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ATPase ACTIVITY AND SODIUM EXCHANGE IN THE RABBIT AORTA

by

© MICHAEL WALTER WOLOWYK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "ATPase Activity and Sodium Exchange in Rabbit Aorta" submitted by Michael Walter Wolowyk in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

It is now generally accepted that the membrane ($Na^+ + K^+$)-ATPase which has been isolated from a wide variety of tissues is intrinsically involved in the active transport of Na^+ across biological membranes. However the isolation of this enzyme has not previously been reported from vascular smooth muscle.

When methods used for other tissues were employed to isolate the ($Na^+ + K^+$)-ATPase of rabbit aortae, then only a Mg^{++} or Ca^{++} activated ATPase activity not stimulated by Na^+ plus K^+ addition or inhibited by ouabain was found in the homogenates and subcellular fractions of rabbit aorta assayed in Tris buffer. When histidine buffer was used to replace Tris buffer in the enzyme assay media Na^+ plus K^+ stimulation of the Mg^{++}-ATPase activity in the total homogenate could be demonstrated. However to demonstrate such activity in the isolated microsomal fractions, a soluble, non-enzymatic, factor remaining in the 100,000 x g supernatant material had to be added.

Since the microsomal fraction isolated by differential centrifugation was found to be contaminated with mitochondrial fragments, a further density gradient centrifugation technique was developed to eliminate most of this contamination and increase the specific activity of the monovalent cation stimulated ATPase.

In the presence of the supernatant factor the Mg^{++}-ATPase activity in the density gradient isolated microsomal fraction could be optimally stimulated by the addition of 50 mM Na^+ and 2 mM K^+. The greater part of this stimulation was due to a ouabain insensitive Na^+

stimulated Mg^{++}-ATPase activity. However the remainder of the total stimulation was ouabain sensitive and attributed to a $(Na^{+} + K^{+})$-ATPase. Ca^{++} could not replace Mg^{++} nor other nucleotides ATP for the monovalent cation stimulation. The ouabain inhibition could be overcome by increasing the K^{+} concentration.

The cellular efflux of ^{22}Na in rabbit aortae suggested the presence of at least two types of Na^{+}-pumps. One may be a ouabain sensitive Na^{+}-pump and the other may be a metabolically dependent Na^{+}-pump, unaffected by ouabain but controlling cell volume.

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In addition, I wish to express my deep appreciation to my wife, Norma, for her wonderful understanding, forbearance, and encouragement.

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CHAPTER I

GENERAL INTRODUCTION

CHAPTER I. GENERAL INTRODUCTION

A. Introduction to the Thesis Project.

The ionic composition of the extracellular fluid of all animal cells differs markedly from that of the fluid within the cells. The external environment of the cell usually contains a high concentration of Na^+ relative to K^+, whereas within the cell these concentrations are almost reversed (Macallum, 1905). Besides the chemical gradient there also exists an electrical gradient across the cell membrane, with the interior of the cell being negative with respect to the outside. These gradients occur across a cell membrane; however, the cell membrane does not maintain this electrochemical gradient by simply being impermeable to the ions involved. Thus the cell must move Na^+ outward against the electrochemical gradient and in so doing utilizes some of its metabolic energy. This phenomenon has been attributed to the "sodium pump" or active transport (Ussing, 1949).

In 1957 Skou identified an adenosine triphosphatase in crab nerve membrane which required Na^+ plus K^+ as well as Mg^{++} for full activity and suggested that this enzyme [$(Na^+ + K^+)$-ATPase] may participate in the active transport of Na^+. Since this time numerous investigators have substantiated this relationship in a variety of tissues (Post and co-workers, 1960; Dunham and Glynn, 1961; Wheeler and Whittam, 1961; Bonting and co-workers, 1961, 1962; Skou, 1962). Studies comparing $(Na^+ + K^+)$-ATPase activity with Na^+ flux measurements

in the erythrocyte (Post and co-workers, 1960), toad bladder (Bonting and Canady, 1964) and in giant axon (Caldwell and Keynes, 1957; Caldwell, 1960; Bonting and Caravaggio, 1962) have supported the association of these two systems. However, little attention has been paid to the possible role of $(Na^+ + K^+)$-ATPase in the transport of cations in vascular smooth muscle.

Ion flux studies on vascular smooth muscle indicate that an active Na^+ pump exists (Barr and co-workers, 1962; Vidlakova and Klenizeller, 1963; Daniel, 1965; Garrahan and co-workers, 1965; Hagemeijer and co-workers, 1965). These studies indicate that the Na^+ efflux in vascular smooth muscle is K^+ dependent and possesses some sensitivity to the cardiac glycoside ouabain (Garrahan and co-workers, 1965). Thus the movement of the monovalent cations against their electrochemical gradients appear to be mediated by a system which has the properties of a $(Na^+ + K^+)$-ATPase.

The object of this thesis was to isolate and characterize the $(Na^+ + K^+)$-ATPase and any other ATPases that may be involved in the movement of cations in vascular smooth muscle. Further studies of the efflux of Na^+ and K^+ were also carried out in order to obtain a better understanding of how the ion movements are related to the ATP hydrolysing enzyme(s) found in vascular smooth muscle.

B. Parallels Between the Properties of the Active Transport System and the $(Na^+ + K^+)$-ATPase.

The work establishing the existence of active cation transport

and the role of a ($Na^+ + K^+$)-ATPase in this system has been well reviewed (Skou, 1964 and 1965; Whittam, 1964; Judah and Ahmed, 1964a; Baker, 1966; Charnock and Opit, 1968). However, it would be profitable to review the criteria relating to the two systems.

1. Both systems require metabolic energy in the form of ATP for activity and ATP cannot be replaced by other trinucleotides.
2. Both systems require Na^+ and K^+ together.
3. Na^+ and ATP are required on the inside of the membrane along with Mg^{++}, while K^+ is required on the outside.
4. In both systems the requirement for K^+ can be substituted by other monovalent cations; however, the Na^+ requirement cannot be substituted.
5. Both systems are inhibited in the presence of the cardiac glycoside ouabain.
6. The inhibitory effects of ouabain can be overcome by increasing the K^+ concentration in both systems.
7. Ca^{++}addition leads to an inhibition of activity in both systems.
8. Both systems are located in the cell membrane.

i. The Requirement of Metabolic Energy in the Form of ATP. The metabolic dependence of active transport has been well established. Most of the early experiments were done on the red blood cell. Maizels and Whittaker (1940) found that during cold storage red cells would lose K^+ and gain Na^+ by passive diffusion and on subsequent incubation at 37°C would recover K^+ while Na^+ is extruded. Also removal of glucose or inhibition of glycolysis by iodoacetate (IAA) was found to inhibit the active net transport in these cells (Maizels, 1951). This led Maizels to suggest that a product of glycolysis may be involved.

That high energy-rich phosphate compounds, generated during glycolysis, were involved in active transport of sodium was evident. With the advent of the reversible haemolysis technique (Gardos, 1954) red cell ghosts could be prepared devoid of the glycolytic enzymes and with any desired chemicals inside them. Using this system Hoffman (1960) found that ATP but not other nucleotides (CTP, UTP, GTP, and ITP) could support active ion transport.

Caldwell and co-workers (1960) were able to inhibit ^{24}Na efflux from squid giant axons with cyanide, dinitrophenol (DNP) or azide. When they injected ATP or compounds readily converted to ATP into a fully poisoned axon, but not if placed in the external medium there was a brief period of increased Na^+ efflux and the magnitude of this depended on the amount of energy-rich phosphate compounds injected. AMP, GTP, and ITP were not effective. Thus ATP could be used in a poisoned axon as the energy source for active transport.

Studies on the properties of the $(Na^+ + K^+)$-ATPase showed that only the hydrolysis of ATP was stimulated by Na^+ and K^+. The hydrolysis of other nucleotides (ADP, ITP, GTP, or UTP) by this enzyme was not stimulated in the presence of Na^+ and K^+ in the medium (Skou, 1960; Post and co-workers, 1960; Rendi and Urh, 1964). Also ADP itself will inhibit the ATPase reaction. This has led Charnock and Opit (1968) to postulate a "feed-back control" of the enzyme by the level of the ATP:ADP ratio within the cell.

ii. Sodium and Potassium Requirements. Both ATP breakdown and sodium transport are stimulated by Na^+ on the internal surface of the red cell membrane; K^+ stimulation of the ATPase and of active Na^+ transport occurs at the external surface. Furthermore ATP is hydrolysed only when present inside the cell and stimulation of the ATPase is regulated by the concentrations of internal Na^+ and external K^+ as is Na^+ transport (Whittam, 1962; Sen and Post, 1964). The concentration of Na^+ and K^+ required to produce half maximal activation of both ATPase and the active transport system were found to be identical (Post and co-workers, 1960; Dunham and Glynn, 1961).

The rate of active Na^+ transport is decreased when external K^+ is removed (Glynn, 1956). Many variables which decrease Na^+ efflux also decrease K^+ influx into cells. This led to the hypothesis that the outward transport of Na^+ was chemically or electrically coupled to the uptake of K^+. For activation of both Na^+ efflux and the stimulated hydrolysis of ATP by $(Na^+ + K^+)$-ATPase the K^+ requirement can be replaced

by other monovalent cations (Li^+, Cs^+, Rb^+, NH_4^+). However, the internal Na^+ requirement for both transport and the ATPase cannot be replaced by the monovalent cations (Skou, 1960; McConaghly and Maizels, 1962). It was also shown that K^+ can displace Na^+ from its activation site, rendering the enzyme less active and therefore the enzyme activity seems to depend on the internal Na^+:K^+ ratio rather than on the concentration of each ion.

iii. Cardiac Glycoside Sensitivity. Cardiac glycosides were first shown to inhibit ion transport in red cells by Schatzmann (1953), and have since then been found effective in many tissues capable of transporting ions. The site of action of these drugs appears to be the sodium pump itself. The glycosides do not inhibit metabolism, or affect cellular ATP levels but do inhibit the ($Na^+ + K^+$)-ATPase and are effective inhibitors of transport in red cell ghosts. It has also been shown that their effect may be reduced by increasing the K^+ concentration in the external medium (Glynn, 1957). That the inhibitory effects of glycosides on Na^+ transport result through an action on the outside of the cell membrane has been demonstrated in nerve (Caldwell and Keynes, 1959) and in the red cell (Whittam, 1958).

Ouabain and related cardiac glycosides in low concentrations were also found to be potent inhibitors of the ($Na^+ + K^+$)-ATPase (Dunham and Glynn, 1961; Caldwell and Keynes, 1959; Judah and Ahmed, 1964b). The Na^+ and K^+ stimulated portion of the ($Na^+ + K^+$)-ATPase activity is completely inhibited by the glycosides. For inhibition of either the transport system

or the $(Na^+ + K^+)$-ATPase of intact red cells the glycoside must be outside the cell membrane (Glynn, 1964). Moreover, the concentration of ouabain required to cause half-maximal inhibition of the Na^+ and K^+ stimulated portion of the $(Na^+ + K^+)$-ATPase is similar to that needed for inhibition of active transport in the red cell (Post and co-workers, 1960). The structural requirements of glycosides for inhibition of the $(Na^+ + K^+)$-ATPase are also the same as those for inhibition of active Na^+ transport (Dunham and Glynn, 1961).

The degree of enzyme inhibition by the glycosides was diminished by increased K^+ concentration outside the red cell membrane as was the inhibition of ion transport (Dunham and Glynn, 1961; Kingsolving and co-workers, 1963). Thus the parallel action of these drugs on both the enzyme and transport system has further substantiated the association between the two systems.

Glycosides were believed to be competitive inhibitors of the $(Na^+ + K^+)$-ATPase and ion transport in competition with K^+ for the K^+ activating site (Glynn, 1957; Skou, 1957). However, Matsui and Schwartz (1966) have presented kinetic evidence that the ouabain inhibition of the $(Na^+ + K^+)$-ATPase in cardiac tissue is not due to a displacement of K^+ from its activating site. Also Hoffman (1966) presented evidence that K^+ prevents the action of glycosides by a non-competitive allosteric manner.

iv. Effects of Other Inhibitors. The concentrations of Mg^{++} and ATP required for maximal $(Na^+ + K^+)$-ATPase activity are usually equal.

Addition of Ca^{++} in low concentrations inhibits the $(Na^{+} + K^{+})$-ATPase, and Epstein and Whittam (1966) have presented evidence that Ca^{++}-ATP rather than ionic Ca^{++} acts as an inhibitor that is competitive with Mg^{++}-ATP. Ca^{++} also inhibits active transport of Na^{+} when present inside the cell (Hoffman, 1962). In a kidney cortex $(Na^{+} + K^{+})$-ATPase preparation substitution of Mg^{++} by Ca^{++} results in an ATPase activity equivalent to or slightly greater than that found with Mg^{++} alone. However, the addition of Na^{+} and K^{+} does not enhance this activity (Charnock and Post, 1963).

The metabolic inhibitors azide, DNP, IAA and cyanide, which do not directly inhibit the active transport of Na^{+}, have no effect upon the $(Na^{+} + K^{+})$-ATPase (Hoffman, 1962). The enzyme is, however, inhibited by sulfhydryl blocking agents (Weed and Berg, 1962). Those reagents which react only with the most readily available sulfhydryl groups, such as n-ethylmaleimide (NEM) and IAA, do not inhibit the enzyme while the more potent inhibitors, $HgCl_2$ and chloromerodin, block the $(Na^{+} + K^{+})$-ATPase (Weed and Berg, 1962). However, preincubation with NEM for 30 minutes has also been reported to be effective (Fahn and co-workers, 1966).

The $(Na^{+} + K^{+})$-ATPase can also be inhibited by mercurial diuretics, ethacrynic acid and furosemide (Landon and Norris, 1963; Taylor, 1963; Hook and Williamson, 1965; Jones and co-workers, 1965). However, a clear connection between their diuretic properties and their ability to inhibit ATPase has not been established. The enzyme has also been found to be inhibited by the lower aliphatic alcohols (Israel and co-workers, 1965, 1966; Israel and Salazor, 1967).

v. Occurrenee and Cellular Localization. Demonstration of the $(Na^+ + K^+)$-ATPase to be a component of the cell membrane, where the hypothetical "sodium pump" is assumed to be located, provides further support for the hypothesis that it is involved in active ion transport. In studies with intact cells the enzyme has been found in the membrane of the erythrocyte (Post and co-workers, 1960; Dunham and Glynn, 1961; Whittam, 1962) and in the sheath of the giant axon of the squid (Bonting and Caravaggio, 1962). ATPase activity has also been demonstrated by histochemical techniques in kidney tubule cells (Spater and co-workers, 1958), liver cell plasma membrane (Essner and co-workers, 1958), HeLa cell membrane (Epstein and Holt, 1963), and in the cell membranes and pinocytotic vesicles of aortic endothelial cells (Hoff and Graf, 1966). However, a great deal of controversy remains as to whether the histochemical lead staining methods used to demonstrate the location of the enzyme are valid (Tormey, 1966; Rosenthal and co-workers, 1966; Novikoff, 1967; Moses and Rosenthal, 1967).

In most tissues it is not as easy to obtain cell membranes as with red cells or giant squid axons. With the use of differential centrifugation techniques it is, however, possible to isolate membrane fractions (microsomes) from broken-cell homogenates. Using these techniques the $(Na^+ + K^+)$-ATPase was found to be located either in the low sedimenting nuclear fraction (Wheeler and Whittam, 1962; Kinsolving and co-workers, 1963; Emmelot and Bos, 1966), in the microsomes (Schwartz and co-workers, 1962; Schwartz, 1964; Glynn, 1963; Whittam and Blond, 1964) or equally distributed in both (Wheeler and Whittam,

1964; Bonting and co-workers, 1962).

The microsomes, which sediment at high gravitational forces (30,000 to 100,000 x g), are thought to be largely composed of endoplasmic reticulum (Hanzon and Toshi, 1959; Siekevitz, 1959) and fragments of the plasma membrane (Hokin and Hokin, 1960; Charnock and Post, 1963). Wallach and his co-workers, using an immunologic technique, concluded that small fragments of plasma membrane sediment are harvested with the "microsomes" and provide the bulk of the $(Na^+ + K^+)$-ATPase activity in the microsomal fraction of cell homogenates of Ehrlich ascites tumor cells (Wallach and Ullrey, 1964; Wallach and Kamat, 1964; Kamat and Wallach, 1965).

In summary the involvement of the $(Na^+ + K^+)$-ATPase with the active transport of Na^+ seems very likely, and has been well correlated to the criteria for an active transport system which were originally set down by Post and co-workers (1960) and elaborated upon more recently by Skou (1964).

