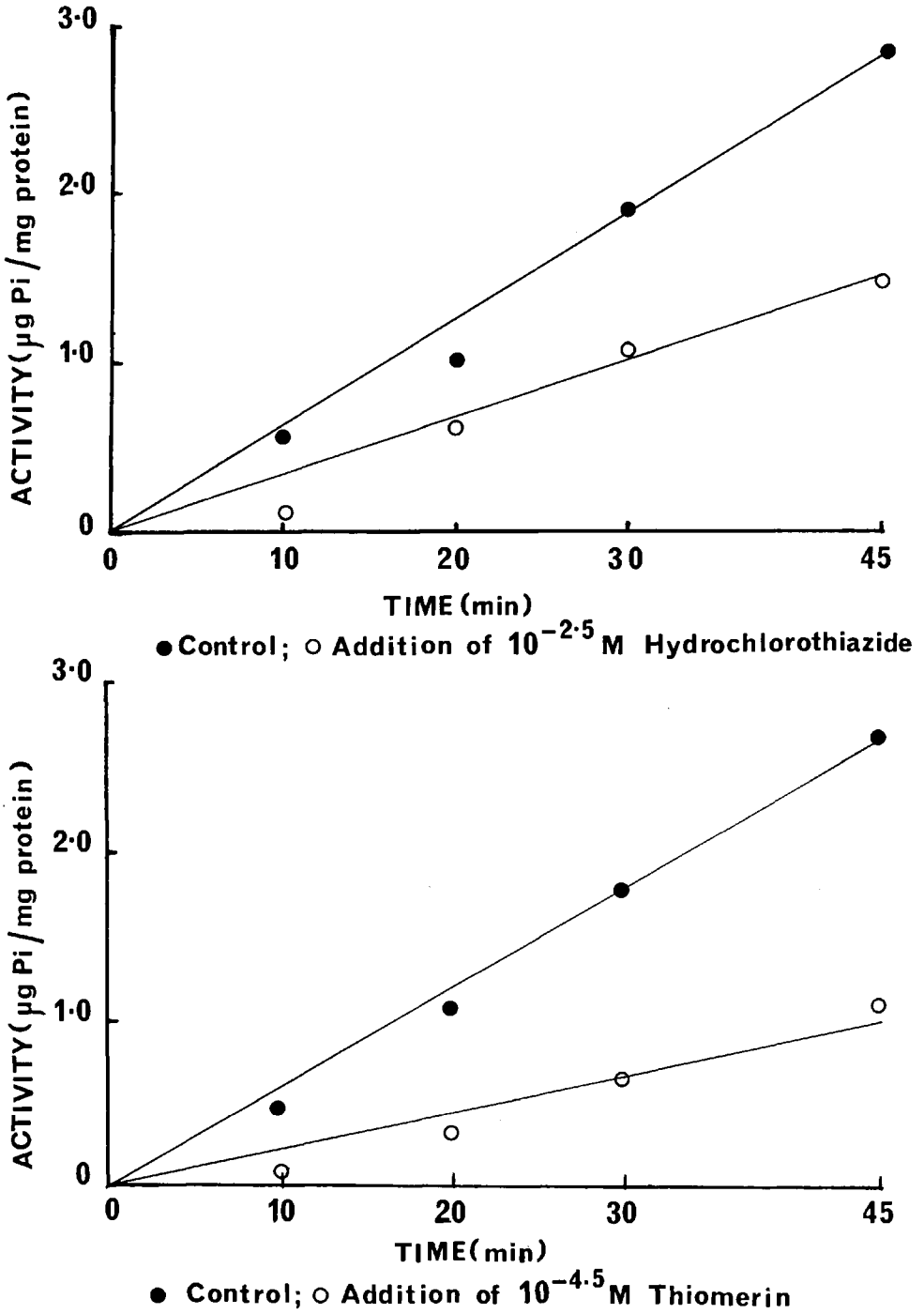


FIG. VIII 3 The effects of diuretics on the time/activity relationship of rat myocardial microsomal (Na^+, K^+) -ATPase.

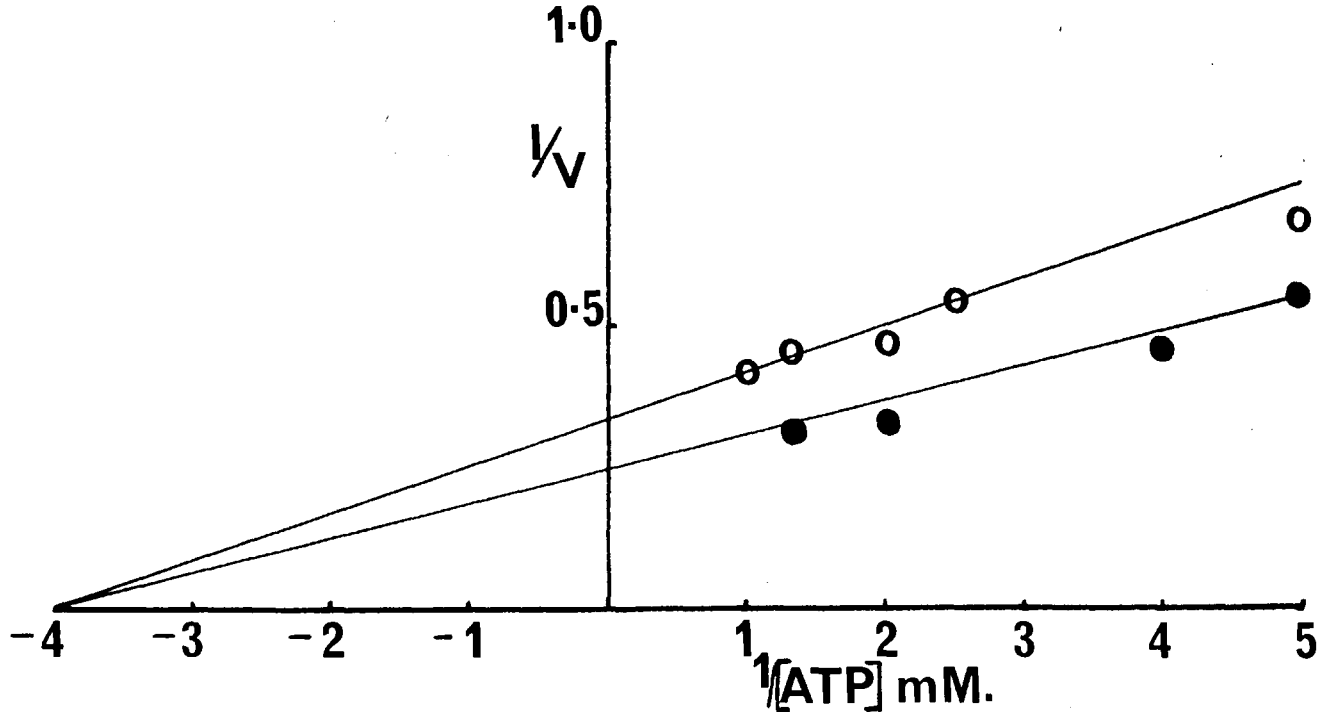


FIG. VIII 4 Enzyme obtained from a rat myocardial microsomal preparation.

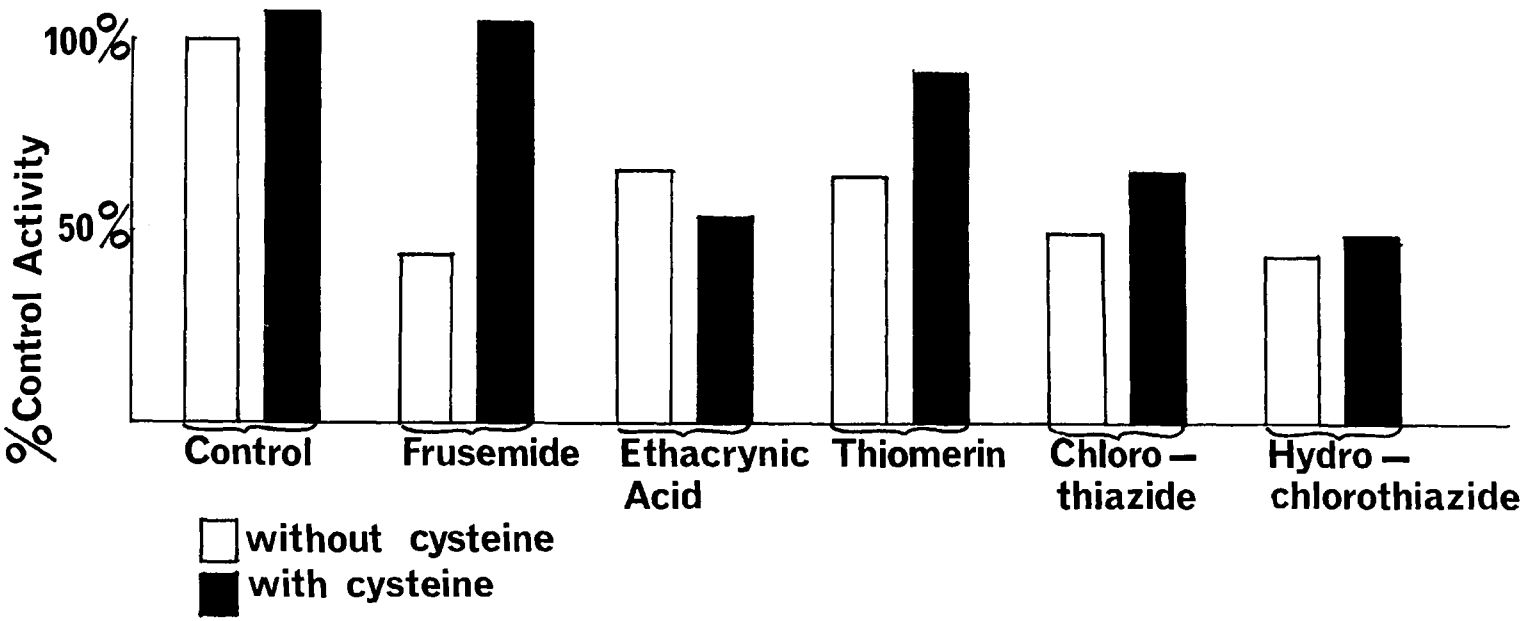


FIG. VIII 5 The effect of cysteine on the inhibitory action of diuretics using rat myocardial microsomal (Na^+, K^+) -ATPase.

only at high concentrations. Figure VIII 3 shows the linear time / activity relationship in the presence of mercaptomerin and hydrochlorothiazide. A linear time / activity was also obtained for the other diuretics. All the diuretics inhibited by a non-competitive mechanism. The inhibition kinetics were examined by the Lineweaver and Burk graphical method (Lineweaver and Burk, 1934). Figure VIII 4 shows the results of frusemide inhibition and is typical of the results obtained with the other diuretics.

The effects of each diuretic were determined with and without the addition of 10^{-2}M cysteine. Fig. VIII 5 shows the results of these experiments. The inhibition by frusemide and mercaptomerin could be almost completely reversed by cysteine. Addition of cysteine had only a slight effect on the inhibitory action of the thiazides, while ethacrynic acid inhibition was unaffected.