C. Separation and Further Purification of the $(Na^+ + K^+)$-ATPase from Other ATPase Activities.

Attempts at further purification by removing the enzyme from the membrane usually are associated with a loss of enzymatic activity. Also during the isolation and purification of membrane fractions with $(Na^+ + K^+)$-ATPase activity, some of the essential features of the sodium pump may be lost.

i. Chemical and Physical Techniques Which Alter the Activity Ratio. When studying preparations of the membrane ATPase two types of ATPase activities are found to exist. One is the ouabain insensitive ATPase activity observed in the presence of Mg^{++} (Mg^{++}-ATPase) and the other is the ouabain sensitive activity in the presence of Mg^{++}, Na^{+} and K^{+}. The activity ratio indicates the proportion of each of these two types of activities present. It is defined as the activity of the enzyme preparation in the presence of Mg^{++}, Na^{+} and K^{+}, divided by the activity of the enzyme in the presence of Mg^{++} alone ($Mg^{++} + Na^{+} + K^{+}/Mg^{++}$). It has been suggested that the two enzyme activities may be either two distinct enzymes (Hoffman, 1962) or two forms of the same enzyme in which the Mg^{++}-ATPase may be ($Na^{+} + K^{+}$)-ATPase material which has become desensitized to Na^{+} and K^{+} during the course of preparation.

The activity ratio can be altered by the method of enzyme preparation (Post and co-workers, 1960; Dunham and Glynn, 1961). In some cases it is believed that the Mg^{++}-ATPase activity is due to the presence of contaminating mitochondrial fragments with this enzyme activity. In such cases the Mg^{++}-ATPase activity could be inhibited selectively by low concentrations of azide, oligomycin and quinidine which inhibit mitochondrial ATPase (Samaha, 1965, 1966). An increase in activity ratio has also been demonstrated by the use of the detergent deoxycholate, which selectively solubilized some of the Mg^{++}-ATPase (Skou, 1962; Järnefelt, 1964) and left the ($Na^{+} + K^{+}$)-ATPase unaffected. On the other hand Charnock and Post (1963) reported that deoxycholate increased the activity ratio not by depression of the Mg^{++}-ATPase activity but by increasing the

specific activity (rate of ATP hydrolysis/mg protein nitrogen) of the Na^+ and K^+ activated component. They also presented results indicating that the effect of deoxycholate treatment was similar to the effects observed after aging of microsomal material.

Schwartz (1962, 1965) and Schwartz and Laseter (1964) have shown that aging of the microsomal preparation of cardiac muscle increases the activity ratio. They also found that the basic protein histone could inhibit the Mg^{++}-ATPase activity more than the $(Na^+ + K^+)$-ATPase activity, and thus postulated that histones, which may not have been released during homogenization, were released and interacted with the enzyme during the aging procedure. Histones were also found to inhibit actomyosin ATPase. It is therefore conceivable that the increase in activity ratio observed, in their microsomal ATPase preparation, was due to inhibition of the actomyosin ATPase which may have been present as a contaminant.

The Mg^{++}-ATPase and $(Na^+ + K^+)$-ATPase components have also been separated by NaI extraction (Nakao and co-workers, 1963) and by ultrasonication (Tosteson and co-workers, 1965). Askari and Fratantoni (1964) found that sonication of erythrocyte membranes resulted in a Mg^{++}-ATPase activity which could be stimulated independently by Na^+ or K^+ alone and this activity was insensitive to ouabain unless the ions were both present. Somogyi (1964) has reported a method which completely removed the Mg^{++}-ATPase activity from the $(Na^+ + K^+)$-ATPase activity in a rat brain preparation. Using consecutive heat and detergent treatments he was able to get an enzyme which did not hydrolyse ATP in the presence of Mg^{++} unless Na^+ and K^+ were added.

Solubilization of the membrane ATPase-system by the use of detergents has allowed further purification (increased enzyme activity) of the enzyme (Medzihradsky and co-workers, 1967; Uesugi and co-workers, 1969). These workers concluded that the enzyme has lipoprotein properties with an apparent molecular weight of 670,000. However, Kepner and Macey (1968) have reported a molecular weight of 250,000. If it is assumed that the ATPase in different tissues is the same, then these different values could be an indication of different purities of preparations. However, the many difficulties involved in molecular weight determinations of macromolecules may also be responsible for the different values.

Thus it is apparent that more extensive purification of the enzyme and studies of its properties are required. Also the answer to the question of whether the enzyme activities observed are in fact separate or simply manifestations of a single enzyme activity has not been solved.

D. Role of Lipoproteins in the $(Na^+ + K^+)$-ATPase Activity.

Recent evidence indicates that phospholipids are involved in maintaining the full activity of the $(Na^+ + K^+)$-ATPase and suggest that the enzyme is a lipoprotein. The activity of this enzyme is reduced by treatment with phospholipases (Skou, 1961; Schatzmann, 1962; Swanson and co-workers, 1964). Swanson and co-workers attributed this loss of activity to the removal of phosphorylcholine from lecithin. Emmelot and Bos (1965) found that neuraminidase treatment, which removed the terminal o-glycosidic linked sialic acid from a membrane glycoprotein, affected

both Mg^{++}-ATPase (inhibition) and the $(Na^+ + K^+)$-ATPase (inhibition or activation). The latter effect depended on the specific activity of the $(Na^+ + K^+)$-ATPase preparation before treatment, ATPase preparations with low specific activities being activated and those with high specific activities being inhibited by the action of neuraminidase.

Tanaka and Abood (1964), and Tanaka and Strickland (1965) found that deoxycholate solubilization of the $(Na^+ + K^+)$-ATPase derived from beef brain, results in a loss of Na^+ and K^+ activity. They could restore the activity by the addition of commercial animal lecithin. The phospholipid addition had no effect on the Mg^{++}-ATPase but could stimulate the activity with Na^+ and K^+ added. Fenster and Copenhaver (1967) carried these studies further and were able to demonstrate that the activating effect obtained from commercial lecithin was due to the presence of phosphatidyl serine. Also Ohnishi and Kawamura (1964) showed that phosphatidyl serine would restore the activity of the $(Na^+ + K^+)$-ATPase in a preparation which had been incubated with phospholipase A.

E. Role of a Phosphorylated Intermediate in the $(Na^+ + K^+)$-ATPase Reaction Sequence.

The molecular mechanism by which the hydrolysis of ATP results in the active transport of Na^+ and K^+ is still obscure. However, recent experimental findings have provided evidence for the involvement of a phosphorylated intermediate. In 1960, on studies using C^{14}-labeled ADP as a substrate for the membrane ATPase in crab nerve, Skou postulated a sequence of events which suggested that a phosphorylated intermediate

was involved. This work was then followed by many others, using ATP labeled with P^{32} in the terminal position, to demonstrate the formation of a phosphate-bound protein complex during the hydrolysis of ATP by the $(Na^+ + K^+)$-ATPase.

In 1963 Charnock and co-workers found that transfer of the terminal phosphate of ATP^{32} to a protein component of the preparation was enhanced by the presence of Na^+, and that upon the subsequent addition of K^+, a reduction in the amount of labeling occurred. No enhancement of labeling occurred with K^+ alone. By directly studying the rate of breakdown of the phosphorylated intermediate, in the presence and absence of K^+, Post and co-workers (1965) showed that K^+ increased dephosphorylation of the intermediate rather than inhibiting its formation by Na^+. Moreover, under these conditions more P^{32} was hydrolysed from ATP than in the presence of Na^+ alone.

The cardiac glycoside ouabain reduced the dephosphorylation of the labeled protein produced by the addition of K^+; however, when conditions were adjusted so that the degree of labeling was sensitive to the Na^+ concentration then ouabain also inhibited the Na^+ dependent labeling to some extent (Post and co-workers, 1965; Rodnight and co-workers, 1966; Charnock and co-workers, 1967). These results have been summarized by Charnock and Opit (1968) as follows, where E = enzyme.

$$\text{I.} \quad E + ATP^{32} \xrightarrow[\text{? ouabain}]{Na^+} E \sim P^{32} + ADP$$

$$\text{II.} \quad E \sim P^{32} + H_2O \xrightarrow[\text{ouabain}]{K^+} E + P^{32}$$

In this reaction sequence Na^+ is required for the formation of the phosphorylated intermediate, and K^+ is necessary for the breakdown of the intermediate.

In 1965 Hokin and co-workers while studying the phosphorylated intermediate compound from brain tissue found that digestion of the protein after previous P^{32} incubation released two major radioactive peptides. Treatment of these peptides with hydroxylamine acetate or with acyl phosphatase liberated most of the radioactivity from these peptides as inorganic phosphate. This observation led these workers to suggest that one of the phosphorylated intermediates in the transport ATPase is probably an acyl phosphate. The stability of the intermediate at various pH's was also compatible with its being an acyl phosphate compound.

A close relationship between the rates of hydrolysis of acyl phosphate and ATP by the transport ATPase of guinea pig kidney cortex preparations has led Bader and co-workers (1966) and Bader and Sen (1966) to suggest that the K^+ dependent acyl phosphatase activity is the same as the K^+ dependent hydrolysis of the phosphorylated intermediate. Rendi (1966) showed that by lipid extraction of a $(Na^+ + K^+)$-ATPase preparation the K^+ dephosphorylation step could be separated from the Na^+ phosphorylation step. Recently the acyl phosphate intermediate in brain microsomal $(Na^+ + K^+)$-ATPase has been identified as an L-glutamyl-γ-phosphate residue (Kahlenberg, Galsworthy and Hokin, 1967).

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Some doubt has been raised as to whether the acyl phosphate intermediate exists (Schoner and co-workers, 1966; Chignell and Titus,

1966). Their hypothesis was that hydroxylamine dephosphorylated the intermediate by irreversibly combining with the active site and therefore should inactivate the $(Na^+ + K^+)$-ATPase reaction. However, the ATPase reaction could not be inhibited even with high concentrations of hydroxylamine.

In 1967 Charnock and co-workers further studied the effects of hydroxylamine and demonstrated that it functions as a substitute for K^+ in the reaction sequence. Also Bader and Broom (1967) found that hydroxylamine will inhibit the $(Na^+ + K^+)$-ATPase, if small amounts of Ca^{++} (10^{-8} M - 10^{-5} M) are present.

Recently Bader and co-workers (1968) examined the possible participation of a phosphorylated intermediate in activity of the $(Na^+ + K^+)$-ATPase from six tissues in eleven species and concluded that the intermediate did participate in the mechanism of the $(Na^+ + K^+)$-ATPase in a similar manner in all cases.

F. Other Modes of Na^+ Transport.

All of the studies referred to above deal with a ouabain sensitive active sodium pump which translocates Na^+ from inside the cell out when potassium is present in the external medium. However, increasing evidence is accumulating that Na^+ can be transported by other means.

i. Hoffman's Three Pump System in Red Blood Cells. In 1966, Hoffman and Kregenow while studying the efflux of Na^+ from red cells demonstrated that more than one pathway for this flux exists and postulated

the existence of separate pumps. The properties of these pumps have been summarized by Hoffman (1966) and are listed below.

Pump IA--A Na^+ outflux that is coupled to and dependent upon the presence of K^+ in the external medium (the classical Na^+-K^+ pump discussed above). It is inhibited by cardiac glycosides and is unaffected by the presence or absence of external Na^+.

Pump IB--A Na^+ outflux that requires the presence of Na^+ in the external medium. This flux is inhibited by cardiac glycosides and is unaffected by the presence or absence of K^+ in the external medium.

Pump II--A Na^+ outflux that like pump IB has an obligatory requirement for external Na^+, is independent of external K^+, but unlike pump IB is insensitive to cardiac glycosides. This flux is inhibited by ethacrynic acid in the presence of maximally inhibiting concentrations of cardiac glycosides.

Pump I, composed of two parts, A and B, is defined as the glycoside-sensitive component of Na^+ efflux, and pump II is defined as the glycoside-insensitive component of Na^+ efflux which is inhibited by ethacrynic acid. Both pumps are active in the sense that they produce a net uphill movement of Na^+ against its electrochemical gradient. Hoffman excluded the involvement of Na^+ exchange diffusion in either pump IB or pump II by showing that the influx of Na^+, in a Na^+ medium, is unaffected by cardiac glycosides or ethacrynic acid under conditions of marked inhibition of Na^+ outflux.

Pumps I and II also differ in their concentration dependencies

on the internal and external concentrations of Na^+. Both parts of pump I depend on ATP for their source of energy but the energy source for pump II is yet unknown and does not appear to be ATP. During continued incubation at 37°C in the absence of substrate pump I is completely inhibited after eight hours, correlating with the disappearance of ATP, but pump II is still active even after fourteen hours. The efflux of Na^+ during operation of pump II can be accompanied by an influx of K^+ equal in magnitude, which depends on Na^+ in the external medium, is glycoside-insensitive but is inhibited by ethacrynic acid. Moreover, the K^+ influx shows a lack of metabolic dependence similar to that of pump II. Hoffman therefore claims that this K^+ influx is distinguishable from that associated with pump IA.

The Na^+ efflux from frog sartorius muscle is composed of three independent and additive components (Mullins and Frumento, 1963; Horowicz, 1965; Keynes, 1966). One component is strophanthidin and K^+ sensitive, the second is not blocked by strophanthidin and requires Na^+ in the external solution, while the last and smallest component is described as a passive leak. The proportion of the two major components of Na^+ efflux depend on the intracellular Na^+ concentration. It was found that in fresh tissue only a small fraction of the Na^+ efflux is ouabain sensitive whereas in Na^+ rich tissues a larger ouabain sensitive component exists (Sjodin and Beauge, 1968; Beauge and Sjodin, 1968).

LeBlanc and Erlij (1969) demonstrated that the Na^+ dependent Na^+ efflux in frog muscle can be inhibited by ethacrynic acid and suggested that it was related to Hoffman's pump II. However, unlike Na^+

flux in the red blood cell, ethacrynic acid was also able to inhibit the Na^+ influx during the operation of this pump and therefore would indicate a Na^+-Na^+ exchange reaction to exist. Garrahan and Glynn (1967b) have also presented evidence for the existence of pump II in red cells but they did not study the effects of ethacrynic acid. Moreover, Garrahan and Glynn (1965, 1967a) have shown that ouabain sensitive Na^+-Na^+ exchange exists in red cells in the absence of external K^+, similar to pump IB. This exchange process requires Na^+ in the external medium for maximum Na^+ efflux and requires ATP, but does not cause any appreciable ATP hydrolysis.

ii. The Na^+-Cl^- Pump. Another active sodium pump which involves the outward movement of Na^+ followed by the passive movement of Cl^- has also been identified. This pump has been shown to exist in kidney tubule and kidney slices and is believed to be involved with maintaining cell volume (Kleinzeller and Knotkova, 1964; Whittembury, 1966; Macknight, 1968a, 1968b). When metabolism is inhibited in these tissues by anoxia and cold, or metabolic inhibitors the tissues swell; upon returning them to a normal medium they lose water and ions. This recovery could also occur in the presence of ouabain and thus suggested that some mechanism other than the Na^+-K^+ pump was responsible for this recovery (Kleinzeller and Knotkova, 1964, 1967).

Whittembury (1968) describing the Na^+-Cl^- pump in kidney cells presents evidence that it results in an active extrusion of Na^+ followed by a concomitant passive efflux of Cl^- which may be occurring by an electrogenic mechanism. This pump is not inhibited by ouabain, does not

need K^+ in the extracellular medium and is inhibited by ethacrynic acid. Thus it is distinguishable from the Na^+-K^+ transport or from Hoffman's pump I. Some of its properties point to a relationship to Hoffman's pump II; however, this Na^+-Cl^- pump does seem to require energy from ATP since it will not operate in anoxia or when DNP is added.

Diamond (1962a,b,c, and 1964) has also reported observations of electrically neutral coupled active transport of Na^+ and Cl^- which is accompanied by a passive movement of water across the wall of gall bladder. This active Na^+-Cl^- pump possesses a dependence for metabolic energy but it differs from the Na^+-Cl^- pump in kidney cells by being inhibited by ouabain and a K^+ free solution on the serosal side. The K^+ requirement for the Na^+-Cl^- transport in the gall bladder was not associated, however, with a transport of K^+ (Wheeler, 1963).

Thus the existence of at least four types of Na^+ pumps are indicated. First, one which is inhibited by ouabain, requires K^+ externally and is probably involved with maintenance of intracellular ion content and maintains the membrane potential. This is Hoffman's pump IA. (Pump IB does not require K^+ externally and may be a Na^+-Na^+ exchange.) The second pump is a Na^+-Cl^- pump which is inhibited by ethacrynic acid, is ATP dependent and controls cell volume. Third, a Na^+-Cl^- pump which is inhibited by ouabain and requires metabolic energy and external K^+. The fourth type is a Na^+ pump not requiring ATP or external K^+, but it does require external Na^+ and is inhibited by ethacrynic acid (Hoffman's pump II).

G. Other Membrane ATPases.

Since these other types of Na^+ pumps exist which may be using ATP as their source of energy, it seems that a membrane ATPase with properties different from the classical $(Na^+ + K^+)$-ATPase may exist. This leads one to wonder if the Mg^{++}-ATPase or the ouabain insensitive ATPase remaining after ouabain inhibition of the $(Na^+ + K^+)$-ATPase may be somehow involved. In 1967 a report appeared demonstrating the presence of another type of ATPase in red cell membrane which required Na^+ for maximal activation, was sensitive to ouabain and inhibited by K^+ (Czerwinski and co-workers, 1967). This enzyme activity could only be demonstrated when low concentrations of ATP (2×10^{-6} M) were used. The chief differences between this Na^+-ATPase and the $(Na^+ + K^+)$-ATPase are its ability to operate maximally only in the absence of K^+ and in the presence of Na^+. These authors believe it operates under the normal conditions used to assay $(Na^+ + K^+)$-ATPase; however, at high ATP concentration the percent contribution by this enzyme becomes trivial and is easily masked. A similar enzyme activity in a calf brain membrane fraction has been described by Neufeld and Levy (1969). Whether this new ATPase activity is another enzyme or a different facet of the $(Na^+ + K^+)$-ATPase and whether the enzyme is part of the same or a different transport system has not been resolved.

Ethacrynic acid has also been shown to inhibit $(Na^+ + K^+)$-ATPase (Duggan and Noll, 1965; Nechay and co-workers, 1967). Ethacrynic acid (10^{-3}M) could inhibit 50% of the $(Na^+ + K^+)$-ATPase and this inhibition could be increased to 79% if the enzyme was pre-incubated with the drug for 60 minutes

prior to enzyme assay (Duggan and Noll, 1965). Nechay and co-workers (1967) also found that the inhibition of the $(Na^+ + K^+)$-ATPase by ethacrynic acid could not be enhanced by ouabain. However, these studies did not consider the possible involvement of ethacrynic acid sensitive Na^+ pumps with a membrane ATPase.

CHAPTER II

METHODS AND MATERIALS

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A. Tissue Preparation.

All the following steps were carried out in the cold (4°C). Female New-Zealand white rabbits weighing 1.5-2.5 Kg were killed by cervical dislocation. The aorta was quickly excised, chilled and rinsed free of blood with cold 0.25 M sucrose. The intima-media layer was separated by slitting the aorta lengthwise, laying it flat on a piece of sucrose wetted tissue paper, and carefully stripping off the inner intima-media muscle layers. The tissue from rabbit aorta usually weighed 450-550 mg. The tissues from two rabbits were blotted, weighed, cut into small pieces and a 5% (w/v) homogenate in 0.25 M sucrose was prepared.

B. Homogenization.

i. Glass-Glass and Teflon-Glass Homogenization. For some experiments the tissue was homogenized in a Potter-Elvehjem ground glass homogenizer of about 17 ml capacity with a clearance of 0.004-0.006 inch between pestle and tube. A 10% (w/v) sucrose homogenate of the tissue was prepared by 20 strokes of the pestle, which was driven by a 1/150 h.p. motor at about 1,550 rpm. The tube was then rinsed with 0.25 M sucrose and the rinse was added to the homogenate to obtain a final 5% (w/v) homogenate. On other occasions a Potter-Elvehjem homogenizer with an unground glass tube and teflon pestle of the same specifications as the all glass homogenizer was used. However, due to the toughness of the tissue it was found necessary to cut the tissue into small pieces before

homogenization by this technique. This was accomplished by freezing the tissue into a block of 0.25 M sucrose which was mounted on a Leitz-Wetzlar freezing microtome, and sectioned at 5 μ. The resulting material was then homogenized in the teflon-glass homogenizer with about 25-35 strokes. This still did not completely disrupt the tissue and only the material which had passed the pestle was decanted off and used.

ii. VirTis Homogenization. It was shown that the ground glass type homogenizer released free Na^+ (about 0.2 mM) into the medium (Allen, 1967) and because of the difficulty of using the teflon homogenizer, the VirTis "23" homogenizer (The VirTis Co. Inc., Gardener, N.Y.) was used for most of the experiments.

The tissue (about 1 gm) was added to a homogenizing flask of 5-30 ml capacity along with 10 ml of cold 0.25 M sucrose. This was attached to the VirTis and surrounded by an ice-water bath. The tissue was homogenized at top speed (23,000 rpm) for one minute, the sides of the flask were then rinsed with an additional 5 cc of sucrose solution to assure complete homogenization which was then carried out for an additional four minutes at top speed. The homogenate was then made up to 5% (w/v) by adding additional sucrose solution used to rinse the flask.

C. Subcellular Fractionation and Membrane Isolation.

i. Differential Centrifugation. The homogenates were fractionated according to the scheme shown in Figure 1 and described in detail below. Equal portions of the tissue homogenate were transferred into two

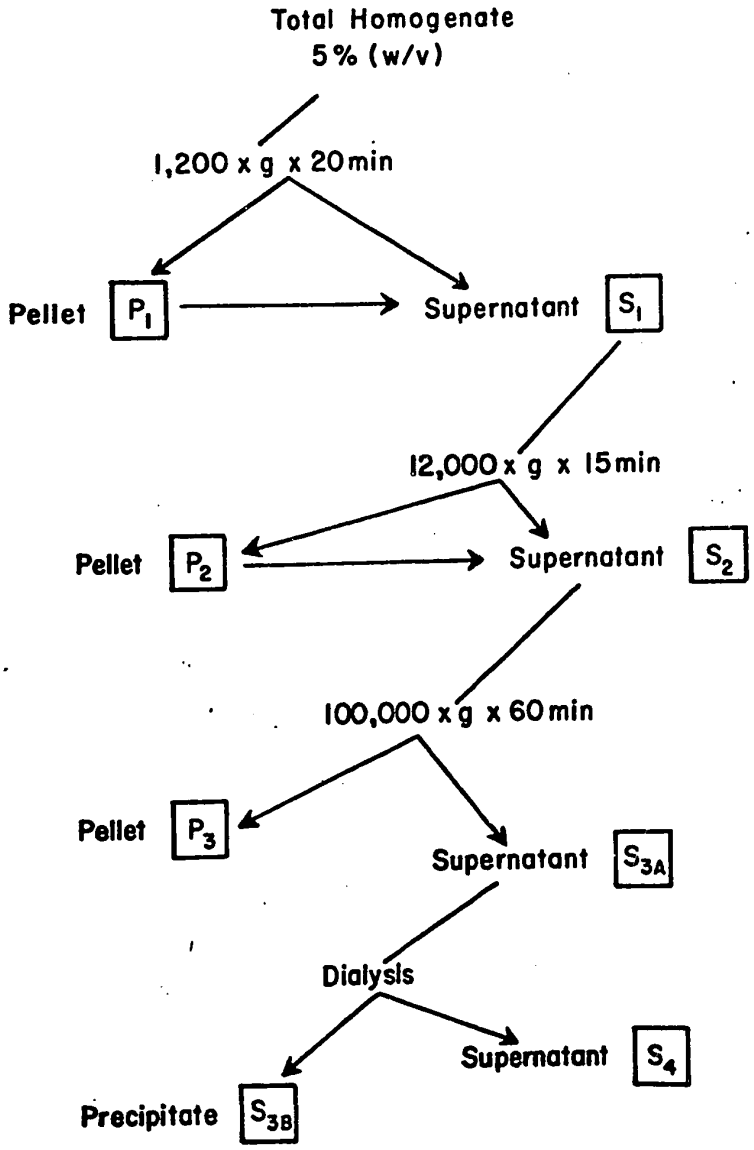

Figure 1. Procedure for isolation of subfractions of aortic homogenates by differential centrifugation.

15 ml cellulose nitrate tubes and centifuged at 1,200 x g for 20 minutes in a clinical centrifuge (International, Model CL), the supernatant saved, the sediment resuspended in 0.25 M sucrose, centrifuged against the same force, and the two supernatants combined (S_1). The pellet was called P_1 (nuclei and cell debris). S_1 was then centrifuged at 12,000 x g for 15 minutes in a Spinco Model L2 ultracentrifuge fitted with a type 40 rotor to obtain a pellet. This pellet was resuspended in 0.25 M sucrose and recentrifuged at 12,000 x g for 15 minutes to obtain pellet P_2 (heavy mitochondrial fraction) and a combined supernatant S_2. S_2 was then centrifuged at 100,000 x g for 60 minutes to obtain pellet P_3 (crude microsomes) and supernatant S_{3A} (soluble material). The S_{3A} supernatant was then separated from the sucrose by dialysis against de-ionized water. The flocculent precipitate (S_{3B}) thus formed was washed once and then re-dissolved in 5 mM histidine buffer. The various pellets isolated were resuspended in de-ionized water if used the same day, or in 5 mM histidine buffer pH 7.6 if kept for longer periods of time.

ii. Density Gradient Centrifugation. Density gradient centrifugation was used to obtain a membrane microsomal preparation less contaminated by mitochondrial material (Figure 2). 10 to 15 ml of fraction S_2 or a resuspension of pellet P_3 in 0.25 M sucrose was layered on top of a discontinuous density gradient of 1.4 M and 2.0 M sucrose and centrifuged for 3 hours at 40,000 rpm in a swinging-bucket type SW40 rotor to obtain two layers P_D (microsomal membranes), M_D (light mitochondria) and a pellet N_D (heavy mitochondria and collagen debris).

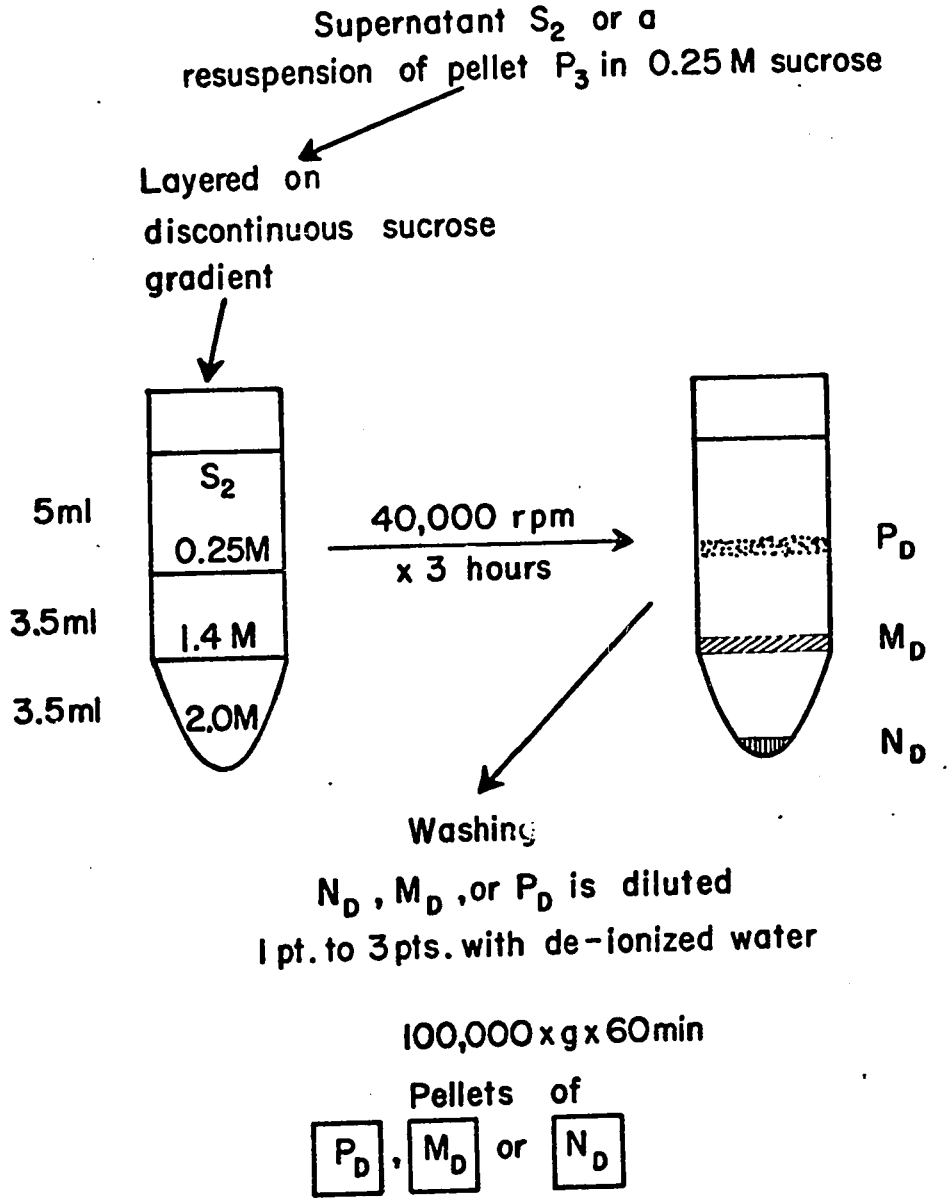

Figure 2. Density gradient isolation scheme. (pt. = part)

The individual layers (P_D and M_D) were carefully removed with a Pasteur pipet, transferred to centrifuge tubes, and diluted with an additional 2 volumes of de-ionized water. The gradient tube was then cut about 2 cm from the bottom, the pellet (N_D) was removed, resuspended in de-ionized water and transferred to another centrifuge tube. The tubes containing P_D, M_D and N_D were then centrifuged at 100,000 x g for 60 minutes in a type 40 rotor to obtain pellets of P_D, M_D and N_D. These pellets were then separately resuspended in de-ionized water or 5 mM histidine at pH 7.6. For a few experiments 1 ml sodium tetraphenylboron was added to 9 ml S_2 (final concentration of 1 mg NaTPhBo/ml S_2) prior to isolation of P_D. All the fractions obtained by the techniques described above were kept cold until used.

iii. Cell Membrane Isolation. This technique was based on the procedure of Rosenthal and co-workers (1965) as modified by Carroll and Sereda (1968).

Solutions:

1. KCl buffer. The composition and final concentrations are as follows:

   45 mM KCl

   30 mM $KHCO_3$

   2.5 mM histidine HCl

   Adjusted to pH 7.8 by the addition of a small amount of Tris. The final volume of the solution was 50 ml.

2. NaOH 2.5×10^{-7} N.

3. $\underline{CaCl_2}$ 50 mM.

The tissue was prepared as previously described.

Glycerol treatment:

The tissue was placed in the following glycerol-Krebs mixtures:

1:9 glycerol-Krebs (w/v) 37°C 2 hrs.

2:8 glycerol-Krebs (w/v) 0°C 1/2 hr.

3:7 glycerol-Krebs (w/v) -4°C 1/2 hr.

To make the glycerol-Krebs solution, the appropriate amount of glycerol (1, 2 or 3 gm) was weighed in a previously tared 10 ml volumetric flask. The solution was then brought to 10 ml with Krebs media (composition in section K). After glycerol treatment the tissue was frozen on a block of CO_2, cut into 1-cm pieces, and set into frozen blocks with 50 mM $CaCl_2$. These blocks were then placed in foil, and stored in liquid nitrogen for varying lengths of time.

Tissue sectioning:

The blocks of tissue were mounted on a Letiz-Wetzlar freezing microtome, and sectioned at 5 μ. The resulting material was placed in cold 50 mM $CaCl_2$.

Centrifugation and NaOH treatment:

All centrifugation was carried out in a clinical centrifuge at maximum speed (1,200 x g).

1. The above material was spun and the supernatant decanted.
2. The pellet was resuspended in 5 ml KCl buffer and centrifuged twice. Each time the supernatant was discarded.

3. The pellet was resuspended in buffer, incubated for 1/2 hr at 37°C and then cooled in ice water.
4. The suspension was centrifuged three times, and each time the pellet was resuspended in buffer, and allowed to settle in ice water before centrifugation.
5. The above procedure was repeated using de-ionized water instead of buffer.
6. The above procedure was repeated five to six times using NaOH instead of water. The volume used was 10 ml.
7. The residue was suspended in 10 ml of 0.2 mM ATP, mixed gently and the total added to 75 ml of de-ionized water.
8. The suspension was rinsed with double distilled water two or three times, each time centrifuging and discarding the supernatant.
9. The pellet was resuspended in double distilled water for storage.

iv. LiBr-Salt Extraction of Muscle. The method designed for isolating the membrane ATPase from cardiac muscle (Potter and co-workers, 1966) was used. The technique was carried out on both cardiac and aortic muscle. In one case 5-10 gm of rabbit ventricles were used and in the other 5-7 gm of rabbit aorta prepared as previously described. The isolation was carried out in the cold (4°C).

Solutions:

1. Tris Buffer.

1 mM Tris-HCl, and

1 mM ethylene diamine tetraacetic acid (EDTA)

adjusted to pH 6.2, pH 6.8 or pH 7.6.

2. LiBr 1 M.

In each case the tissue was cut into small pieces with scissors and homogenized in a ground glass Potter-Elvehjem apparatus. Sufficient Tris buffer (pH 6.2) was used to give a 10% (w/v) homogenate. The homogenate was strained through gauze to remove large tissue debris, and the pH of the filtrate re-adjusted to 6.8 by the addition of 0.2 M Tris solution.

The homogenate was centrifuged at 1,200 x g for 20 minutes in a clinical centrifuge. The supernatant was discarded and the sediment resuspended in approximately 100 ml of Tris buffer (pH 6.8) and centrifuged again at 1,000 x g for 20 minutes. The supernatant was discarded and the pellet resuspended in 1 M LiBr (5 ml of LiBr solution per gm wet weight of original tissue). This was allowed to stand in the cold for 18 hours. The mixture was then diluted 1:1 with de-ionized water slightly buffered to pH 7.0 with Tris-HCl. The material was then centrifuged at 5,000 x g for 10 minutes (Servall centrifuge and a swinging bucket rotor with 50 ml tubes). The supernatant was discarded and the pellet washed three times by resuspending in Tris-buffer pH 7.6 and centrifugation at 5,000 x g for 10 minutes, to remove excess lithium salts. Finally the pellet was resuspended in 15 ml of de-ionized water.