Human myocardial microsomal (Na^+, K^+) - ATPase

All the diuretics inhibited human microsomal (Na^+, K^+) - ATPase activity in vitro. The results were similar to those obtained for the rat myocardial microsomal (Na^+, K^+) - ATPase. Figure VIII 6 shows the effects of adding various concentrations of mercaptomerin and is typical of the results obtained for the other diuretics. The Ki values together with the diuretic concentration at the start and at complete inhibition are summarized in Table VIII 3. Figure VIII 7 shows the linear time /

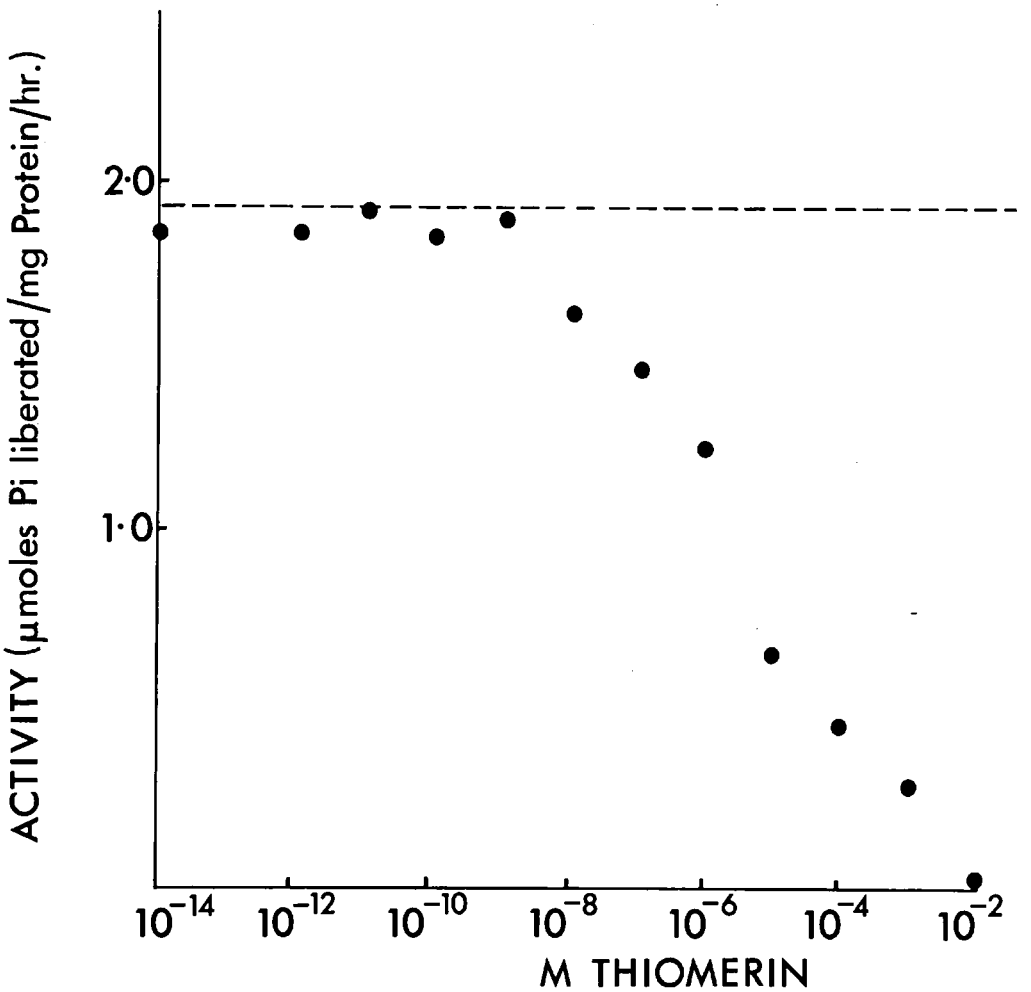


FIG. VIII 6 The effects of various concentrations of thiomerin on human post mortem myocardial microsomal (Na^+, K^+) -ATPase activity. The broken line represents the control activity.

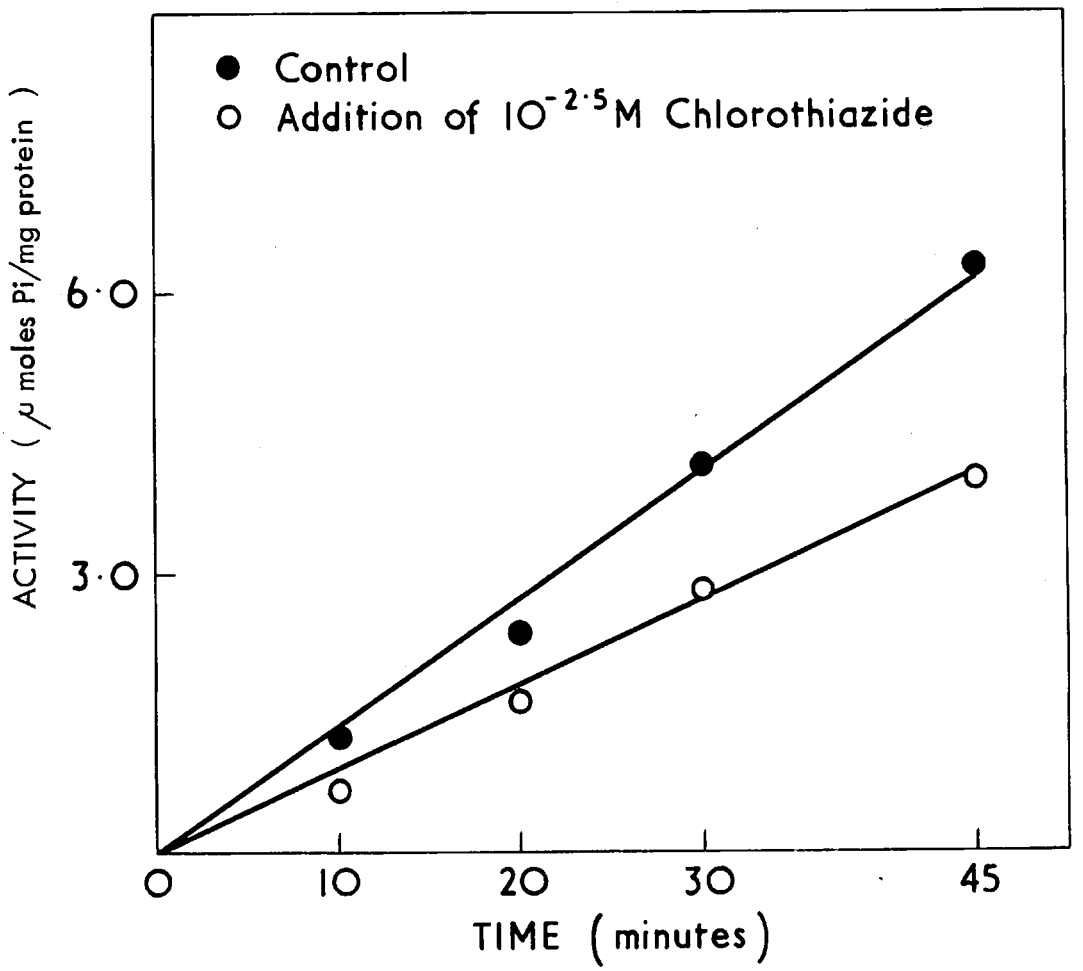


FIG. VIII 7 The effects of chlorothiazide on the time/activity relationship of human post mortem myocardial microsomal (Na^+, K^+) -ATPase activity.

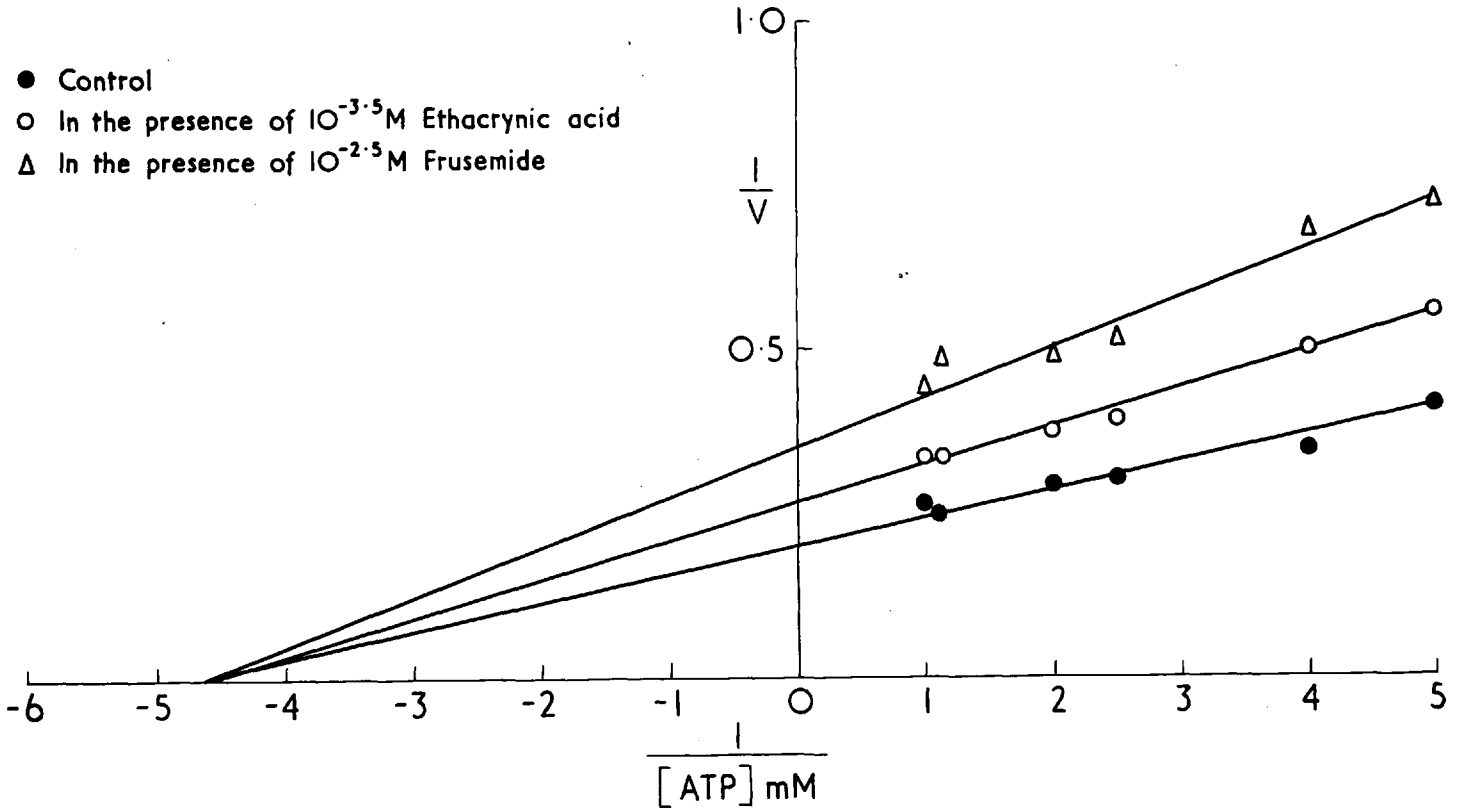


FIG. VIII 8 Non-competitive inhibition of ethacrynic acid and frusemide on human post mortem myocardial microsomal (Na^+, K^+) -ATPase.

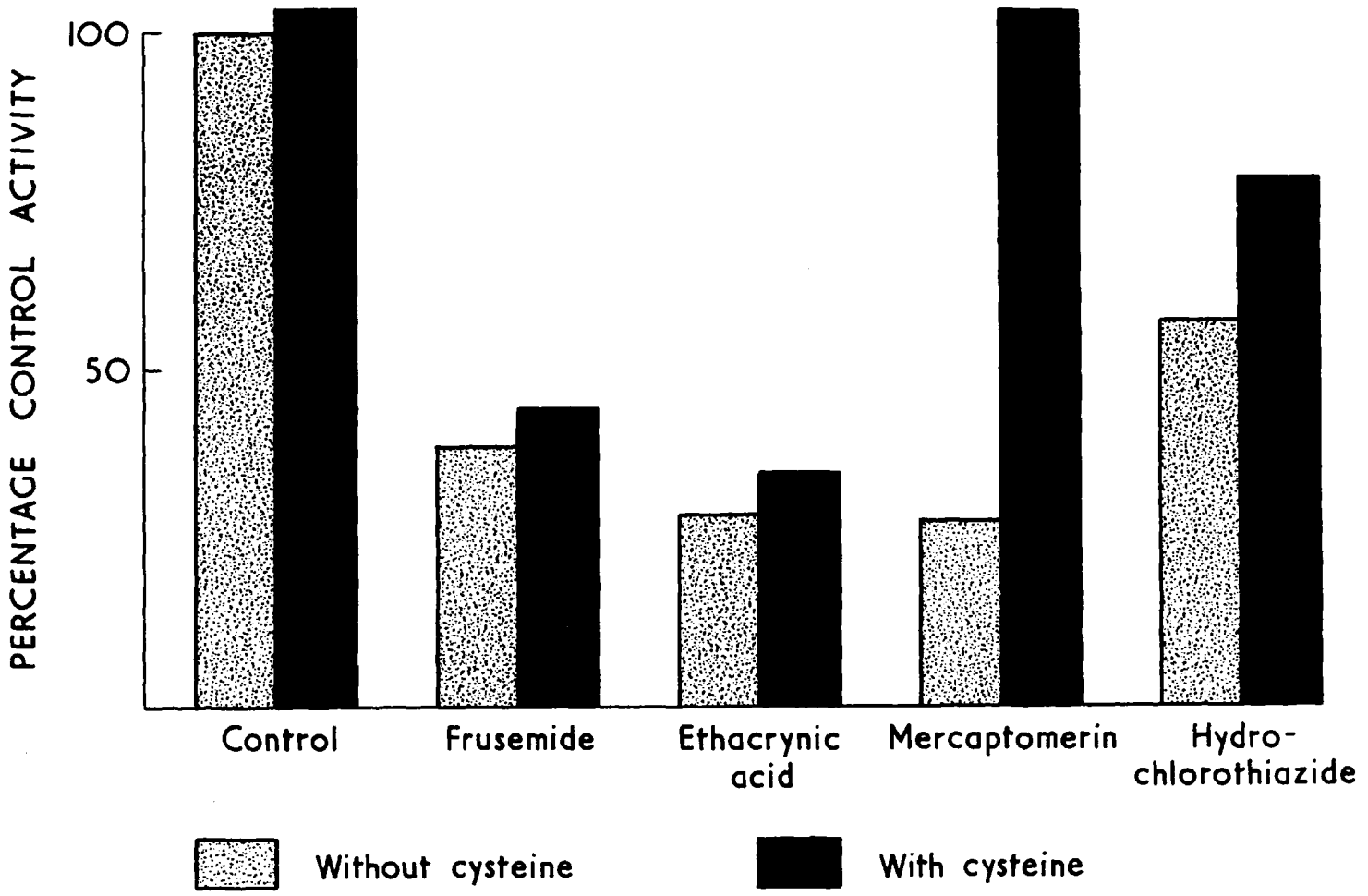


FIG. VIII 9 The effects of cysteine on the inhibitory action of diuretics using human myocardial microsomal (Na^+, K^+) -ATPase.

activity relationship in the presence of chlorothiazide. All the diuretics showed a similar linear time / activity relationship. The diuretics inhibited by a non-competitive mechanism. Figure VIII 8 shows the Lineweaver and Burk plot of ethacrynic acid and frusemide inhibition and is typcial of the results obtained for the other diuretics.

The effects of adding 10^{-2}M cysteine and 10^{-2} M cysteine plus various diuretics on (Na^+, K^+) - ATPase activity is shown in Fig. VIII 9. The inhibitory action of mercaptomerin could be completely reversed by addition of cysteine. Addition of cysteine to hydrochlorothiazide had only a slight effect. The effect of cysteine on frusemide and ethacrynic acid inhibition was negligible.

The inhibitory effect of 10^{-4}M ethacrynic acid could not be reversed by addition of excess potassium ions up to 30 mM, by the addition of excess sodium ions up to 150 mM, by the addition of excess magnesium ions up to 12 mM or by increasing the substrate concentration up to 4 mM. Myocardial Mg^{++} - ATPase activity was not affected by the addition of diuretics in vitro in both the rat and human microsomal preparations.

Discussion

Prolonged in vivo administration of the diuretics studied did not significantly effect the level of (Na^+, K^+) - ATPase activity in the myocardium or kidney of

rats. It is possible that there may have been some transient effect similar to that observed after digoxin treatment but this would not have been apparent from the present studies in which no observations were made before two weeks.

The renal results are at variance with those reported by Hook and Williamson (1965) who found that a single dose of ethacrynic acid or frusemide was sufficient to cause an inhibition of potassium stimulated renal microsomal (Na^+, K^+) - ATPase in the rat. Jones et al. (1965) also found an in vivo inhibition by mercurial diuretics. This discrepancy may possibly be related to the difference in the duration of administration of the drugs.

In vitro, all the diuretics inhibited myocardial microsomal (Na^+, K^+) - ATPase from rat and human preparations. Mercaptomerin was the most potent inhibitor followed by ethacrynic acid. Frusemide and the thiazides inhibited only at very high concentrations. Taylor (1963) could not find inhibition of rabbit kidney (Na^+, K^+) - ATPase by chlorothiazide in vitro. The inhibition found in the present experiment did not effect the linear time / activity relationship. This would indicate that binding of the diuretic to the enzyme was rapidly completed so that during incubation no further increase in binding took place. It was important to establish this before kinetic studies were made. Duggan and Noll (1965) compared the binding time of ethacrynic acid to cysteine with other compounds and found that only 0.8 min was required to complete 50% of the reaction.

An analysis of the enzyme kinetics showed that in all cases the inhibition was of the non-competitive type. This was supported by the results of increasing both ion and substrate concentrations in the presence of ethacrynic acid. Neither increasing ions nor substrate could reverse the inhibition. This would indicate a binding site on the enzyme not associated with either the substrate or ion stimulating sites. It has been reported that inhibition of (Na^+, K^+) - ATPase by ouabain in high concentrations (10^{-4}M) cannot be reversed by K^+ ions whereas in lower concentrations inhibition can be reversed by K^+ ions (Bonting et al., 1964). In the present studies with diuretics low inhibitor concentrations were difficult to select because in most cases the inhibition curve was very steep. Therefore the possibility that lower concentrations of diuretic can be reversed by sodium or potassium ions cannot be excluded.

The presence of cysteine completely reversed mercurial diuretic inhibition. This may be expected from the data relating to the formation of mercaptides by mercurial compounds and the fact that sulphydryl groups are required for (Na^+, K^+) - ATPase activity. It should be noted however that not all sulphydryl reacting compounds inhibit (Na^+, K^+) - ATPase. For example iodoacetate and iodacetamide do not inhibit (Na^+, K^+) - ATPase activity in vitro (Taylor, 1963).

In the rat frusemide inhibition could be reversed by addition of cysteine but this was not so in the case of human preparations. This possibly represents a species

difference in the mechanism of action of frusemide. As previously mentioned there is a species difference in the effects of ethacrynic acid which is not a diuretic in rats but is in humans. The inhibition by the thiazides was little affected by cysteine in both species. It would appear that the inhibitory mechanism of the diuretics studied is different in each case but the exact nature and site of reaction remains to be elucidated.

The inhibitory action of diuretics on (Na^+, K^+) - ATPase has formed the basis for a hypothesis regarding their physiological action in the kidney (Taylor, 1963). However, in the myocardium it is difficult to explain how an inhibitory mechanism would help in a situation such as congestive heart failure where sodium is accumulating intracellularly while potassium is being lost (Flear, 1966). Inhibition of (Na^+, K^+) - ATPase would only exacerbate such a situation. It is most unlikely that the high concentrations required to inhibit (Na^+, K^+) - ATPase in vitro would reach such levels in vivo.

It was surprising in view of the in vitro inhibition of diuretics that no effect on (Na^+, K^+) - ATPase activity was seen in either the myocardium or kidney after prolonged administration.

The relationship between Mg^{++} - ATPase and (Na^+, K^+) - ATPase is not known. It is, therefore, difficult to understand the relevance of a significantly increased renal and ventricular Mg^{++} - ATPase activity in the absence of any significant

changes in the (Na^+, K^+) - ATPase activity in vivo after the administration of ethacrynic acid.

CHAPTER IX

CATECHOLAMINES

Membrane bound ATPases have been implicated as possible receptor sites for catecholamines in the myocardium (Belleau, 1967) and in smooth muscle (Jenkinson and Morton, 1967).

Glitsch et al. (1965) found that catecholamines had little effect on the action potential of the frogs heart in terms of amplitude and duration but increased sodium and potassium ion fluxes. Brady (1967) however, found that in the presence of adrenalin the duration of the action potential is elongated in cardiac fibres of the guinea pig atria. Brady suggests that this is due to the catecholamine coupling site and the membrane potential generating regions having the cell membrane as a common site of action. The most characteristic and clearly defined effect of catecholamines, on electrical activity, is on the automatic cells of the myocardium. There is an increase in the slope of phase four depolarization (Hoffman and Singer, 1967). This means that the threshold for depolarization is reached more rapidly, in the presence of adrenalin, and consequently the heart rate is increased.

Jenkinson and Morton (1967) found that adequate doses of adrenalin would abolish the action potentials in smooth muscle cells and reduce or abolish electrical excitability.

Both catecholamines and sympathetic blocking agents have been shown to

inhibit (Na^+, K^+) - ATPase of guinea pig brain but when catecholamines and sympathetic blocking agents are combined they do not inhibit (Lee and Yu, 1963). In view of their importance in the function of the myocardium a study of the action of catecholamines on myocardial microsomal (Na^+,K^+) - ATPase has been made. The in vivo effects of ephedrine on rat myocardial (Na^+, K^+) - ATPase have been investigated together with the in vitro effects of catecholamines and their metabolites on human myocardial (Na^+, K^+) - ATPase.