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D. Further Separation of Microsomal Supernatant Material.

Through the kind assistance and suggestions offered by Dr. A. M. Kidwai of the Department of Pharmacology, University of Alberta, the following procedures were employed to further study the nature of the S_{3B} supernatant material.

i. Isoelectric Focusing. The procedure described by Vesterberg and co-workers (1967) was followed. Isoelectric focusing and separation of the S_{3B} supernatant material was carried out at 25°C in a special vertical electrolysis column of 110 ml capacity equipped with a cooling jacket (LKB-Produkter AB, Stockholm-Bromma, Sweden).

The electrolysis column was filled with a gradient of sucrose and ampholytes (polyamino-polycarboxylic acids with different pI's) selected to give a pH gradient between pH 1.0 and 12. Dialyzed S_{3B} material was dissolved in 1 ml 5 mM histidine and applied to the top of the gradient. The central tube surrounding the anode was filled with a 50% sucrose solution containing 0.05 ml of concentrated phosphoric acid. This prevents acidic proteins and carrier ampholytes from coming into contact with the anode (Vesterberg and Svensson, 1966). For prevention of contact between the ampholytes and the cathode, 0.05 ml of diethylene-triamine was added at the top of the column.

After focusing for 48 hours with a final potential of about 600 V, the contents of the column were eluted into 4 ml fractions, after which the pH of the fractions was measured with a Fisher Accumet pH meter. The amount of protein in the fractions was estimated by measurements

of absorbance at 280 mμ with a Beckman DU spectrophotometer. The fractions with high absorbance at 280 mμ were then dialyzed for 12 hours against de-ionized water at 4°C with two changes. These fractions were then separated and redissolved in 5 mM histidine pH 7.6 and kept cold until used.

ii. Ion Exchange Resin Treatment.

Preparation of cellulose ion exchangers:

1 gm of DEAE-cellulose (Serva, Gallard-Schlesinger Chem. Corp.) or CM-cellulose (Serva) was stirred into 5 ml of de-ionized water and allowed to settle for 45 minutes. The slightly turbid supernatant was discarded. The anion exchange resin (DEAE) was washed with 5 ml of 0.1N HCl and subsequently washed two times with 5 ml of de-ionized water. Washing was carried out by suspending the resin and centrifuging it down in a clinical centrifuge. The cation exchange resin (CM) was washed the same way using 5 ml of 0.1 N NaOH and then de-ionized water.

Treatment of supernatant material:

5 ml of S_{3A} supernatant material (0.085 mg protein N/ml) was suspended with each of the ion exchange resin sediments and allowed to stand in the cold for 30 minutes with occasional shaking. The resin in each case was then centrifuged down and the clear supernatant was collected and used for further studies.

iii. Sephadex Gel Filtration. 3 gm Sephadex G-100 (particle size 40-120 μ, Pharmacia Ltd.) was allowed to swell in 200 ml hot

de-ionized water on a boiling water bath for 5 hours. A 100 ml glass column (2.0 cm diameter) with a sintered glass base and fitted with a narrow piece of rubber tubing at the outlet was used. The column was carefully packed with the swollen slurry avoiding the trapping of air bubbles. 500 ml of de-ionized water was then allowed to flow through the column to aid packing. The final dimensions of the column bed were 2.0 cm x 19.0 cm.

The bed was then equilibrated with 200 ml of 5 mM histidine buffer pH 7.6. The eluant was drained until the meniscus reached the top of the bed and the outlet was closed. Without disturbing the bed surface 4 ml of CM-cellulose treated supernatant material was applied with a Pasteur pipette. The column outlet was opened to allow draining of the sample into the bed. The column was then filled with an additional 20 ml of 5 mM histidine eluant and connected to an eluant reservoir.

The elution at a rate of 4 ml/5 minutes was carried out at 4°C. Eighteen to twenty 4 ml fractions of the elution were collected with the aid of a fraction collector (LKB Radi Rac). The amount of protein in the fractions was estimated by measurements of absorbance at 280 mμ with a Beckman DU spectrophotometer. Some of the protein containing fractions were then kept for ATPase studies.

iv. Lipid Extraction. The method of Folch and co-workers (1957) was used to extract the lipids from the S_{3B} supernatant material. The S_{3B} material was suspended and extracted with chloroform:methanol

(2:1 v/v) at room temperature and then washed with 0.2 volumes of deionized water to remove non-lipid material. The mixture was then centrifuged at 300 x g for 10 minutes in a clinical centrifuge to separate the two phases. Most of the upper phase, which consisted of 40% of the total volume of the system, was removed by means of a Pasteur pipette. The remaining portion of the upper phase was removed in the following manner: A solution containing the same solvent composition as the upper phase; viz., chloroform:methanol:water (3:48:47 v/v) was prepared and added carefully on top of the lower phase. The upper phase was then again removed. This procedure was repeated twice and the washed lower phase was then freeze-dried with the aid of a VirTis freeze-drying apparatus. The combined upper phases were concentrated by removing the solvent under reduced pressure at room temperature.

E. Electron Microscopic Procedures.

I am indebted to Dr. R. M. Henderson, Mr. G. Duchon and Mrs. M. Podesta of the Department of Pharmacology, University of Alberta, for preparing the various subcellular fractions for electron microscopic observations by the procedure described below.

The pellets of material obtained from subcellular fractionation were fixed with phosphate buffered glutaraldehyde and kept overnight in the cold. The pellets were then washed three to four times in Millonig's buffer, and post fixed with 1 ml of 1% osmium tetroxide solution for one hour. After a water rinse, the pellets were dehydrated by passage through a graded ethanol series and were finally washed in propylene oxide. Epon

resin was used for embedding. Thin sections were cut with a diamond knife. The sections were mounted on 200 mesh copper grids and after staining with lead citrate and uranyl acetate, were examined in a JEM-7A electron microscope.

F. Protein Nitrogen Determinations.

The method of Lowry and co-workers (1951) was used.

Reagents:

1. 10% Na_2CO_3.
2. 2% sodium potassium tartrate.
3. 4% and 20% NaOH.
4. 0.5% $CuSO_4 \cdot 5H_2O$.
5. Dilute Folin-Ciocalteau (F-C) reagent was prepared by diluting 1 volume of phenol reagent (B.D.H.) with 2 volumes of de-ionized water.
6. Copper reagent was freshly prepared by taking 20 ml of (1), 1 ml of (2), adding de-ionized water to a volume of 100 ml and adding 2 ml of (4).
7. A standard stock solution of crystalline bovine albumin (Armour) of known protein nitrogen content was used to prepare a series of dilute solutions containing 1.6-32 µg protein N/ml.

Procedure:

A sample of the cell fraction was dissolved in 1 ml of 20% NaOH and diluted to 10 ml. The blank was prepared by mixing 1 ml of 4% NaOH with 1 ml de-ionized water. The standards were prepared

by mixing 1 ml of protein N standards with 1 ml of 4% NaOH.

1. To 1 ml of each of the above preparations was added 5 ml of copper reagent and the tubes were then incubated at 37°C for 30 minutes.
2. The tubes were agitated with the aid of an omnimixer and 0.5 ml of F-C reagent was quickly added to each tube.
3. The color was allowed to develop for 30 minutes and then the optical density at 540 mμ was measured with the Hitachi Perkin-Elmer spectrophotometer (Model 130). A standard curve was constructed (Figure 3).

Calculations:

mg Protein N/ml of unknown sample

$$= \frac{\mu g \text{ Protein N/ml value from graph}}{100 \times \text{volume of unknown sample digested.}}$$

G. Determination of Inorganic Phosphate (P_i).

For some of the initial work the method of Fiske and SubbaRow (1925) was used. Most of the work described involved the method of LeCocq and Inesi (1966). The latter method was used the most since it was simpler, more sensitive and the color formed was more stable.

i. Fiske-SubbaRow Method. The reducing agent was prepared by thoroughly triturating the following with a mortar and pestle.

0.2 gm 1-amino-w-naphthol-4-sulfonic acid,

1.2 gm $NaHSO_4$, and

1.2 gm $NaSO_4$.

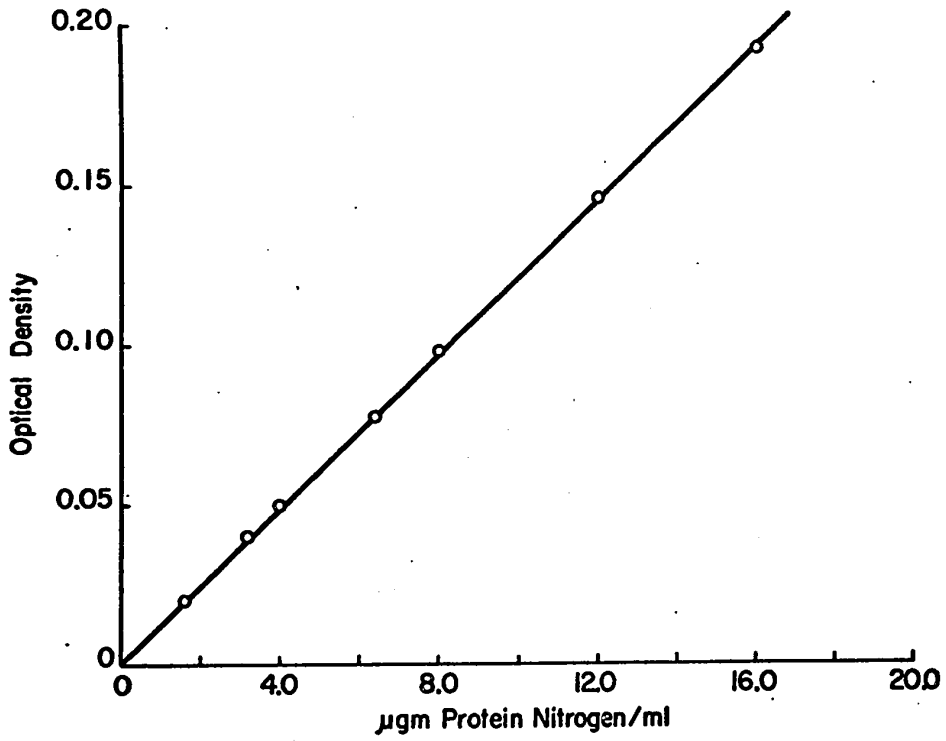


Figure 3. Standard curve for protein N determination by the method of Lowry and co-workers (1951).

This powdered mixture was stored in a dark glass bottle, away from light. For use 0.25 gm of this mixture was dissolved in 10 ml of de-ionized water.

Procedure:

1. A 1 ml aliquot of the enzyme reaction medium was added to 1 ml of cold 15% TCA.
2. The mixture was then centrifuged at maximum speed in a clinical centrifuge and 1 ml of the supernatant was used for P_i determination.
3. 1 ml of 5 N H_2SO_4, 1 ml 2.5% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 0.1 ml of reducing agent were added to 1 ml of above supernatant. The volume was adjusted to 10 ml with addition of de-ionized water. The tubes were well mixed and after 10 minutes the optical density of the color was read on a Beckman DU spectrophotometer at 660 mμ.
4. The standard curve (Figure 4) was prepared by substituting 1 ml of the enzyme reaction medium in the above procedure with 1 ml of a standard phosphate solution prepared from a stock solution of 10 μM/ml KH_2PO_4.

ii. LeCoeq and Inesi Method.

Reagents:

1. Ammonium molybdate $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$, 100 gm, and NH_4OH (specific gravity 0.90), 10 ml, were dissolved in de-ionized water to make 1 liter. (R-I)
2. 2.35 gm ammonium vanadate (NH_4VO_3) were dissolved in 400 ml

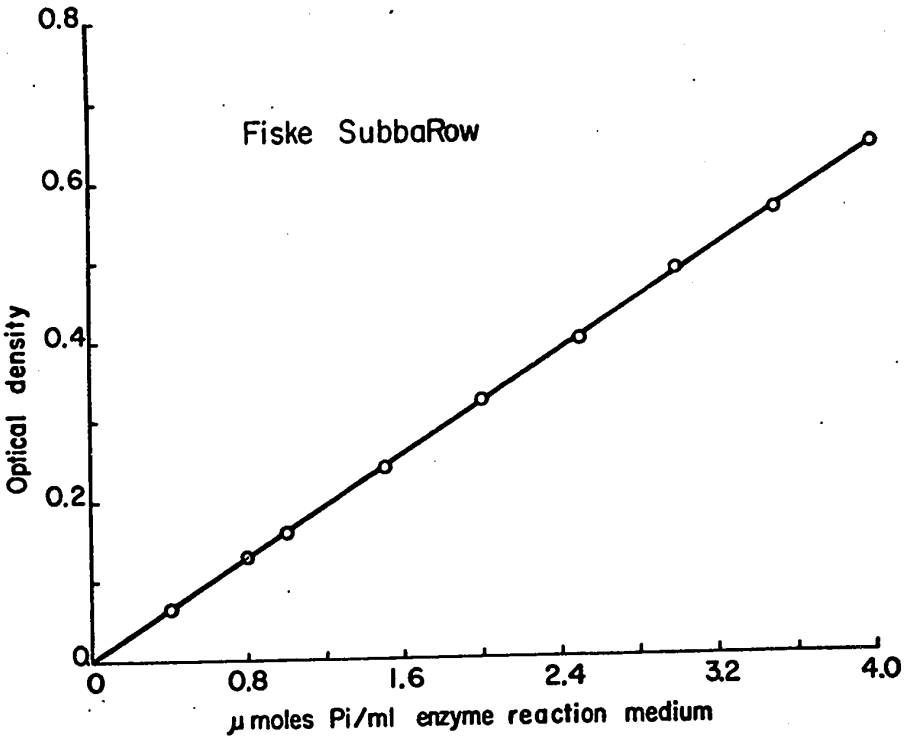


Figure 4. Standard curve for inorganic phosphate (Pi) determination by the method of Fiske SubbaRow (1925).

hot de-ionized water; cooled, and then 6.16 ml HNO_3 (specific gravity 1.42) diluted with 14 ml de-ionized water to make 1 liter. (R-II)

3. 150.5 gm TCA were added to 100 ml R-I and 100 ml R-II, and made up to 500 ml with de-ionized water. (R-III)

Procedure:

1. 1.5 ml of cold R-III were added to 1.5 ml of enzyme reaction medium; this was then cooled on ice.
2. The solution was centrifuged at top speed in a clinical centrifuge for about three minutes, and 1 ml of the clear supernatant was used for P_i determination.
3. 1 ml of this supernatant was made up to 10 ml with de-ionized water. The tube contents were well mixed and the optical density of the color formed was measured at 350 mμ on a Beckman DU spectrophotometer against a blank of 0.5 ml R-III and 9.5 ml de-ionized water.
4. The standard curve (Figure 5) was prepared by mixing 1 ml of R-III with 1 ml of a known phosphate standard solution and following the same steps as with the enzyme sample.

H. ATPase Assay.

i. Assay Media and Procedure. Various assay media were prepared so that all the chemical additions and the enzyme preparation were in a final volume of 1.5 ml unless otherwise stated. Each medium was made up in a stock solution containing all the chemicals, except the

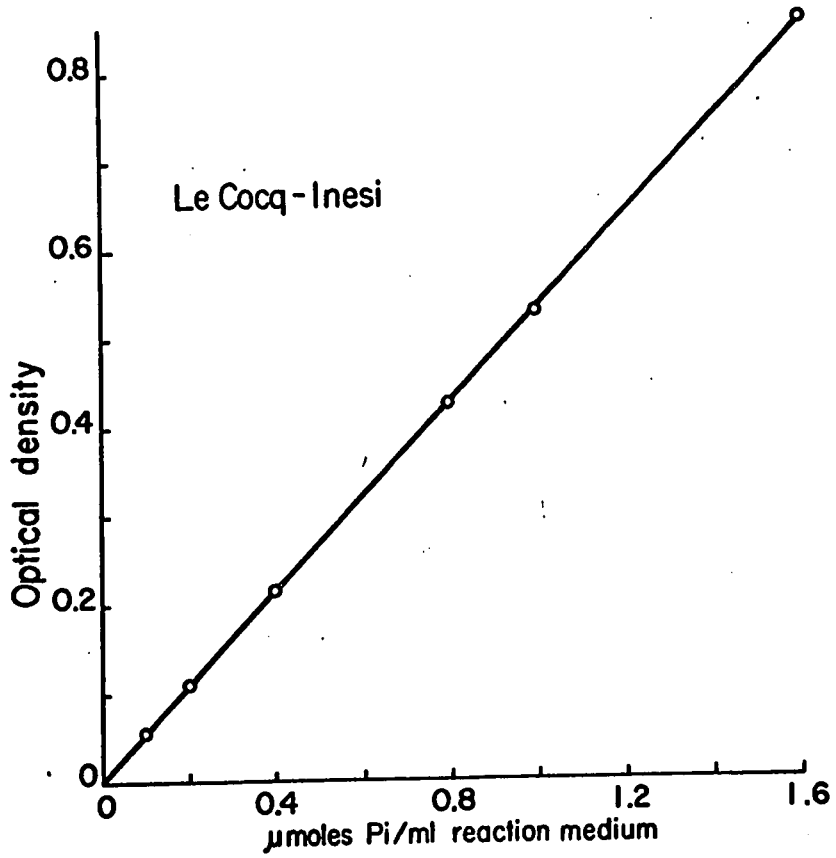


Figure 5. Standard curve for inorganic phosphate (Pi) determination by the method of LeCocq and Inesi (1966).

enzyme and substrate, in a final volume of 18 ml. To 0.9 ml of the stock solution 0.1 ml of substrate solution and 0.5 ml of enzyme preparation were added to yield the final concentrations listed below.