Materials and Methods

In vivo experiment

One group of 10 rats was treated with ephedrine, 1 mg / Kg body weight / day, by gastric intubation for two weeks. A control group of 20 rats was given water by gastric intubation.

The strain of rats, microsomal preparative procedures and assay methods are the same as those described previously in Chapter VII.

In vitro experiment

The human post mortem myocardial microsomal preparation was used. Details of preparation are given in Chapter V.

Chemicals

Ephedrine was obtained in 30 mg tablets from the hospital dispensary. All the

TABLE IX 1

THE EFFECTS OF TREATING RATS WITH EPHEDRINE ON MYOCARDIAL AND RENAL MICROSOMAL ATPases

		(Na^+, K^+) - ATPase						Mg^{++} - ATPase					
	No. of	ATRIUM		VENTRICLE		KIDNEY		ATRIUM		VENTRICLE		KIDNEY	
	Animals	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
CONTROL	20	3.2	2.3	4.9	2.5	21.9	5.8	5.1	2.5	7.0	2.1	11.7	5.5
EPHEDRINE	10	3.3	2.1	5.8	2.0	28.6	3.8	3.1	2.1	4.5	2.4	12.2	2.2

TABLE IX 2

THE EFFECTS OF CATECHOLAMINES AND SOME OTHER RELATED COMPOUNDS ON HUMAN POST MORTEM MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase ACTIVITY

COMPOUND	CONC. AT START OF INHIBITION	KI VALUE	CONC. AT MAXIMAL INHIBITION
PHENYL ALANINE	DID NOT INHIBIT		
TYRAMINE	10^{-3}	7.0×10^{-3}	10^{-1}
TYROSINE	10^{-3}	5.0×10^{-3}	10^{-2}
DOPA	10^{-4}	2.0×10^{-2}	*
DOPAMINE	10^{-4}	5.0×10^{-3}	10^{-1}
NORADRENALIN	10^{-4}	7.1×10^{-3}	10^{-1}
ADRENALIN	10^{-4}	5.2×10^{-3}	10^{-2}
3,4 - DIHYDROXYMANDELIC ACID	10^{-4}	4.0×10^{-3}	*
NOR METANEPHRINE	10^{-5}	3.2×10^{-3}	*
VANILLYLMANDELIC ACID	5×10^{-5}	3.2×10^{-3}	10^{-2}
MANDELIC ACID	DID NOT INHIBIT		

* These compounds were insoluble at concentrations below that required for maximal inhibition

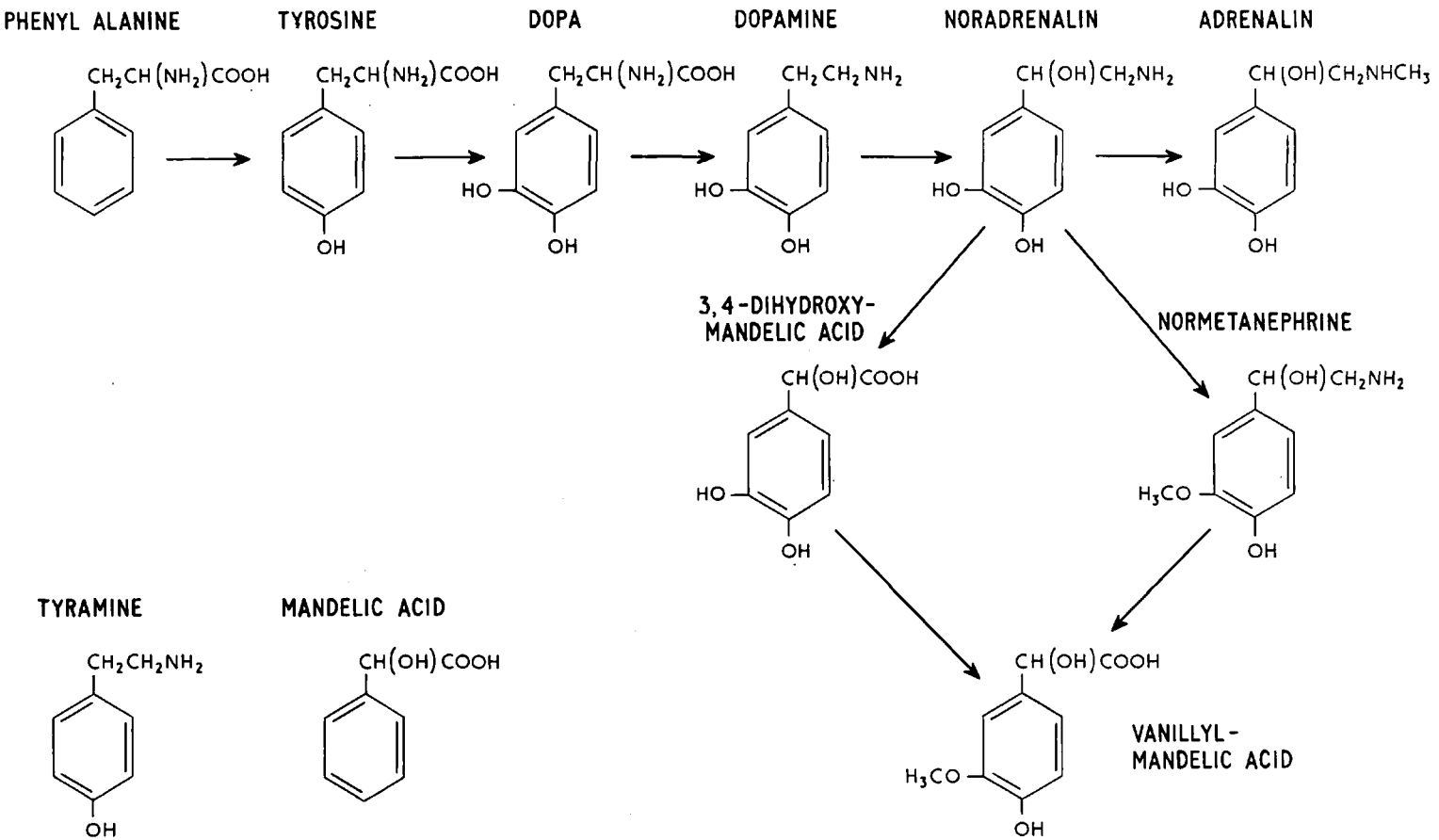


FIG. IX 1 The structural formulae of the compounds studied and the metabolic pathway of noradrenalin and adrenalin.

catecholamines were obtained from the Sigma Chemical Company London. Phenylalanine and mandelic acid were obtained from Hopkin and Williams Ltd.

Results

In vivo experiment

Table IX 1 shows the microsomal ATPase activities of myocardium and kidney of rats after treatment with ephedrine. There was no significant difference in myocardial (Na^+, K^+) - ATPase activity between the control and ephedrine treated rats. The increase in renal (Na^+, K^+) - ATPase activity of the ephedrine treated rats compared to the control animals was statistically significant ($P =< 0.01$). Mg^{++} - ATPase activity was not significantly altered in the ephedrine treated rat myocardial or renal tissues.

In vitro experiments using human post mortem myocardial microsomal (Na^+, K^+) - ATPase

Fig. IX 1 shows the metabolic pathway of the catecholamines (Axelrod, 1965) together with the structural formulae of two other compounds used in this study.

All the catecholamines were inhibitors of (Na^+, K^+) - ATPase activity. The Ki values ofsthe compounds are shown in Table IX 2 along with the concentrations at the start of the inhibition and at complete inhibition.

The type of inhibition was determined by the Lineweaver and Burk (1934) graphical method. Fig. IX 2 shows the results of inhibition by adrenalin and nor adrenalin,

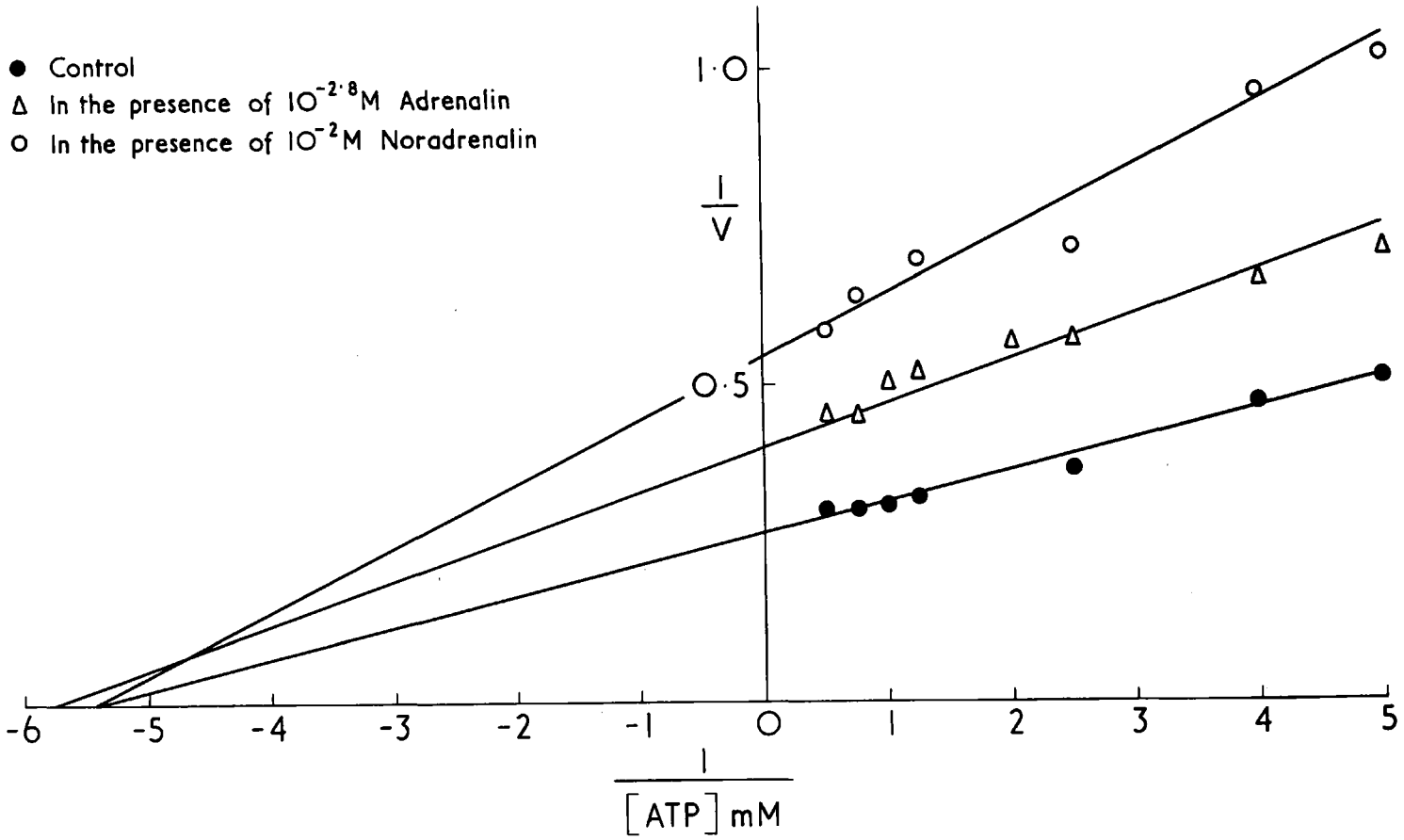


FIG. IX 2 Non-competitive inhibition of human myocardial (Na^+, K^+) -ATPase by adrenalin and noradrenalin.