Media:

Medium A was based on the medium used by Bonting (1961), and contained 2 mM $MgCl_2$, 2 mM substrate, 0.1 mM Na_2EDTA, ± varying concentrations of NaCl and KCl, ± 0.1 mM ouabain (Nutritional Biochem. Co.), and Tris-HCl buffer at a final pH of 7.5.

Medium B: Medium A was varied and replaced by medium B which contained 4 mM $MgCl_2$, 4 mM substrate, ± 50 mM NaCl, ± 2 mM KCl, ± 0.1 mM ouabain, and 50 mM histidine (Nutritional Biochem. Co.) buffer pH 7.6.

Substrates:

The following nucleoside phosphates were used: Tris ATP, MgATP, and the sodium salts of ATP, ADP, AMP, ITP, GTP, CTP, UTP and UMP. All were purchased from the Sigma Biochemical Co.

Procedure:

0.5 ml of tissue homogenate or subcellular fraction were added to 10 ml Erlenmeyer flasks each containing 0.9 ml of assay medium of the desired composition for the experiment. The flasks were then lightly stoppered with a one hole No. 00 rubber stopper. The flasks were then placed into a Labline shaker-water bath (Labline Instruments Co., Melrose Park, Ill.), and allowed to warm

up at 37°C for 15 minutes. The enzyme reaction was started by adding 0.1 ml of substrate to each flask at 0.5 minute intervals. The flasks were then slowly agitated for 15 minutes. The enzyme reaction was then stopped at 0.5 minute intervals in each flask, either by addition of 1.5 ml of cold 15% TCA or 1.5 ml of cold R-III and then the flasks were placed on ice and assayed for inorganic phosphate as described earlier. Two blanks were also included in this procedure. The tissue homogenate or fraction blank contained everything except substrate which was replaced by 0.5 ml of de-ionized water. When time course experiments were carried out the final volume of the enzyme reaction mixture was 4.5 ml and all above additions were proportionally increased (2.7 ml medium, 0.3 ml substrate and 1.5 ml fraction). In this type of experiment the reaction was stopped by removing 1 ml of the reaction mixture at different time intervals and adding to 1 ml of 15% cold TCA or cold R-III.

iii. Calculation and Expression of Results.

1. The amount of P_i in the reaction mixtures with enzyme and the blanks was determined as the μM P_i/ml reaction medium. The values in the blanks were then subtracted from each of the experimental values to obtain the actual amount of P_i hydrolyzed from the substrate.
2. This amount of P_i was in a volume of reaction medium which was 1/3 original enzyme fraction. By multiplying this P_i value by 3 the amount of P_i liberated from the substrate by 1 ml of enzyme fraction was obtained.

3. This value was then divided by the mg of protein nitrogen in 1 ml of enzyme fraction (determined as described earlier), to obtain the P_i liberated/mg protein N.

4. This value was then divided by the time of reaction in minutes and multiplied by 60 to obtain the enzyme specific activity (ESA) as the μM P_i liberated from the substrate/mg protein nitrogen/hour. In general the following formula was used to calculate the ESA.

$$ESA = \frac{(\mu M\ P_i \text{ liberated from substrate - blank values}) \times 12}{(\text{mg protein N/ml fraction})}$$

5. The activity ratio was calculated as follows.

$$\text{Activity Ratio} = \frac{(\text{ESA in presence of buffer, } Mg^{++} \text{ plus other ions, } \pm \text{ ouabain})}{(\text{ESA in the presence of buffer and } Mg^{++}}$$

6. The per cent stimulation or inhibition of enzyme activity was the (activity ratio - 1.0) x 100.

For example, if the ESA in the presence of Mg^{++} was 68.0, and the ESA in the presence of Mg^{++}, $Na^{+} + K^{+}$ was 82.0, then the activity ratio would be $\frac{82.0}{68.0} = 1.206$ and the per cent stimulation = 20.6.

I. Other Enzyme Assays.

i. Succinate Dehydrogenase. Succinate dehydrogenase activity was determined by measuring the rate of oxygen consumption, based on the method of Opit and Charnock (1965).

Reaction medium:

A stock solution was prepared such that when 2.5 ml was

diluted to 2.86 ml the following concentrations were obtained.

20 mM K_2HPO_4

3 mM $MgCl_2$

10 mM glucose

10 mM succinic acid (Nutr. Biochem. Co.)

2 mM ADP (Sigma)

150 mM sucrose

15 mM Tris-HCl

The pH was adjusted to 6.9.

Measurement of O_2 consumption:

O_2 utilization was determined with a polarographic membrane covered Pt-Ag micro electrode (Beckman), immersed into a glass chamber. The contents of the chamber were mixed by rotation of a teflon coated bar driven by an external magnetic stirrer. The capacity of the chamber with electrode and magnet in it was 2.86 ml. The chamber was surrounded by a glass water jacket which was used to keep the temperature constant at 25°C. The electrode was polarized at -690 mV. The output from the electrode was coupled to a type R-Dynograph recorder with a Beckman Type 9871 coupler. The reaction was initiated by the addition of 0.36 ml of subcellular fraction suspension in 0.25 M sucrose. Both the reaction medium and the fraction suspension were saturated with 19.54% O_2 before the experiment.

Calculations and expression of results:

Since the solubility of a gas in a liquid is directly proportional

to the partial pressure of the gas (Henry's Law), it was necessary to determine the partial pressure of O_2 (P_{O_2}) as follows:

P_{O_2} = total pressure (P_t) - x % O_2 in the gas.

P_t was determined as follows:

The barometric pressure (B.P.) in mm Hg was measured on a glass Hg barometer and corrected for the vapor pressure of water at 25°C ($V_{P_{H_2O}}$), which was taken as 23.76 mm Hg.

Therefore P_t = B.P. - $V_{P_{H_2O}}$

The recorder was calibrated before each experiment by saturating the solution in the chamber with known dilutions of O_2 (4.68%, 9.79%, 19.54% O_2 in N_2, Liquid Carbonic Corp. Ltd.). A standard curve (Figure 6) was compiled which shows that the change in P_{O_2} (ΔP_{O_2}) was in a linear relation to the pen deflection.

The O_2 consumption during the reaction was recorded as ΔP_{O_2} in the reaction chamber.

ΔP_{O_2} was then converted to μMoles O_2 consumed/2.86 ml as follows:

$$\Delta P_{O_2} = \frac{\Delta N_{O_2}}{K'} \qquad \text{(Henry's Law)} \qquad (1)$$

where N_{O_2} = mole fraction of O_2 in water, and

K' = Henry's Law constant and = 2.3×10^{-5} atm^{-1} at 25°C.

$$\text{Since } \Delta N_{O_2} = \frac{\Delta n_{O_2}}{n_{H_2O}} \qquad (2)$$

where n = moles.

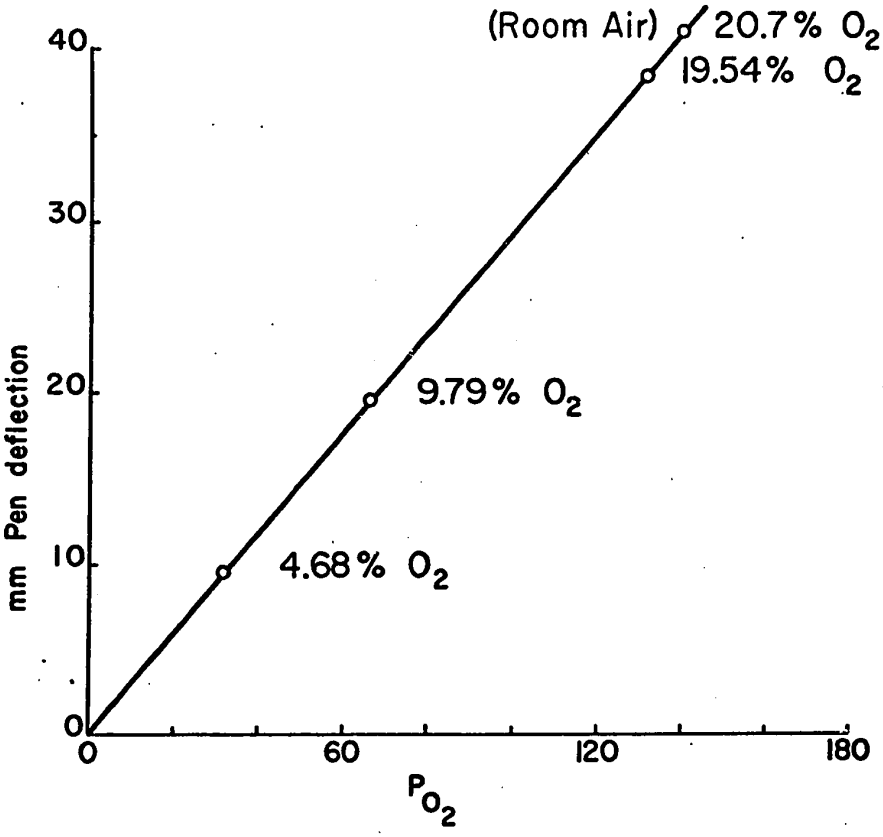


Figure 6. Standard calibration curve for O_2 uptake measurements.

Then by substituting equation (2) into (1)

$$\Delta P_{O_2} = \frac{\Delta n_{O_2}}{K' \times n_{H_2O}}$$

and by rearranging:

$$\Delta N_{O_2} = \Delta P_{O_2} \times K' \times n_{H_2O} .$$

Therefore at 25°C, and assuming the solubility of O_2 in this solution to be the same as that in water, for a volume of 2.86 ml we have:

$$\Delta n_{O_2} = \frac{\Delta P_{O_2} \times 2.3 \times 10^{-5} \times 2.86}{760 \times 18} \text{ moles consumed/2.86 ml.}$$

This was then simplified to:

$$\mu M\ O_2 \text{ consumed/2.86 ml} = \Delta P_{O_2} \times 4.8085 \times 10^{-3}.$$

The results were then finally converted to $\mu M\ O_2\Delta$/mg protein N/minute.

ii. Acetylcholinesterase. A modified procedure of Biggs and co-workers (1958) was followed using a constant pH titration apparatus (Radiometer; Emdrupvej, Copenhagen).

Reagents:

1. KH phthalate stock standard 0.01 M.
2. Stock titrant 1.0 M NaOH.
3. Working titrant 0.005 M NaOH.
4. Substrate 0.40 M acetylcholine bromide.

Procedure:

1.5 ml of homogenate or subcellular fraction suspended in 0.25 M sucrose was introduced into the sample chamber. 0.5 ml acetylcholine were added to start the reaction. The mixture was stirred at 37°C and titrated to a constant pH of 8.0 for five minutes. A standard curve was prepared (Figure 7) using dilutions of KH phthalate as standard.

iii. Glucose-6-Phosphatase. The method of Harper (1963) was used.

Solutions:

Citrate buffer.

100 mM citric acid

Adjusted to pH 6.5 with NaOH.

Substrate:

90 mM glucose-6-phosphate.

Adjusted to pH 6.5 with NaOH.

Stored frozen in 5 ml quantities until used.

Procedure:

The subcellular fractions to be tested were suspended in the citrate buffer and 1 ml of the suspension was used. After a 15 minute pre-warming at 37°C the reaction was started by the addition of 0.5 ml substrate. After a 15 minute incubation at 37°C the reaction was stopped by the addition of 1.5 ml cold R-III. The phosphate hydrolyzed was determined by the LeCocq-Inesi method

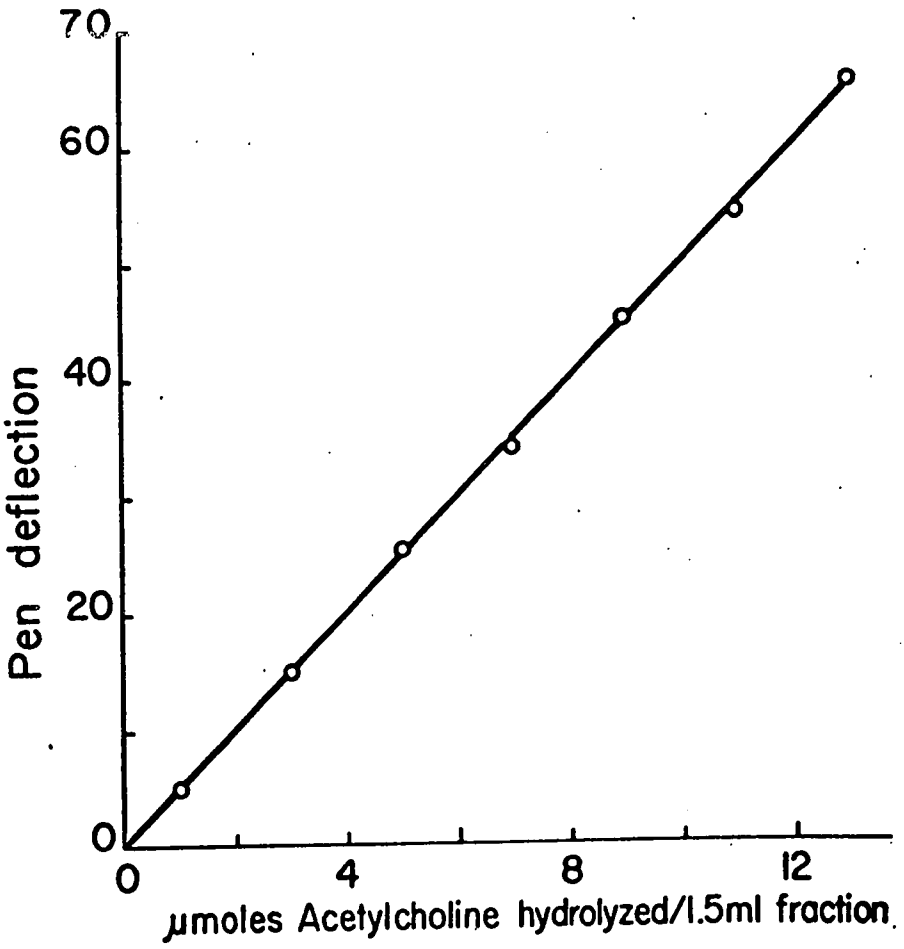


Figure 7. Standard curve for acetylcholinesterase activity.

described previously. As with ATPase the enzyme activity was expressed as the μM P_i liberated from glucose-6-phosphate/mg protein N/hour.

iv. p-Nitrophenyl Phosphatase. The method described by Sachs and co-workers (1967) was used. The reaction was started by adding 0.5 ml of subcellular fraction to 1 ml of pre-warmed reaction medium. The final concentrations in the mixture were as follows:

10 mM disodium p-nitrophenyl phosphate (Sigma).

10 mM $MgCl_2$.

50 mM histidine buffer pH 7.6.

± KCl (2-20 mM).

± ouabain 10^{-4} M.

Procedure:

After 15 minutes incubation at 37°C in a shaking water bath, the reaction was stopped by the addition of 1.5 ml cold 10% TCA. The protein was centrifuged down with a clinical centrifuge at top speed for 5 minutes. One ml of the clear supernatant was then transferred to a graduated tube, 1 ml of 350 mM Tris was added to bring the pH to 7.8. The mixture was then made up to 10 ml with de-ionized water and the color read on the DU spectrophotometer at 405 mμ. Blanks and expression of activity followed the same format as described for ATPase assay. The activity was expressed as μM p-nitrophenyl phosphate hydrolyzed/mg protein N/hour. A standard curve using various concentrations of p-nitrophenol was prepared (Figure 8).