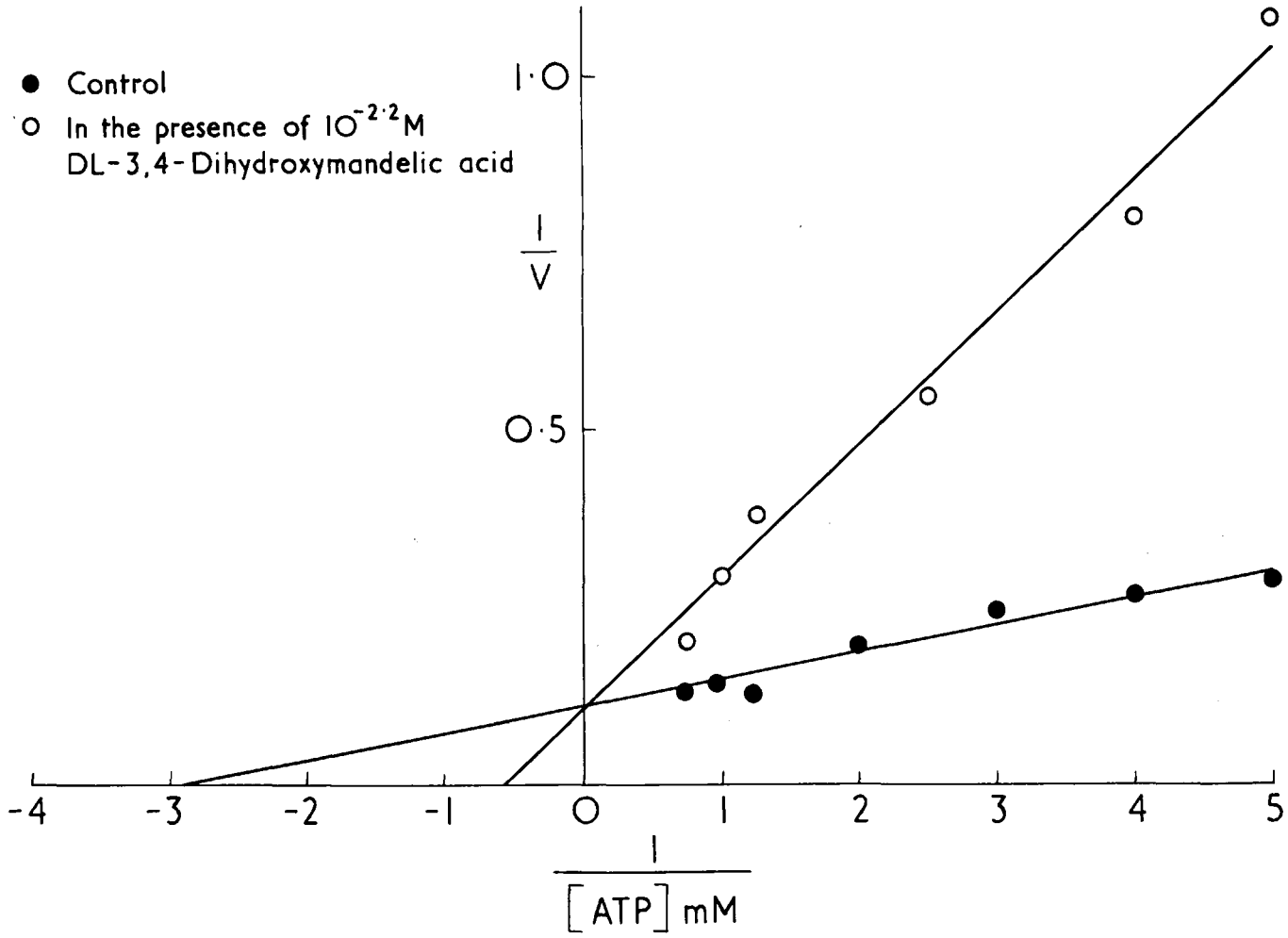
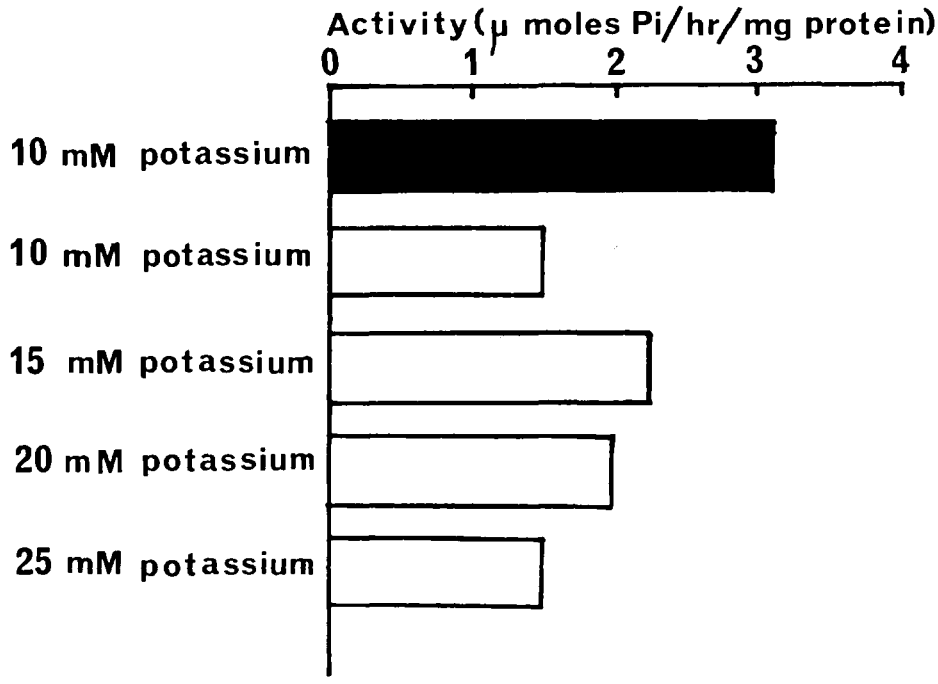


FIG. IX 3 Competitive inhibition of human myocardial microsomal (Na^+, K^+) -ATPase by DL - 3, 4 - dihydroxymandelic acid.



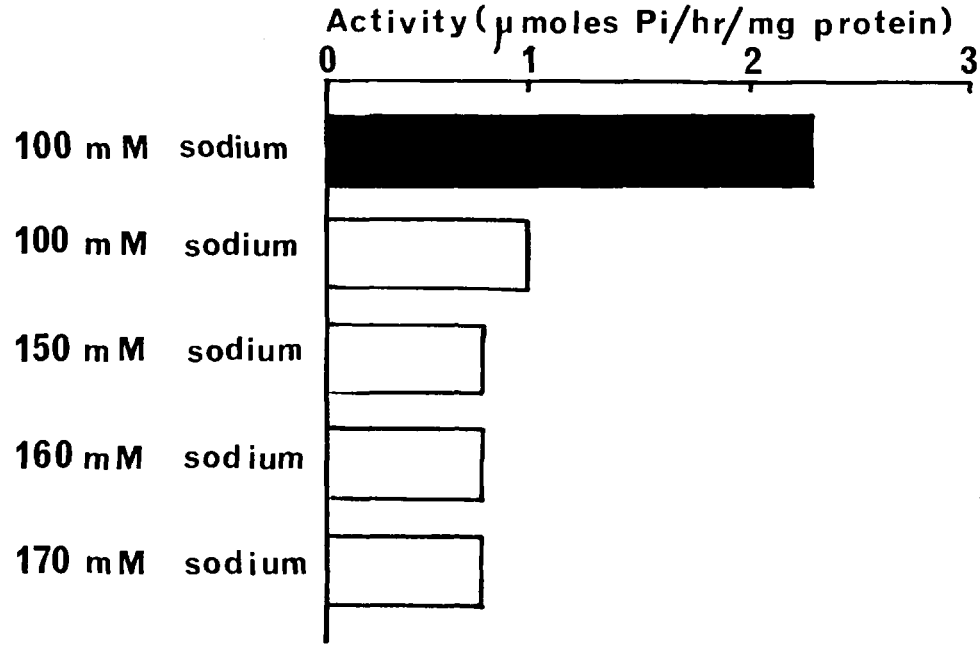


FIG. IX 4 (Upper Diagram) The effects of potassium ions on 10^{-3} M noradrenalin inhibition of (Na^+, K^+) -ATPase.

(Lower Diagram) The effects of sodium ions on 10^{-3} M noradrenalin inhibition of (Na^+, K^+) -ATPase.

■,control ; □ , plus 10^{-3} M noradrenalin

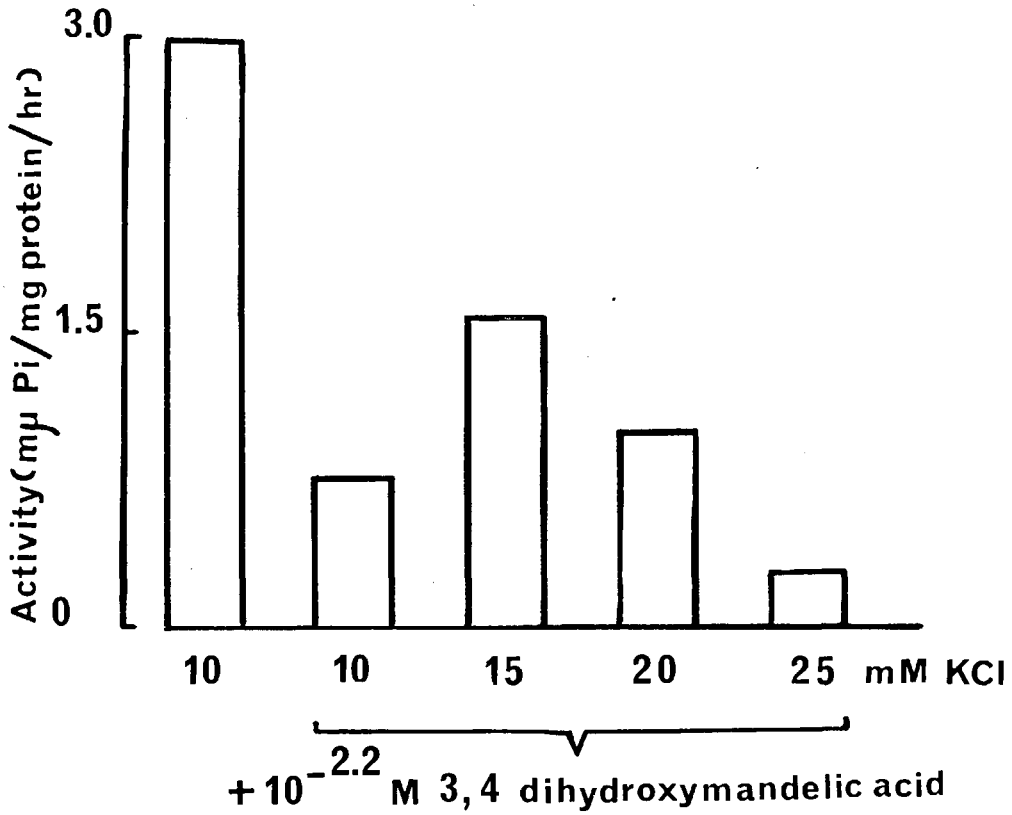


FIG. IX 5 The effect of potassium ions on $10^{-2.2}$ M 3, 4 - dihydroxymandelic acid inhibition.

which are non-competitive inhibitors. This type of curve was also found with tyramine, tyrosine and all the catecholamines with the exception of 3, 4 - dihydroxymandelic acid. Fig. IX 3 shows the Lineweaver and Burk plot of (Na^+, K^+) - ATPase activity with and without the addition of 3, 4 - dihydroxymandelic acid. The curve is typical of a competitive type of inhibitor. Neither phenylalanine nor mandelic acid inhibited (Na^+, K^+) - ATPase activity.