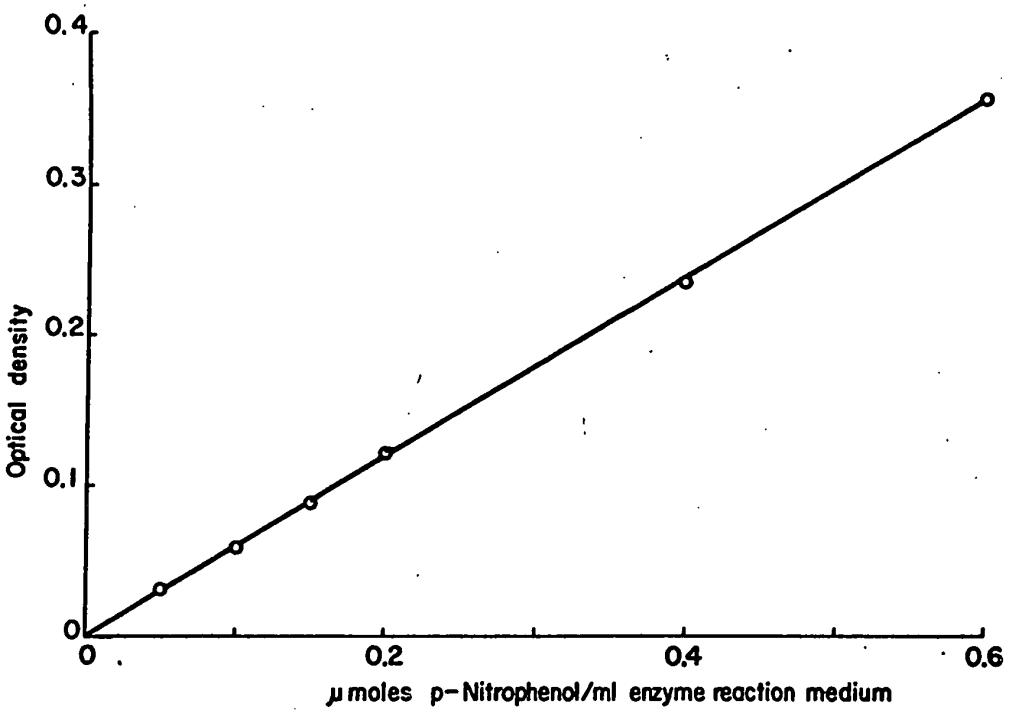


Figure 8. Standard curve for p-Nitrophenyl phosphatase activity.

v. Adenylate Kinase. Adenylate kinase activity was studied qualitatively by thin layer chromatography following the procedure below.

Preparation of chromatoplates:

A slurry of 82 ml of de-ionized water added to 11 gm Ecteola Resin (Macherey, Nagel and Co.; Duren, Germany) was prepared and shaken vigorously for two minutes. The slurry was applied to the carrier glass plates (20 cm x 20 cm) on the spreading template with the aid of a coating applicator (Desaga; Heidelberg, Germany) adjusted to a thickness of 500 μ. Chromatoplates were allowed to dry on the template at room temperature for one hour and then activated at 50°C for 60 minutes. The chromatoplates were then inserted into chromatographic chambers containing developing solvent and allowed to develop completely. The solvent system consisted of a mixture of tert. butanol, formic acid and water in the proportions 10:8:10 by volume. The chromatoplates were then dried in a fume hood at room temperature for 30 minutes.

Preparation of samples and application to chromatoplates:

In order to test for the presence of adenylate kinase advantage was taken of this enzyme's resistance to boiling at pH 1.0 (Dixon and Webb, 1964). This treatment destroyed all other enzymes hydrolyzing ATP and ADP in the tissue, so addition of ADP to such a preparation should result in the formation of ATP and AMP by adenylate kinase.

The total aortic homogenate and the subcellular fractions were suspended in 1 mM Tris-HCl at pH 1.0 and boiled for 10 minutes. The suspensions were then cooled and adjusted to pH 8.2 with Tris. A

0.5 ml volume of the suspensions were then incubated at 37°C with 1 ml of reaction medium. The final composition of the medium was 4 mM $MgCl_2$, 8 mM ADP, and 50 mM Tris-HCl pH 8.2. The reaction was stopped by adding 1.5 ml of cold TCA. The protein was centrifuged out at top speed in a clinical centrifuge and the clear supernatant was collected. Controls consisting of ATP, ADP and AMP with and without homogenate but always without Mg^{++} were also used.

Using capillary tubes, four drops of the supernatant from each sample were applied to the chromatoplates at a distance 2.5 cm from the bottom. Each drop was dried with a Master Appliance Corp. heat gun, Model MG-201, before the next drop was applied. The chromatoplates were then inserted into the developing chambers again and allowed to develop in the same solvent for 80 to 90 minutes at room temperature. The plates were then dried with a heat gun and examined under ultraviolet light.

vi. Actomyosin ATPase. The procedure of Mallin (1965) was followed for extraction of actomyosin. The aortic homogenates were prepared in the manner previously described, using the VirTis homogenizer. The homogenates were divided into two equal portions and each half was then used for subcellular isolation. One set of fractions acted as comparative controls and were re-suspended in 5 ml of 0.25 M sucrose. The experimental set were re-suspended in an equal volume of Weber-Edsall solution composed of 0.6 M KCl, 0.04 M $NaHCO_3$, and 0.01 M Na_2CO_3. The suspensions were then placed into polycarbonate ultracentrifuge tubes,

capped and shaken for 20 hours in the cold with a wrist action shaker (Burrell Corp.). The tubes were then centrifuged at the speeds used for the particular fractions. The resulting supernatants and pellets were then assayed for ATPase activity using the procedure previously described. ATPase activity was determined in a reaction medium which contained 4 mM Ca, 4 mM ATP, 50 mM histidine pH 7.6 plus or minus 425 mM KCl. The ionic strength (I) of the medium without KCl was I = 0.074 and for that with KCl I = 0.5.

J. Nucleic Acid Determinations (DNA and RNA).

The method of Schmidt and Thannhauser (1945) as modified by Fleck and Munro (1962) was used.

To 5 ml of 5% tissue homogenate or 5 ml of tissue fraction in de-ionized water 2.5 ml of cold 2.1 N perchloric acid (PCA) were added. This mixture was allowed to stand for 15 minutes, then the precipitate was centrifuged down in a clinical centrifuge and subsequently washed two times with 2.5 ml of 0.7 N PCA. The PCA was carefully drained off and discarded.

The precipitate was then digested at 37°C in 4 ml 0.3 N KOH for 20 minutes. After cooling the pH was adjusted to pH 1.0 with 10 N PCA. The precipitate was centrifuged down and further washed two times with cold 0.5 N PCA. The supernatant fluids were pooled and made up to 100 ml with de-ionized water and to a final concentration of 0.1 N PCA. The precipitate was saved for the DNA assay.

The optical density of the supernatant was read at 260 mμ on

a Beckman DB spectrophotometer against a blank of 0.1 N PCA. A standard curve was prepared (Figure 9) with yeast RNA (Sigma type XI) as standard.

The DNA in the precipitate was determined by the method of Cariotti (1952) with the modification of Blobel and Potter (1966). To the precipitate were added 2 ml de-ionized water, 1 ml of 0.04% indole solution and 1 ml of 2.5 N HCl. The tube was well agitated and placed into a boiling water bath for 20 minutes. The tube was kept covered with a clean glass marble. After cooling to room temperature the solution was extracted three times with 4 ml of chloroform (spectranalyzed, A.C.S.).

The optical density of the aqueous phase was then read at 490 mμ in a Beckman DB spectrophotometer against a blank prepared in the same way with de-ionized water. A standard curve (Figure 10) was prepared using calf thymus DNA (Sigma type I) as standard.

K. Tracer Ion Efflux Measurements.

The methods used for studying the efflux of ^{22}Na and ^{42}K were those of Daniel and Robinson (1969a,b,c) and are described below.

i. Media. Two basic media, Krebs-Ringer and K-free Krebs-Ringer, were used throughout the experiments. Drugs and inhibitors were incorporated when necessary: Iodoacetate (IAA) 10^{-3} M, dinitrophenol (DNP) 10^{-3} M, IAA and DNP both 10^{-3} and ouabain 10^{-3} M. All media were aerated with 95% oxygen and 5% carbon dioxide.

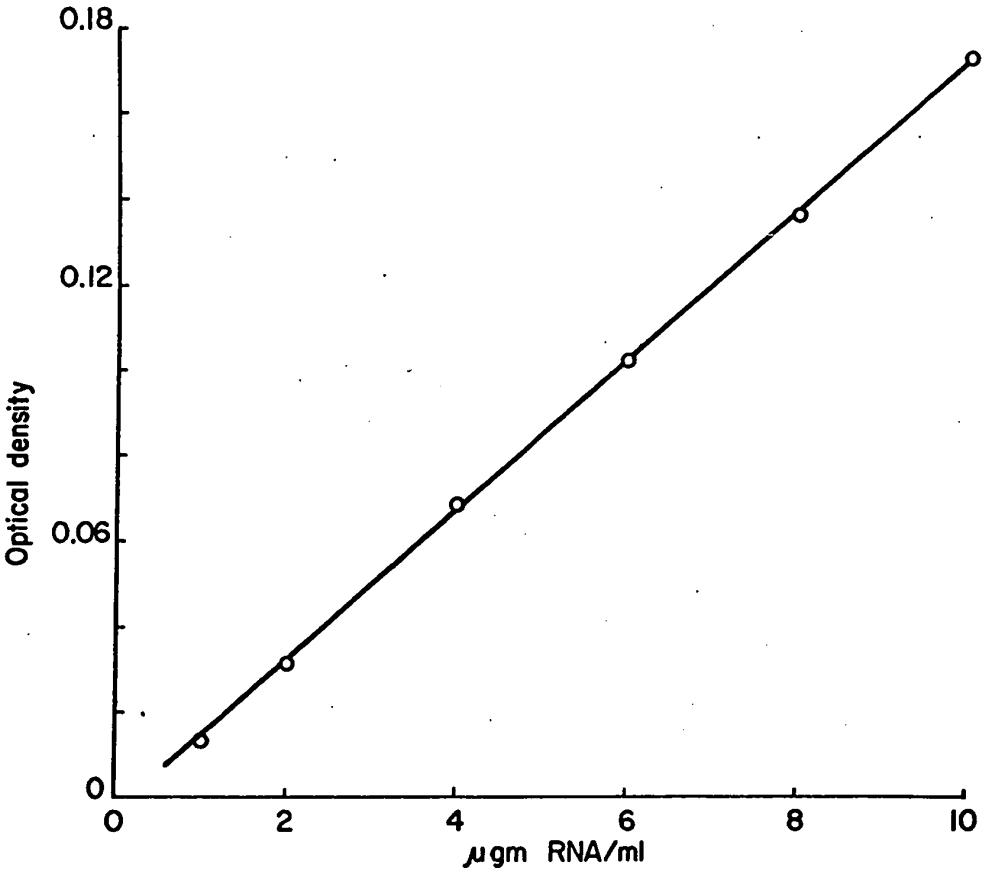


Figure 9. Standard curve for RNA determination.

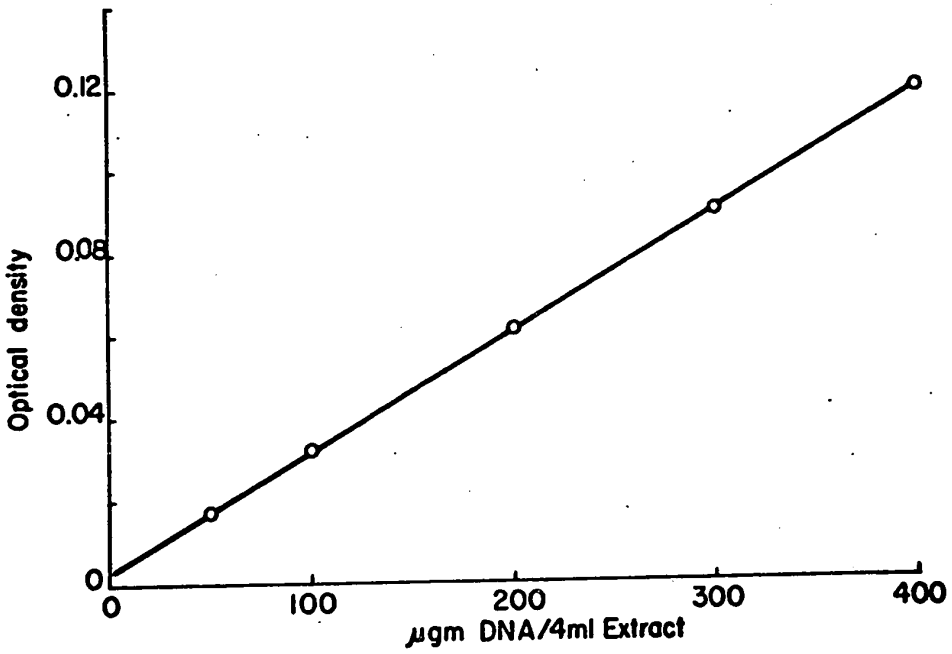


Figure 10. Standard curve for DNA determination.

Composition of media:

	Krebs-Ringer mM/l	K-free Krebs-Ringer mM/l
NaCl	115.48	120.10
$NaHCO_3$	21.91	21.91
NaH_2PO_4	1.16	1.16
KCl	4.63	--
$CaCl_2$	2.47	2.47
$MgSO_4$	1.16	1.16
Glucose	49.20	49.20

^{22}Na, obtained from Nuclear Science and Engineering Corp. (Pittsburgh, Penna.) as NaCl in 0.5 N HCl, was neutralized and diluted with de-ionized water so that 1 ml contained 50 μc. ^{42}K was received as K_2CO_3 from the Atomic Energy of Canada Limited (Ottawa, Canada). This powder was neutralized with 0.1 N HCl and diluted further with de-ionized water. The total sodium or potassium of the Krebs-Ringer media was adjusted to allow for the addition of ^{22}Na or ^{42}K to the media.

ii. Tissues Studied. The two types of tissues studied were fresh tissues and Na-rich tissues. The dissected aorta from a rabbit was slit lengthwise, cut into pieces (0.6 cm x 0.4 cm), and allowed to recover in oxygenated Krebs-Ringer solution at 37°C for one hour. These tissues were designated as fresh tissues. Fresh tissues to be used in efflux studies were incubated for two hours with ^{22}Na or ^{42}K-containing

Krebs-Ringer solution at 37°C. The experimental pieces were incubated a further 30 minutes in ^{22}Na or ^{42}K Krebs-Ringer with drugs added.

Tissues dissected and incubated overnight at 4°C in unoxygenated K-free Krebs-Ringer solution were designated as Na-rich tissues. When efflux was to be studied, ^{22}Na was added to the overnight medium. The following day, Na-rich tissues to be used for efflux studies were transferred into fresh, but still K-free ^{22}Na media (with drugs included for experimental pieces) for 30 minutes at 25°C. Na-rich tissues were not used for potassium efflux.

iii. Experimental Procedures. The tissue, either fresh or Na-Rich, was rinsed one to two seconds in isotonic choline chloride, attached to a hollow stainless steel hook which was inserted through a rubber cork. This was immediately placed in the first tube of the Krebs-Ringer medium for the efflux procedure. Media were aerated through the hollow tube. Efflux from each tissue was followed for 240 minutes; transfers to 10 ml fresh medium were made at 2, 4, 6, 8, 10, 15, 30, 45, 60, 75, 90, 120, 150, 180, and 210 minutes. Some tissues were not effluxed but rinsed in isotonic choline chloride, blotted lightly and weighed to determine the extent of initial exchange of labeled cation with the tissue cation.

At the end of the experiment, the tissues were blotted lightly, weighed and then placed in the oven overnight at 105°C. A dry weight was obtained the following day, and the tissues transferred to digestion tubes which were placed in a heated sand bath. 0.4 ml concentrated nitric

acid and 0.1 ml H_2O_2 (30-35%) were added for a quick digestion of the tissue. When only a fine white ash remained, it was dissolved and diluted to a 10 ml volume with de-ionized water and counted for ^{22}Na or ^{42}K. A further dilution to 25 ml was necessary for readings on the Eel Flame Photometer (Evans Electroselenium) to measure Na^+ and K^+ contents. The standard curves for Na^+ and K^+ are presented in Figure 11. A new standard curve was prepared for each set of samples analyzed.

Counting:

The 10 ml efflux samples and digested tissue samples were counted in a Well Type Scintillation Detector (Picker X-Ray Engineering Ltd., Canada) in conjunction with an all-transistorized Spectroscaler III. All samples were read for 200,000 counts or 10 minutes, whichever came first. A printer system recorded the sample number, count and time information.

For counting ^{22}Na, the Spectroscaler III was set with a lower level of 0.423 MeV and a 0.175 MeV window to reach an upper level of 0.51 MeV. For ^{42}K a recorder (Rostrak$^{(R)}$) and transistorized rate-meter (Model 5812) were added to the Picker unit. The time over which each sample was counted was recorded automatically on a strip chart calibrated in 2-minute intervals. The Spectroscaler III was set with a lower level of 1.35 MeV with a 0.300 MeV window to reach an upper level of 1.65 MeV. The decay rate of ^{42}K was followed for three weeks and shown to be that predicted for pure ^{42}K within the error of measurement. The decay factor of 10 minutes = 0.00932 was used to calculate the radioactivity in each sample at a zero time. The efflux data obtained were analyzed by a curve peeling technique which is described in Chapter V.

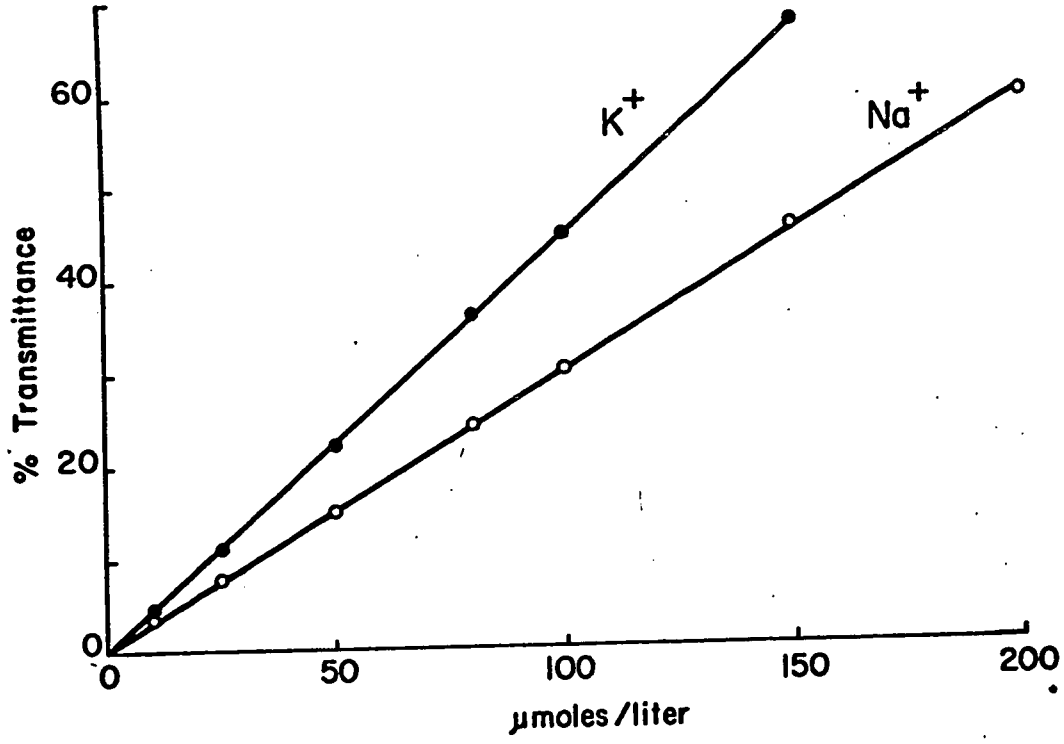


Figure 11. Standard curves for electrolyte (Na^+ and K^+) determinations.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF THE SUBCELLULAR FRACTIONS OF RABBIT AORTA

CHAPTER III. ISOLATION AND CHARACTERIZATION OF THE SUBCELLULAR FRACTIONS OF RABBIT AORTA.

A. Introduction.

When studying the $(Na^+ + K^+)$-ATPase in membrane material from tissues, contamination with ATPases from other cell organelles should be avoided. To obtain membrane preparations from tissues, tissue homogenates are fractionated by differential centrifugation. By layering the centrifuged particles onto a sucrose density gradient and centrifuging, a separation can be achieved according to size and density of the particles.

The nature of the various fractions thus obtained can be determined by morphological examination and identification of the cell organelles. However, since some organelles may have been disrupted into smaller fragments and rendered unidentifiable, other methods must also be used. The distribution of DNA and RNA in particulate fractions may help establish the distribution of various cell components. The DNA of the cell is almost entirely in the cell nucleus, while a very large part of the RNA is present in the ribosomes of the endoplasmic reticulum, some being present also in both mitochondria and nucleus. However, in conjunction with morphological examination enzyme marker studies have been most useful in characterization of subcellular fractions. Many enzymes are now known to be located or bound to specific cell organelles (DeDuve and co-workers, 1962) and are used as markers for the presence of these particular components.

In this study homogenates of the intima-media smooth muscle

layers of rabbit aortae were fractionated and characterized. Liver homogenates were also studied as a familiar control tissue.

B. Results.

The fractionation procedure presented in the Methods was developed to obtain a membrane ATPase preparation from rabbit aorta with minimal contamination from other cell structures. Since the next chapter is devoted to the distribution and properties of the ATPase, the results in this section deal with the characterization of the fractions most used for the ATPase studies.

i. Differential Centrifugation. Table I lists the concentrations of protein nitrogen in the various subcellular fractions obtained by differential centrifugation of aortic homogenates prepared by two different methods. The greatest portion of the protein of aorta was present in the 1,200 x g P_1 pellet. The remainder of the protein nitrogen was distributed among the other fractions. VirTis homogenization, although more disruptive, only reduced the amount of material in fraction P_2 and did not reduce the amount in P_1 as compared to glass-glass homogenization. Since Na^+ may be released from the ground glass homogenizer, most of the enzyme studies were carried out on VirTis homogenized preparations.

Table II lists the distribution of the nucleic acids (RNA and DNA) in the subcellular fractions. Most of the total tissue RNA was found in the P_1, P_3 and S_{3A} fractions respectively, with the P_2 fraction containing the least. When expressed per mg protein N, then the P_1 fraction

TABLE I

Distribution of Total Protein Nitrogen in Different Subcellular Fractions of the Aortic Homogenates Prepared by Two Different Homogenization Techniques.

Fraction	mg Protein N/gm Equivalent of Tissue	
	Glass-Glass	VirTis
Total Homogenate	20.88 ± 0.18	20.86 ± 0.22
P_1	14.67 ± 0.26	15.51 ± 0.14
P_2	3.77 ± 0.056	2.33 ± 0.035
P_3	1.21 ± 0.056	1.39 ± 0.072
S_{3A}	2.07 ± 0.051	1.95 ± 0.099

Values are means ± S.E.M. with No. = 5 for the glass-glass values and No. = 6 for the VirTis values.

TABLE II

Distribution of the Nucleic Acids RNA and DNA in the Subcellular Fractions of Rabbit Aorta.

Fraction	RNA		DNA		RNA/DNA
	µgm/gm Equivalent of Tissue	µgm/mg Protein N	µgm/gm Equivalent of Tissue	µgm/mg Protein N	
P_1	549.4 ± 24.8	35.42 ± 1.59	1228.1 ± 120.4	79.2 ± 7.96	0.447
P_2	292.4 ± 22.6	125.5 ± 9.72	164.1 ± 16.6	70.4 ± 7.20	1.783
P_3	514.4 ± 24.9	370.1 ± 17.93	256.4 ± 30.6	184.4 ± 22.0	2.006
S_{3A}	403.5 ± 44.6	207.4 ± 21.5	160.6 ± 20.7	86.6 ± 10.6	2.512
TOTAL	1759.7		1804.2		0.973

Aortae were homogenized with the VirTis homogenizer. Values are means ± S.E.M. with No.= 6 for each determination.

contained the least with fraction P_3 containing the most. More than one-half of the total tissue DNA was localized to the P_1 fraction with the remainder being distributed in the other fractions. The ratios of RNA/DNA of the various fractions indicate that DNA was primarily in P_1 whereas RNA was predominant in the more slowly sedimenting fractions.

Table III lists the distribution of three enzymes which are associated with the cell membrane. Acetylcholinesterase activity was highest in fraction P_3 and the 100,000 x g supernatant S_{3A}. This distribution was also true for the glucose-6-phosphatase and p-nitrophenyl phosphatase activities. The p-nitrophenyl phosphatase activity of P_3 was also stimulated by K^+ and this K^+ stimulation was ouabain sensitive.

The distribution of mitochondria and fragments thereof was determined by measuring succinate dehydrogenase activity by O_2 consumption. The aortic fractions were found to have very low succinate dehydrogenase activity. For comparison, fractions from rabbit liver were prepared in an identical manner. The results listed in Table IV indicate that differences in the distribution of the mitochondrial enzyme depend on the initial homogenization technique employed. Aortic fractions prepared using the VirTis homogenizer resulted in a high O_2 uptake in fraction P_3 with 58.7% of the total activity. The glass homogenized aortae resulted in fractions which contained most of the O_2 consumption in P_2 (50%); however, fraction P_3 still contained 32% of the succinate dehydrogenase activity.

When liver was homogenized with the VirTis the P_3 fraction contained 51% of the total O_2 consumption and P_2 34.4% (Table V). Less disruptive homogenization of the liver with a teflon-glass homogenizer

TABLE III

Specific Activities of Enzymes in the Subcellular Fractions of VirTis Homogenized Rabbit Aorta.

Fraction	Acetylcholin-esterase	Glucose-6-phosphatase	p-Nitrophenyl Phosphatase		
			4 mM Mg^{++}	+ 20 mM K^{+}	+ 20 mM K^{+} + 10^{-3} M ouabain
P_1	0.664 ± 0.013	0.077 ± 0.01	0.064±0.015	0.086±0.021	--
P_2	2.10 ± 0.053	0.815 ± 0.07	0.736±0.20	0.958±0.31	--
P_3	20.29 ± 0.435	2.45 ± 0.09	2.47 ±0.35	3.705±0.43	2.63 ± 0.39
S_{3A}	16.2 ± 0.378	--	1.87 ±0.63	2.95 ±0.57	2.13 ± 0.55

Specific activity was expressed as: µM acetylcholine hydrolyzed/mg protein N/hour, for acetylcholinesterase, No. = 3; µM P_i liberated/mg protein N/hour for glucose-6-phosphatase, No.= 2; µM p-nitrophenol liberated/mg protein N/hour for p-nitrophenyl phosphatase, No.= 6.

TABLE IV

Distribution of O_2 Uptake in Subcellular Fractions of Aorta Prepared by Two Different Homogenization Techniques.

Fraction of Aorta	μM O_2 Δ x 10^{-3}/gm Equivalent of Tissue/minute		μM O_2 Δ x 10^{-3}/mg Protein N /minute	
	VirTis	Glass-Glass	VirTis	Glass-Glass
P_1	9.22 ± 1.5 (16.1%)	10.74 ± 1.15 (18.4%)	0.594 ± 0.08	0.732 ± 0.07
P_2	14.40 ± 1.36 (25.2%)	29.17 ± 6.4 (50.0%)	6.18 ± 0.58	7.83 ± 1.72
P_3	33.54 ± 1.34 (58.7%)	18.44 ± 6.6 (31.6%)	24.13 ± 0.97	15.24 ± 5.5
TOTAL	57.16	58.35		

Values in parentheses represent the per cent of the total O_2 uptake. Values are means ± S.E.M. with No.= 5 for each of the VirTis homogenized fractions and No.= 3 for each of the glass-glass homogenized fractions.

TABLE V

O_2 Uptake of Subcellular Fractions of Liver Prepared by Two Different Homogenization Techniques.

Fraction of Liver	O_2 Uptake/gm Equivalent of Tissue/minute ($\mu M\ O_2\ \Delta \times 10^{-3}$)	
	VirTis	Teflon-Glass
P_1	157.4 ± 19.8 (14.6%)	383.8 ± 66.2 (34.1%)
P_2	371.3 ± 29.3 (34.4%)	677.2 ± 78.4 (60.1%)
P_3	550.6 ± 25.8 (51.0%)	65.8 ± 8.7 (5.84%)
TOTAL	1079.3	1126.8

Values in parentheses represent the per cent of the total O_2 uptake. Values are the means ± S.E.M. with No. = 3 for each determination.

resulted in the largest mitochondrial enzyme activity in fractions P_2 (60.1%) and P_1 (34.1%), with a very low activity in fraction P_3 (5.8%).

In order to further characterize the fractions the various pellets obtained were prepared for electron microscopic observation. A section of intact intima-media layer of the rabbit aorta (Figure 12) shows the type of smooth muscle cells mostly seen. The cells are very long and are surrounded by a great deal of collagen and elastic connective tissue. The cell membranes bear many pinocytotic vesicles. Inside the cells a large nucleus and the myofibrillar material occupy most of the space. Mitochondria were few in number but when seen were usually located alongside or at the ends of the nucleus. Figure 13 shows a group of mitochondria and also some granular endoplasmic reticulum in the aortic smooth muscle cell.

After homogenization of the arterial smooth muscle some of the fractions isolated were also observed with the electron microscope. It was difficult to obtain good sections of fraction P_1 and therefore few pictures were taken. In general Figure 14a and 14b show the type of material seen. the fraction contained many masses of material which may be elastin or myofibrillar material. On some occasions nuclear material was seen (Figure 14b) but in general the fraction contained cell debris and much collagen. With the aid of a phase contrast microscope, groups of unbroken cells were also seen in fraction P_1.

Figure 15 is an electron micrograph of the P_2 fraction. This fraction was composed of smaller cell fragments which included vacuolar-appearing mitochondria, vesicular material, some rough endoplasmic

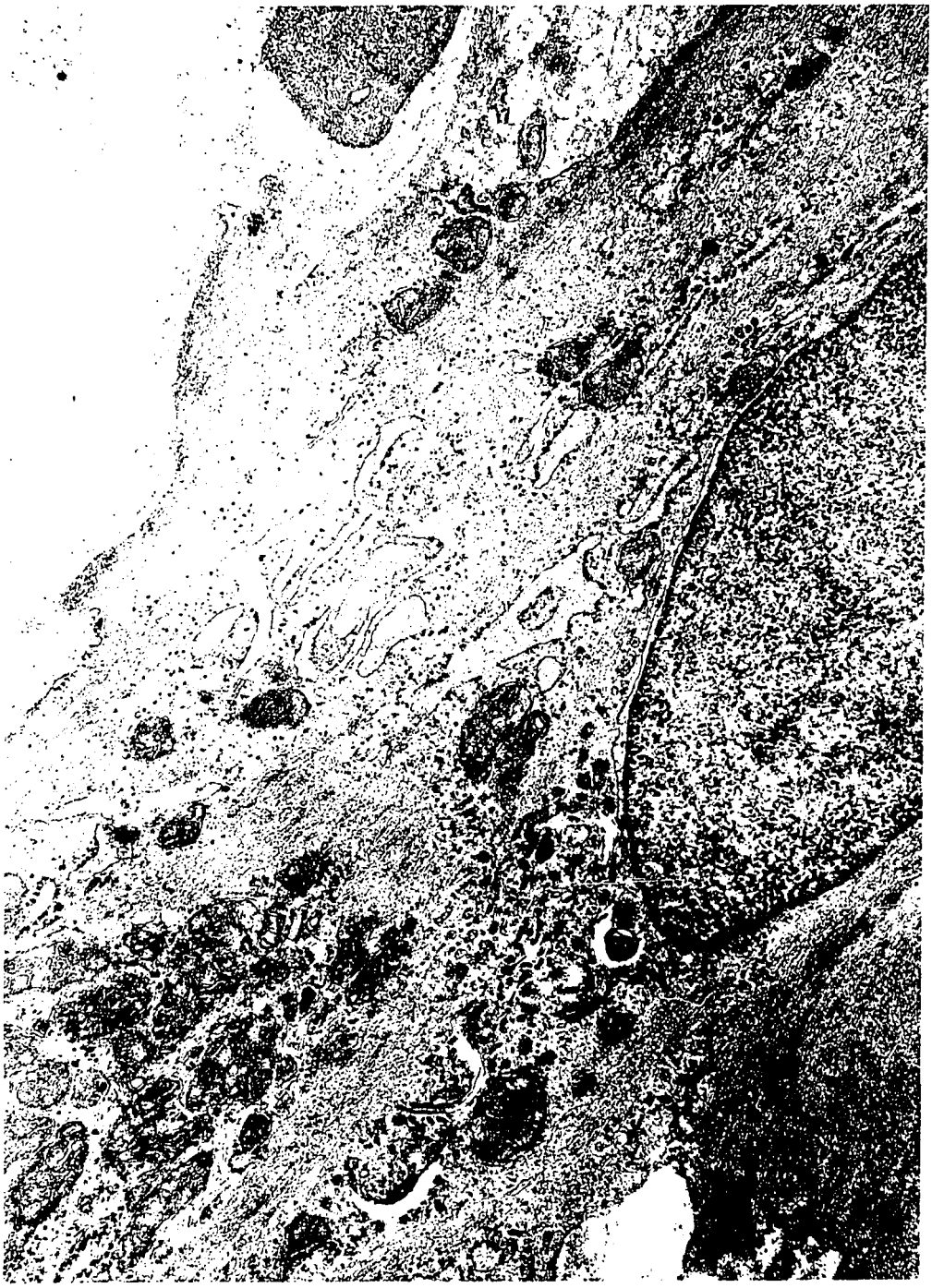


Figure 13. Electron micrograph of smooth muscle cell from rabbit aorta. x 26,000.