Nor adrenalin and 3, 4 - dihydroxymandelic acid inhibition could be partly reversed by increasing the potassium ion concentration but not by increasing the sodium ion concentration, fig. IX 4 and IX 5. Neither increasing the ATP concentration to 4 mM nor increasing the Mg^{++} ion concentration to 12 mM had any effect on nor adrenalin or 3, 4 - dihydroxymandelic acid inhibition.

Discussion

In vivo experiment

Ephedrine has a cardiac stimulating action and can increase cardiac metabolism (Aviado, 1959). Vasopressor amines, including ephedrine, act at the same receptor sites as catecholamines (Watson, 1965). The results from the present study show a lack of in vivo effect of ephedrine on (Na^+, K^+) - ATPase activity in the myocardium but an increased renal (Na^+, K^+) - ATPase activity. The complexity of ion transport processes in the kidney makes an interpretation of increased

renal (Na^+, K^+) - ATPase activity difficult, without considerably more experimental data.

In vitro experiments

A considerable amount of work has been reported on the relationship between structure and activity with regard to catecholamines and the so-called alpha and beta receptor sites (for example, Sutherland and Robinson, 1966 and Belleau, 1967). In the present study the presence of a phenolic hydroxyl group was found to be necessary for an inhibitory action of (Na^+, K^+) - ATPase activity. Ariens (1967) studying the relationship between structure and activity of beta adrenergic drugs found that elimination of both hydroxyl groups resulted in a strong decrease in the affinity of catecholamines for the beta receptors. It may also be relevant that hydroxyl groups in the C3 and C14 positions have been found to be a structural requirement of the inotropic action of cardiac glycosides which also have an inhibitory action on microsomal (Na^+, K^+) - ATPase (Fieser and Fieser, 1959; Duggan and Noll, 1965). In the relationship between structure and activity of (Na^+, K^+) - ATPase neither phenylalanine nor mandelic acid have phenolic hydroxyl groups and both compounds failed to inhibit (Na^+, K^+) - ATPase. It would appear that hydroxyl groups play an important part in the relationship between the structure of catecholamines and their inhibitory effect on (Na^+, K^+) - ATPase.

It should be noted that the concentrations of all the substances which were studied were higher than might be expected to occur physiologically. It is possible that the preparation of the enzyme, which is necessary to unmask (Na^+, K^+) - ATPase, modifies the catecholamine binding sites so that they require higher concentrations to affect the enzyme in vitro than would be necessary in vivo.

Nor adrenalin inhibition was partly reversed by increasing the potassium ion concentration but not by increasing the Na^+ or Mg^{++} ion concentration or substrate ATP. The formation of the Mg^{++}, ATP enzyme complex requires Na^+ ions while the hydrolysis of the enzyme substrate complex requires K^+ ions (Skou, 1964). Therefore nor adrenalin probably interferes with the hydrolysis of the enzyme substrate complex.

The difference in the inhibitory action if 3, 4 - dihydroxymandelic acid compared to the other compounds studied may be attributed to the small difference in molecular structure causing a steric hindrance to either ion or ATP binding. This would mean that 3, 4 - dihydroxymandelic acid is not a true competitive inhibitor because it may not be competing for the ion or ATP binding sites. However, the inhibition due to 3, 4 - dihydroxymandelic acid was partially reversed by potassium ions which would suggest that it is the potassium binding site which is involved in the inhibitory mechanism of 3, 4 - dihydroxymandelic acid. The difference in the

molecular structure between 3, 4 - dihydroxymandelic acid and its analogues is in the carboxylic group and the meta hydroxyl group. It is the meta hydroxyl group which is the more important for the action of alpha and beta receptors (Ariens, 1967). 3, 4 - dihydroxymandelic acid is formed within the myocardium by amine oxidase activity and its properties might, therefore, be thought to be of more immediate importance in the heart.

Adrenalin was a more effective inhibitor of myocardial (Na^+, K^+) - ATPase than nor adrenalin in vitro. In vivo adrenalin has a greater effect on the irritability, rate and strength of cardiac muscle contraction.

CHAPTER X

OTHER DRUGS OF CARDIOLOGICAL INTEREST

In this chapter a number of drugs which affect the myocardium or the cellular exchange of sodium and potassium have been tested for their effects on myocardial and, in some experiments, renal microsomal (Na^+, K^+) - ATPase.

The drugs studied were, atropine and acetylcholine which act on the parasympathetic system, propranolol and ergotamine, drugs causing adrenergic blockade and diphenylhydantoin and quinidine which have antidysrrhythmic properties. The effects of deoxycortiosterone have also been studied. Most of the experiments have been carried out in vitro but the in vivo effects of two compounds, atropine and spironolactone, was also tested.

The electrical properties of the cardiac cell are the result of an unequal distribution of Na and K ions inside and outside the cell membrane (see Chapter I for further details). Treatment of cardiac dysrrythmias by antidysrrythmic drugs is, basically, dependent on modifying the electrical properties of the cell. As the cellular Na and K ion concentrations inside the cell are regulated by the activity of (Na^+, K^+) - ATPase it was of interest to study the effects of drugs having antidysrrythmic properties, and other compounds effecting the distribution of Na and K ions, on (Na^+, K^+) - ATPase activity.

Atropine and acetylcholine

Atropine increases the heart rate in therapeutic doses. Its major action is by a competitive antagonism to acetylcholine and atropine can also prevent potassium loss induced by acetylcholine (Holland et al., 1952). Acetylcholine decreases the heart rate and can cause hyperpolarization of the cardiac cell membrane due to an increase in the selective permeability to potassium ions (Goodman and Gilman, 1965)

Propranolol and ergotamine

Propranolol is an adrenergic beta receptor blocking drug counteracting the cardiac effects of sympathetic activity. In normal subjects and patients with heart disease propranolol treatment altered the diurnal pattern of sodium excretion, causing it to take place mainly at night (Epstein and Braunwald, 1967). Propranolol also tends to relieve digitalis induced arrhythmias although the mechanism is not understood (Roberts, 1967). The height and rate of rise of the cardiac action potential is reduced after propranolol treatment (Morales-Aguilerá and Vaughan-Williams, 1965). Vaughan-Williams, (1966) attributes the antiarrhythmic properties of propranolol to a reduction in sodium conductance on excitation.

Ergot alkaloids block alpha adrenergic receptors and are known to be

antagonists of the metabolic effects of catecholamines, at least in smooth muscle (Innes, 1962). Ergotamine can cause bradycardia by central vagal stimulation and can also depress cardiac arrhythmias (Goodman and Gilman, 1965).

Diphenylhydantoin and quinidine

Both diphenylhydantoin and quinidine act on the membrane of the cell. Diphenylhydantoin is closely related in structure to the barbiturates. It is thought to increase sodium efflux which stabilizes the threshold of the cell against hyperexcitability (Woodbury, 1955). Dysrhythmias induced by digitalis toxicity can be reversed by diphenylhydantoin (Bernstein et al., 1965; Conn, 1965; Ruthan, 1965; Moshal et al., 1967). There is a reversal of cellular potassium loss, induced by digitalis, when diphenylhydantoin is administered (Helfant et al., 1968). It was suggested by Helfant et al. (1968) that diphenylhydantoin acts on the myocardial cell by reversing the inhibition of (Na^+, K^+) - ATPase caused by digitalis. An in vitro study of this hypothesis was tested.

Quinidine is widely used as a antidysrythmic drug. In therapeutic concentrations the spike phase of the action potential is prolonged and the entire action potential may also be prolonged (Conn, 1965). This has the effect of slowing the heart rate.

Deoxycorticosterone and spironolactone

The mineralocorticoids, aldosterone and deoxycorticosterone, augment renal

tubular reabsorption of sodium and chloride and increase the excretion of potassium. The in vitro effects of deoxycorticosterone on myocardial (Na^+, K^+) – ATPase have been studied in view of its importance in the control of cellular electrolyte levels.

Spironolactone is a synthetic compound which is a competitive antagonist of aldosterone. The primary site of action is on the renal tubular reabsorptive regions of the nephron. Spironolactone may be used to prevent potassium loss during diuretic therapy.

Materials and Methods

In vivo experiments

The strain of rats, dosing method and keeping conditions were similar to those reported in previous chapters. One group of 10 rats was treated with 0.02 mg atropine / Kg body weight twice daily for two weeks and a second group of 14 treated with 20 mg spironolactone / Kg body weight / day for three weeks. A control group of 20 rats for the atropine experiment and a control group of 5 rats for the spironolactone experiment were given water by gastric intubation. The dissection of samples, preparation of microsomal fractions and assay procedures have been described in Chapter VII.

TABLE X 1

THE EFFECTS OF FEEDING ATROPINE AND SPIRONOLACTONE ON MYOCARDIAL AND RENAL MICROSOMAL ATPase

		(Na^+, K^+) - ATPase						Mg^{++} - ATPase					
	No. of rats	ATRIUM		VENTRICLE		KIDNEY		ATRIUM		VENTRICLE		KIDNEY	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
ATROPINE													
Control	20	3.2	2.3	4.9	2.5	21.9	5.8	5.1	2.5	6.9	2.1	11.7	5.5
Test	10	3.4	2.5	4.5	3.4	25.2*	6.7	3.6	2.2	6.3	1.3	11.9	1.7
SPIRONOLACTONE													
Control	5	5.6	2.4	4.6	1.8	20.5	2.6	12.1	0.6	11.7	3.2	14.9	5.0
Test	14	7.0	5.6	5.9	4.5	27.7*	6.3	12.9	3.6	13.3	4.8	19.5	3.3

* $P = < 0.01$

Chemicals

Atropine, deoxycorticosterone, eserine, acetylcholine and ergotamine were obtained from the Sigma Chemical Company, London. Spironolactone was obtained from the hospital dispensary in tablet form. Diphenylhydantoin was a gift from Dr. J.A.L. Gorringe of Parke Davis and Company and propranolol from Dr. J.J. Armstrong of I.C.I., Limited, Cheshire.

In vitro experiments

Human post mortem myocardial microsomal fraction was used for all the in vitro experiments in this chapter. For the details of preparation see Chapter V.

Results

In vivo experiments

Table X 1 summarizes the results of the effects of feeding atropine or spironolactone to rats on myocardial and renal microsomal ATPases. There was no significant difference in myocardial (Na^+, K^+) - ATPase or Mg^{++} - ATPase activity between the control and treated rats. There was a significant increase in the renal (Na^+, K^+) - ATPase activity of both the atropine and spironolactone treated rats ($P < 0.01$). The renal Mg^{++} - ATPase activity was not significantly affected by atropine or spironolactone treatment.

TABLE X 2

IN VITRO EFFECTS OF ACETYLCHOLINE, ESERINE AND ATROPINE ON HUMAN POST MORTEM MYOCARDIAL (Na^+, K^+) - ATPase AND Mg^{++} - ATPase

COMPOUND		(Na^+, K^+) - ATPase	Mg^{++} - ATPase
Control		5.4	2.2
Acetylcholine	10^{-2}M	5.5	1.9
	10^{-3}M	4.2	1.8
	10^{-4}M	4.8	2.4
	10^{-5}M	4.5	2.0
	10^{-6}M	5.1	2.4
Control		4.6	2.0
Eserine	10^{-4}M	4.5	2.1
	10^{-5}M	4.9	2.0
	10^{-6}M	4.8	2.5
Eserine 10^{-4}M + Acetylcholine 10^{-2}M		4.3	2.3
(Eserine was dissolved in ethyl alcohol and in concentrations above 10^{-4}M the alcohol strongly inhibited (Na^+, K^+) - ATPase activity.)			
Control		4.6	2.0
Atropine	10^{-2}M	4.7	2.2
	10^{-3}M	4.1	2.3
	10^{-4}M	5.0	2.0
	10^{-5}M	4.4	2.3
Control		3.6	2.2
10^{-2}M Acetylcholine + 10^{-2}M Atropine		3.9	1.9
10^{-3}M Acetylcholine + 10^{-3}M Atropine		3.7	2.6
10^{-4}M Acetylcholine + 10^{-4}M Atropine		3.3	2.4

TABLE X 3

EFFECTS OF VARIOUS CONCENTRATIONS OF DIPHENYLHYDANTOIN ON HUMAN MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase AND Mg^{++} - ATPase

Concentration of Diphenylhydantoin	(Na^+, K^+) - ATPase	Mg^{++} - ATPase
0	4.0	1.4
10^{-4}M	3.9	1.5
10^{-3}M	4.1	1.4
10^{-2}M	3.9	1.4

TABLE X 4

THE EFFECTS OF DIPHENYLHYDANTOIN ON THE INHIBITION OF HUMAN MYOCARDIAL MICROSOMAL (Na^+, K^+) - ATPase BY OUABAIN

Concentration of Ouabain	Activity without Diphenylhydantoin	Activity with 10^{-2}M Diphenylhydantoin
0	1.5	1.5
10^{-6}M	0.9	1.0
10^{-5}M	0.8	0.8
10^{-4}M	0.0	0.0

In vitro experiments

Atropine and acetylcholine

Neither acetylcholine nor atropine had any effect on (Na^+, K^+) - ATPase or Mg^{++} - ATPase activity, Table X 2. Eserine, an anticholinesterase, did not make any difference to the results (Table X 2). Combinations of atropine and acetylcholine were also without effect. Concentrations of atropine higher than 10^{-2}M interfered with the inorganic phosphate assay.

Propranolol and ergotamine

DL - propranolol inhibited (Na^+, K^+) - ATPase activity. Maximal inhibition occurred at 10^{-2}M while inhibition started at 5×10^{-4}M. Half maximal inhibition was 5×10^{-3}M. The inhibition was of the non-competitive type. The Mg^{++} - ATPase activity was not affected.

Ergotamine also inhibited myocardial (Na^+, K^+) - ATPase activity. Inhibition started at 6×10^{-5}M and was complete by 5×10^{-3}M. Half maximal inhibiton was at 2×10^{-4}M. The inhibition was non-competitive.

Diphenylhydantoin and Quinidine

The in vitro effects of various concentrations of diphenylhydantoin on human post mortem myocardial ATPase activity is shown in Table X 3. Diphenylhydantoin had no effect on (Na^+, K^+) - ATPase of Mg^{++} - ATPase activity. Neither did

diphenylhydantoin have any influence on the inhibitory effect of ouabain tested in various concentrations (Table X 4).

It was found impracticable to study the effects of quinidine because the drug interfered strongly with the method for determination of inorganic phosphate.

Deoxycorticosterone

Deoxycorticosterone could only be dissolved in ethyl alcohol before dilutions were made with distilled water. Ethyl alcohol in the deoxycorticosterone dilutions down to 10^{-4}M inhibited (Na^+, K^+) - ATPase activity. Therefore the effects of deoxycorticosterone could only be measured from concentrations of 10^{-4}M down to 10^{-10}M. From 10^{-4}M deoxycorticosterone concentrations down to 10^{-10}M concentration there was no inhibition of either (Na^+, K^+) - ATPase or Mg^{++} - ATPase activity.

Discussion

In vivo experiments

The pesent studies indicate that neither atropine nor spironolactone strongly influence myocardial microsomal ATPases in vivo. This is also supported by the lack of in vitro effects of atropine on (Na^+, K^+) - ATPase activity. The significant increase in renal (Na^+,K^+) - ATPase activity of rats after spironolactone treatment may explain the diuretic properties of spironolactone. Atropine also caused a

significant decrease in renal (Na^+, K^+) - ATPase activity. Interpretation of the renal results is complicated by the fact that, in the kidney, there are several regions each having its own characteristic transport properties.

In vitro experiments

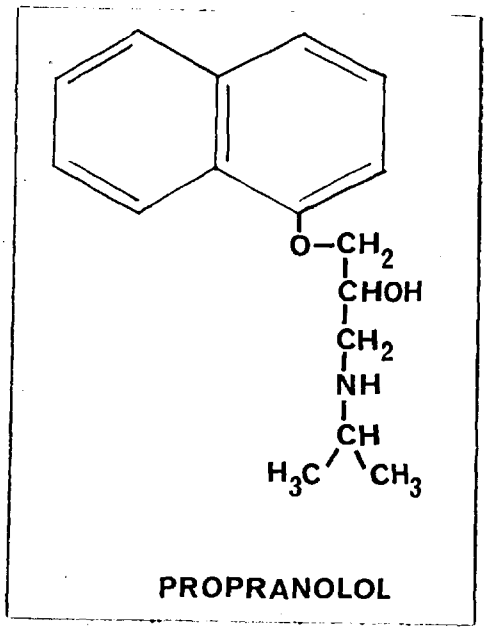
Atropine and acetylcholine

The lack of any inhibitory effects of atropine or acetylcholine is in contradiction to the results of Lee and Yu (1963). These authors found that both substances inhibited (Na^+, K^+) - ATPase activity but that, when both were present together, no inhibiton occurred. It was thought that the presence of an acetylcholinesterase may break down the acetylcholine added to the assay. However, addition of eserine to acetylcholine had no effect on the negative results observed without the presence of eserine. Addition of both atropine and acetylcholine to the same assay mixture did not alter (Na^+, K^+) - ATPase activity.

Propranolol and ergotamine

The non-competitive inhibitory action of propranolol and ergotamine is of interest in view of the inhibitory effects of catecholamines described in Chapter IX. The molecular conformation of ergotamine bears little relation to that of the catecholamines and pharmacologically it is antagonistic. In Chapter IX the inhibition of (Na^+, K^+) - ATPase by catecholamines was shown to be due to the presence of

phenolic OH - groups. However, propranolol, an analog of isoprotorenol in which the phenolic OH - groups are replaced by a benzene ring, inhibited (Na^+, K^+) - ATPase activity. It is the catecholnucleus, especially the phenolic OH - groups that are important for the intrinsic activity on the beta receptors (Ariens, 1967).



PROPRANOLOL

The present results confirm the view that the inhibition of (Na^+, K^+) - ATPase by catecholamines does not occur at an alpha or beta receptor site.

Diphenylhydantoin and quinidine

The results of the diphenylhydantoin experiments suggest that the properties

of this drug in reversing cardiac dysrhythmias caused by digitalis toxicity are not mediated by any influence on myocardial microsomal (Na^+, K^+) - ATPase. Rawson and Pincus (1968) found that diphenylhydantoin inhibited microsomal (Na^+, K^+) - ATPase prepared from rat and guinea pig brain. This may be related to the anticonvulsant properties of the compound and a difference in the properties of the compound and a difference in the properties of the (Na^+, K^+) - ATPase prepared from brain and myocardial tissue. The interference of the inorganic phosphate assay by quinidine is of interest in the light of a reported inhibitory action of quinidine on (Na^+, K^+) - ATPase. (Kennedy and Nayler, 1965)

Deoxycorticosterone

At a concentration of 10^{-6} M deoxycorticosterone has a strong influence on the toad bladder increasing the permeability to sodium ions (Dalton and Smart, 1967). In the present study no change in (Na^+, K^+) - ATPase activity was found at this or higher concentrations. This finding would tend to favour a mechanism by which deoxycorticosterone increases the membrane permeability rather than influencing the activity of (Na^+,K^+) - ATPase transport enzyme.

CHAPTER XI

HYPERTROPHY

Hypertrophy of the myocardium has been much investigated biochemically from both the metabolic and protein synthetic aspects (Meerson, 1965). There are rapid changes in nucleic acid synthesis during the early stages of hypertrophy. RNA polymerase activity of rat myocardium is increased only 12 hours after aortic banding (Nair et al., 1968). During the first five to ten days the concentration of RNA is increased by 32% and protein synthesis, as measured by the incorporation of tagged ^{35}S methionine, is increased two fold (Meerson, 1962). No specific RNA species has been found to increase at a greater rate than any other (Fanburg and Posner, 1968).

During the second stage of hypertrophy, after ten days, the protein synthetic rate returns to normal. The third and final stage of hypertrophy is represented by gradual progressive cardiosclerosis, inhibition of genetic apparatus and a fall in DNA. There is also a decrease in ATP concentration.

De Champlain et al. (1968) found an increase in the activity of monoamine oxidase in the hearts of rats suffering from cardiac hypertrophy, resulting from experimental hypertension produced by either deoxycorticosterone acetate or renal artery stenosis. Monoamine oxidase has been located mainly in the membrane fraction of myocardial tissues (de Champlain et al., 1968). During hypertrophy of the myocardium there is

an increase in the cell volume and hence the cell membrane. The (Na^+, K^+) - ATPase enzyme system is membrane bound and it was thought that some defect may possibly occur during hypertrophy.

Although electrocardiographic changes do occur during hypertrophy they are often difficult to interpret and may simply be the result of the extra bulk of tissue causing conduction disturbances (Hurst and Logue, 1966). There are no changes in action potential, either amplitude or duration, in hypertrophied cardiac tissue (Uhley, 1961).

A convenient method of obtaining hypertrophied myocardial tissue without the need of surgical procedure was to feed rats with ground seeds of Crotalaria spectabilis. Feeding young rats a diet containing Crotalaria spectabilis seeds causes pulmonary hypertension. After about three weeks on the diet the animals die from congestive heart failure. On post mortem examination they are found to have cardiac enlargement due to right ventricular hypertrophy (Kay et al., 1967).

Materials and Methods

Ground Crotalaria spectabilis seed was mixed with powdered MRC 41B diet to give a 0.1% concentration. The diet was mixed into a thick paste with tap water to prevent the rats from selecting the diet only. The control rats were fed a paste of powdered diet 41B. Tap water was given ad libitum throughout the experiment.

The Crotalaria treated rats began to die after about three weeks on the diet.

TABLE XI 1

EFFECTS OF INDUCTING RIGHT VENTRICULAR HYPERTROPHY WITH CROTALARIA ON BODY WEIGHT AND MYOCARDIAL WEIGHT

in g.

CONTROLS	NO.	BODY WEIGHT Mean	BODY WEIGHT S.D.	ATRIUM Mean	ATRIUM S.D.	LEFT VENTRICLE Mean	LEFT VENTRICLE S.D.	RIGHT VENTRICLE Mean	RIGHT VENTRICLE S.D.
GROUP I	9	109	14	0.08	0	0.38	0	0.12	0
GROUP II	7	188	5	0.04	0	0.50	0.01	0.15	0
TEST									
GROUP I	5	105	16	0.06	0	0.36	0.05	0.20	0.04
GROUP II	4	144	23	0.03	0	0.39	0.01	0.15	0

In one test group two animals died and in a second test group one animal died when the experiment was terminated. The remaining rats were killed by a sharp blow to the head. The hearts were dissected and the atria, left and right ventricles separated, blotted free of blood, weighed and placed in bottles surrounded by ice. The interventricular septum was included in the left ventricle sample. The samples were transported from The London Hospital Medical College to the laboratory in a large Thermos container.

The preparative methods for microsomal fractions and assay methods have been given previously.

Results

The relative increase in weight of the right ventricle compared to the left ventricle (Table XI 1) confirmed that right ventricular hypertrophy had occurred in the test group. The body weight of the Crotalaria treated rats had increased only slightly during the experimental period while the control rats had more than doubled their body weights during the same period.

The body weights of the rats were small because of the young age of the animals and the effects of feeding Crotalaria seed. Therefore the myocardial samples of two or three animals were pooled to obtain sufficient tissue to process for the microsomal fractions and assays. The same pooled groups were used for all three chambers.

TABLE XI 2

THE EFFECTS OF INDUCTING RIGHT VENTRICULAR HYPERTROPHY WITH CROTALARIA ON MYOCARDIAL MICROSOMAL ATPase ACTIVITY. (Activity expressed as μ moles Pi/mg protein/hour)

		(Na^+, K^+) - ATPase		Mg^{++} - ATPase	
	Number of Observations	Mean	Standard Deviation	Mean	Standard Deviation
CONTROLS					
Atria	10	4.0	1.3	7.2	3.2
Left Ventricle	10	2.9	0.9	6.8	3.5
Right Ventricle	10	3.0	1.1	7.4	1.7
CROTALARIA					
Atria	9	2.7	0.7	4.1	2.4
Left Ventricle	9	3.6	1.8	5.3	3.8
Right Ventricle	9	3.3	1.8	5.7	4.1

The results of the ATPase assays are shown in Table XI 2. There was no significant difference in (Na^+, K^+) - ATPase activities between the control and test rats in either atria, left or right ventricles. The activity of Mg^{++} - ATPase was not significantly changed in the hypertrophied hearts.

Discussion

Right ventricular hypertrophy was conveniently produced in rats as a result of pulmonary hypertension caused by feeding Crotalaria spectabilis seeds. There was no significant change in (Na^+, K^+) - ATPase activity or Mg^{++} - ATPase activity as a result of hypertrophy. (Na^+, K^+) - ATPase has been used as a membrane marker enzyme in brain tissue (Lim and Adams, 1967). (Na^+, K^+) - ATPase appears to be located in a similar sub-cellular component in the brain and myocardium. Therefore it was used in the present study to measure disturbances in the constitution of the myocardial cell membrane. The negative results would indicate that as new membranes are synthesised during hypertrophy then (Na^+, K^+) - ATPase and Mg^{++} - ATPase enzyme protein is synthesised at a similar rate.

The myocardium was not only hypertrophied but failing. It would appear also, therefore, that (Na^+, K^+) does not play a significant part, if in fact any part, in the failure mechanism of the hypertrophied myocardium.

CHAPTER XII

CONCLUSIONS

A study of myocardial microsomal (Na^+, K^+) - ATPase from various animals has been made and the in vitro and in vivo effects of various drugs and compounds tested. It was difficult to obtain a microsomal enzyme preparation with a low level of Mg^{++} - ATPase activity. A controversy prevails regarding the derivation of Mg^{++} - ATPase and whether or not it is associated with (Na^+, K^+) - ATPase. It would appear from the present study that a close association does exist considering the great difficulty in separating them, although there is some evidence to suggest they are different enzymes. The following facts may be considered as points in favour of the presence of two separate enzymes. Mg^{++} - ATPase has a higher optimum pH than (Na^+, K^+) - ATPase; Mg^{++} - ATPase activity rapidly decreases in activity during storage at + 5°C whereas (Na^+, K^+) - ATPase activity does not; many drugs inhibit (Na^+, K^+) - ATPase but not Mg^{++} - ATPase (for example ouabain); some degree of separation can be achieved by sucrose density gradient centrifugation (Chapter IV p. 70). The presence of both Mg^{++} - ATPase and (Na^+, K^+) - ATPase activities in the microsomal preparations presented difficulties in assay procedure as both activities had to be measured simultaneously. A suitable assay procedure was developed (Chapter III). The overall reaction of (Na^+, K^+) - ATPase can be divided into various stages (see p. 14). It is possible that each stage is catalysed

by a separate enzyme. In the present study however the kinetic experiments are interpreted on the results of the overall reaction, that is on the assumption of a single enzyme reaction. The interpretation may therefore be an oversimplification of a complex reaction.

Addition of 0.1% deoxycholate was found to be the simplest method of treating microsomal preparation to 'unmask' (Na^+, K^+) - ATPase activity. Several other methods were found to unmask (Na^+, K^+) - ATPase activity: sucrose density gradient centrifugation; Sephadex gel columns; glass bead columns and sodium iodide treatment. The results of the sucrose density gradient methods supported evidence for an inhibitor of (Na^+, K^+) - ATPase in myocardial microsomal fractions. The inhibitor was probably not tightly bound to the enzyme because it could be removed by sucrose density gradient centrifugation, remaining in the sample layer or the least dense layer of sucrose after centrifugation. It seems, therefore, that the inhibitor was almost certainly a low molecular weight compound.

The treatment of microsomal fractions or homogenates to release (Na^+, K^+) - ATPase activity by a modification to the membrane with deoxycholate may not be a simple process. Disc electrophoresis showed that protein patterns of microsomal fractions were markedly altered after deoxycholate treatment. Therefore something more than a simple 'loosening' of the membrane is involved in the unmasking of (Na^+, K^+) - ATPase activity of myocardial microsomal fractions. The nature and

extent of these changes awaits elucidation. Attempts to purify the enzyme by preparative disc electrophoresis were disappointing.

Some of the properties of myocardial (Na^+, K^+) - ATPase from human biopsy and post mortem tissue have been described in Chapter V. The properties and specificity of post mortem (Na^+, K^+) - ATPase were not significantly different from the enzyme prepared from biopsy tissue. The characteristics of human myocardial (Na^+, K^+) - ATPase were found to be similar to those reported from a wide variety of other species with only small quantitative differences between them.

Microsomal (Na^+, K^+) - ATPase activity could be measured in human post mortem tissue without the need to pre treat the microsomal fraction with deoxycholate. Autolysis during storage of the tissue may have caused a breakdown of an inhibitory factor or a structural rearrangement of the membrane proteins or lipids.

Many drugs and compounds used in the treatment of cardiovascular diseases had an inhibitory action, in vitro, on(Na^+, K^+) - ATPase and generally had no effect on the Mg^{++} - ATPase activity. With some notable exceptions, for example, ouabain and thiomerin, most of the compounds tested inhibited only at very high concentrations. Whether they reach such high concentrations in vivo after therapeutic doses seems very unlikely although at membrane surfaces drugs may attain fairly high local concentrations. The only compound that increased (Na^+, K^+) - ATPase activity

in vitro was cysteine.

All the diuretics tested were non-competitive inhibitors of human and rat myocardial (Na^+, K^+) - ATPase but the exact inhibitory mechanism was found to be different for each diuretic (Chapter VIII). The diuretics did not affect either the renal or myocardial (Na^+, K^+) - ATPase activity in vivo. It cannot be concluded from the present studies whether or not there was a transient effect on (Na^+,K^+) - ATPase.

An in vitro investigation of the structure / activity relationship of catecholamines and related compounds revealed the requirement of hydroxy phenol groups before inhibition of (Na^+, K^+) - ATPase could take place. With one exception all the catecholamines studied, inhibited by a non-competitive mechanism. The exception was 3, 4 - dihydroxymandelic acid which inhibited by an apparent competitive mechanism. The inhibition could be partly reversed by addition of potassium ions. This indicates that the inhibition of (Na^+,K^+) - ATPase probably involves the potassium binding site on the enzyme. Whether or not the apparent competitive inhibitory mechanism has any physiological significance is not known. 3, 4 - dihydroxymandelic acid is normally regarded as an intermediary in the metabolism of adrenalin to VMA.

The transient stimulation of rat myocardial (Na^+,K^+) - ATPase in vivo after feeding digitalis may necessitate a re-assessment of the hypothesis concerning

the inotropic action of digitalis glycosides. The results of the present study favour a hypothesis which incorporates a stimulatory mechanism of (Na^+, K^+) - ATPase rather than an inhibitory action, despite the convincing hypothesis of Lee (1967). (Na^+, K^+) - ATPase was not stimulated in vitro by low concentrations of ouabain. During the preparation of the microsomal fractions the sensitivity of the cell membranes to low concentrations of ouabain could be altered. Thus, in vitro, obscuring the in vivo effects of ouabain. The binding of ouabain in vitro is not a simple process and possibly involves an allosteric mechanism (Schwartz, 1968). This may also be the situation with regard to catecholamines. Allosteric pumping mechanisms for sodium and potassium ions have been described by Jardetzky (1966). The involvement of compounds in such allosteric systems in myocardial cells would be difficult to examine by techniques used in the present study.

It would seem unlikely that a major defect in (Na^+, K^+) - ATPase enzyme system occurs in myocardial disease in view of the experiments on hypertrophy (Chapter XI) and from the limited number of experiments on human post mortem myocardium (Chapter V).

Feeding potassium chloride to rats significantly increased myocardial (Na^+, K^+) - ATPase activity, while feeding sodium chloride tended to decrease (Na^+, K^+) - ATPase activity. An elevated sodium or potassium ion environment may influence

human myocardial (Na^+, K^+) - ATPase, a fact which could have clinical implications.

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