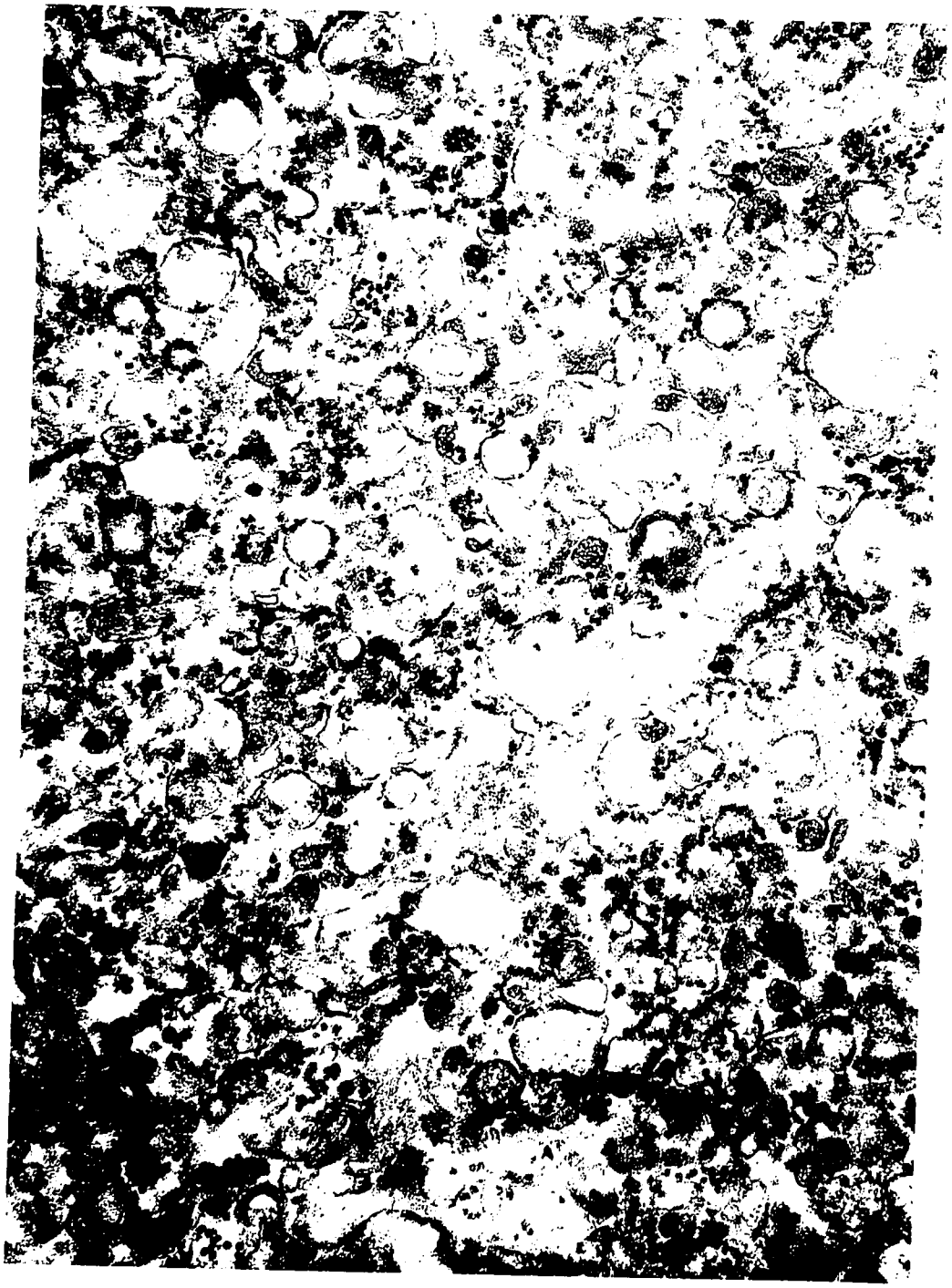


Figure 16. Electron micrograph of the P_3 fraction from rabbit aorta. x 38,000.

This was accompanied by the odd small vesicle which probably was washed over from the microsomal P_3 fraction.

ii. Sucrose Density Gradient Centrifugation of the P_3 Microsomal Fraction. In order to further separate the components of the microsomal fraction P_3 of aorta a resuspension of P_3 in 0.25 M sucrose or the S_2 supernatant which contained the microsomes, was layered on top of a discontinuous sucrose gradient (Figure 18). The first gradient used (I) of 1.0, 1.2, 1.4, and 1.6 M sucrose layers resulted in two fractions after centrifugation at 40,000 rpm for one hour. One fraction was a loose and disperse band in the vicinity of the 1.0 M sucrose layer, and the other was a pellet at the bottom of the tube. The second gradient used (II, Figure 18) of higher sucrose concentration resulted in a separation of the P_3 material into three fractions. One was again a loose band in the 1.0 M sucrose layer, the second was a loose band in the 1.7 M sucrose layer, and the third a small pellet at the bottom of the tube. In order to pack the fractions into a closer band the last gradient (III, Figure 18) of 1.4 and 2.0 M sucrose was used. After centrifuging the S_2 supernatant material layered on the top of this gradient two more concentrated bands on top of each sucrose layer were obtained, and a small pellet at the bottom of the 2.0 M sucrose layer. The three fractions were then labelled as P_D, M_D and N_D respectively.

The protein nitrogen distribution among the density gradient fractions was determined and listed in Table VI. Fraction P_D was found to have p-nitrophenyl phosphatase activity; however, the activities of

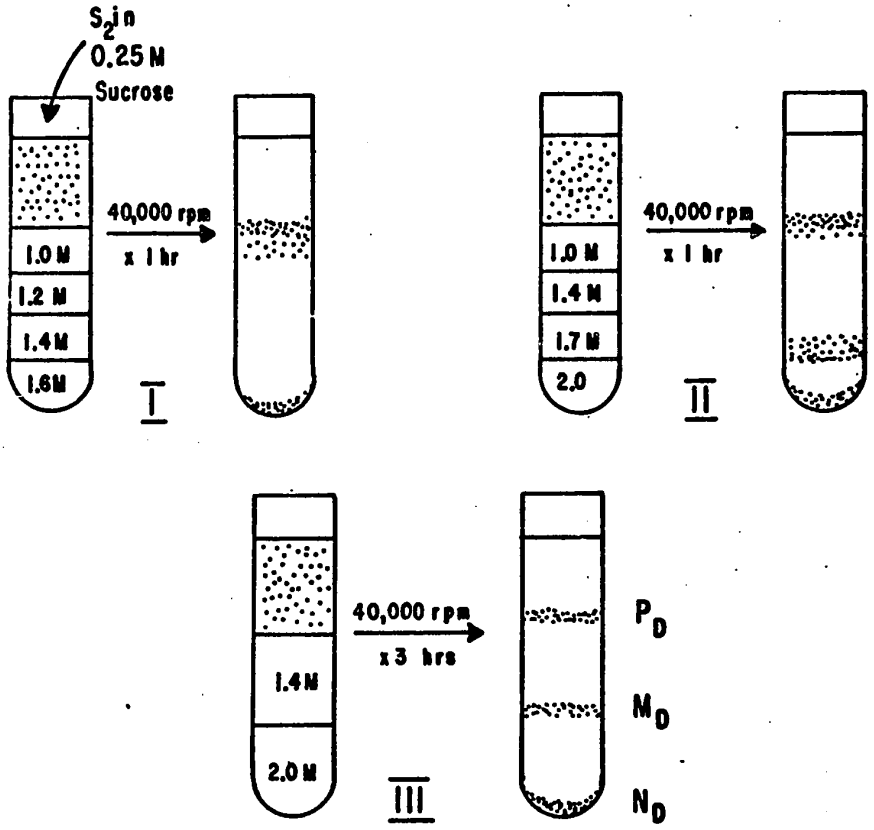


Figure 18. Diagrams of separations achieved by density gradient centrifugation.

this enzyme in M_D and N_D were too low to allow quantitative comparison.

TABLE VI. Distribution of the Protein Nitrogen in the Density Gradient Fractions.

Fraction	mg Protein N/gm equivalent of tissue
P_D	0.4604 ± 0.0055
M_D	0.337 ± 0.0079
N_D	0.588 ± 0.0554

S_2 supernatant material from VirTis homogenized aorta was layered on a discontinuous sucrose density gradient. Values are the means ± S.E.M. from 5 experiments.

O_2 uptake studies of the density gradient fractions revealed that some further separation of the mitochondrial fragments from the microsomal material was obtained (Table VII). Most of the O_2 was taken up by the M_D fraction and the least was taken up by P_D. Also the amount of O_2 uptake/mg protein N/minute in the P_D fraction was lower than that in fraction P_3 (Table IV) before density gradient separation. Since the O_2 uptake of the aortic fractions were all low, suspensions of S_2 from liver were also subjected to the gradient separation. O_2 uptake studies of the liver density gradient fractions (Table VIII) indicate that again most of the uptake was in the M_D fraction regardless of the

TABLE VII

O_2 Uptake of Density Gradient Fractions of VirTis Homogenized Aorta.

Fraction	$\mu M\ O_2\ \Delta \times 10^{-3}$ per gm equivalent of tissue/minute	per mg protein N/hr
N_D	9.43 ± 1.53 (27.6%)	16.04 ± 2.60
M_D	18.28 ± 0.61 (53.6%)	54.24 ± 1.81
P_D	6.41 ± 0.895 (18.7%)	13.92 ± 1.94
TOTAL	34.12	

Fractions were obtained by layering S_2 supernatant material on a discontinuous density gradient of 1.4 M and 2.0 M sucrose. Values in parentheses represent the per cent of the total O_2 uptake. Each value is the mean ± S.E.M. from 3 different preparations.

TABLE VIII

O_2 Uptake of Density Gradient Subcellular Fractions of VirTis and Teflon-Glass Homogenized Liver.

Fraction	VirTis	Teflon-Glass
N_D	78.91 ± 8.3 (14.69%)	16.5 ± 3.7 (23.88%)
M_D	352.93 ± 42.4 (65.7%)	41.82 ± 8.1 (60.34%)
P_D	105.34 ± 19.8 19.61%)	10.94 ± 2.7 (15.78%)
TOTAL	537.18	69.3

Fractions were obtained by layering liver S_2 supernatant material on a discontinuous density gradient of 1.4 M and 2.0 M sucrose. Values in parentheses represent the per cent of the total O_2 uptake. Each value is the mean ± S.E.M. from 2 different preparations.

homogenization technique employed. The per cent of the total O_2 uptake by the N_D fraction differed with the homogenization technique.

Electron micrographs of the density gradient fractions from the VirTis homogenized aorta show that fraction P_D was composed of small vesicular membranes (Figure 19a and 19b) with less contamination by collagen fragments and rough endoplasmic reticulum compared to that seen in the P_3 fraction (Figure 16). The M_D fraction (Figure 19c) was composed of material which was more granular in nature and less vesicular. Some of the M_D material was composed of small chains of ribosomal material; however, whole mitochondria were not seen. The N_D fraction was of similar appearance to that of the M_D fraction but it also contained fragments of collagen material.

When the liver density gradient fractions were examined some whole mitochondria were also seen. Most of these mitochondria were found in the N_D and M_D fractions (Figure 19d). No mitochondria were seen in the liver P_D fraction.

C. Discussion.

The method of initial homogenization of the intima-media layers of smooth muscle from rabbit aorta cannot be varied as much as for other tissues and generally a more disruptive method is required. Since the use of a VirTis type of homogenization is more disruptive it became more difficult to obtain a microsomal membrane preparation with minimal contamination from other cell components. The use of glass-glass homogenizers was less destructive to the mitochondria which were found to be in fraction

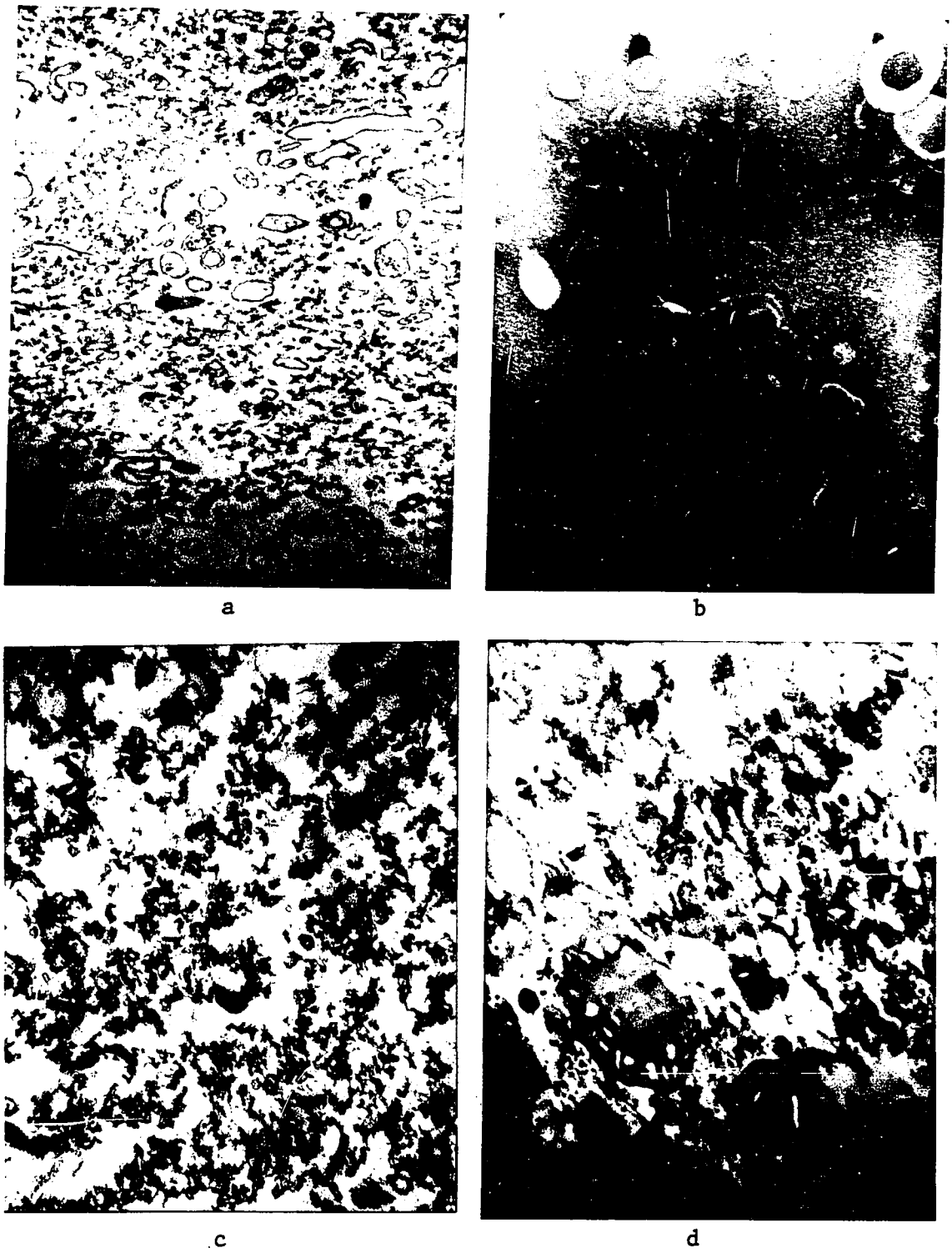


Figure 19. Electron micrographs of various density gradient fractions. a and b, fraction P_D from rabbit aorta; a x 18,000, b x 52,000; c, fraction M_D from rabbit aorta x 30,000; d, fraction M_D from rabbit liver x 29,500.

P_2. However, the VirTis homogenizer was used for most of the studies on ATPase since it has been shown that extra Na^+ contamination of the fractions can arise from the glass-glass homogenizers (Allen, 1967).

The P_1 fraction obtained by differential centrifugation of the VirTis homogenized aorta contained most of the total protein nitrogen. The electron micrographs and the RNA and DNA distributions indicate that fraction P_1 is composed primarily of nuclei and other cell debris. The RNA/DNA ratio for purified nuclei from skeletal muscle, liver, thymus and spleen have been reported to range from 0.1 to 0.3; however, the ratio in the corresponding tissue homogenates was greater than 1.0 (Edelman and co-workers, 1965). The RNA/DNA ratio in fraction P_1 was 0.447, whereas the ratio in the other fractions was greater than 1.0. Most of the cell debris in fraction P_1 consisted of fragments of connective tissue and collagen.

The P_3 microsomal material was most concentrated with RNA which suggests that this fraction contains a lot of the ribosomal material or endoplasmic reticulum. The electron micrographs of P_3 also show many granular membrane structures comparable to endoplasmic reticulum.

The enzymes acetylcholinesterase, glucose-6-phosphatase and potassium-activated-p-nitrophenyl phosphatase have been shown to be plasma membrane bound enzymes (DeDuve and co-workers, 1962; Emmelot and co-workers, 1964; Coleman and co-workers, 1967; Lansing and co-workers, 1967) and were used as enzyme markers for the membrane. The distribution of the membrane enzymes in the aortic fractions indicated that most of the membrane material sedimented in the P_3 fraction.

The potassium-activated-p-nitrophenyl phosphatase activity was highest in fraction P_3 and S_{3A} and also showed a sensitivity to ouabain. This enzyme activity has been associated with the membrane $(Na^+ + K^+)$-ATPase by Albers and co-workers (1965). The distribution of the $(Na^+ + K^+)$-ATPase in the aortic fractions were also found to be similar; these results are presented and discussed in the next chapter.

The subcellular distribution of mitochondria in the aortic fractions was determined by measuring the O_2 uptake. The results show that most of the mitochondria are located in the microsomal P_3 fraction of VirTis homogenized tissue. However, electron microscopy only revealed whole mitochondria in fraction P_2. This inconsistency led to the assumption that the homogenization technique must have disrupted mitochondria into smaller fragments which then sedimented at the higher gravitational forces into the P_3 fraction. This was further substantiated by showing that less disruptive homogenization techniques increased the amount of O_2 uptake in fraction P_2 with less O_2 uptake from mitochondrial fragments in fraction P_3.

By further fractionating the aortic P_3 fraction, prepared from a VirTis homogenate, in a sucrose density gradient, it was possible to obtain a microsomal fraction P_D which was much less contaminated by mitochondrial fragments. The final gradient used separated the P_3 fraction into three components (P_D, M_D, and N_D).

The M_D fraction seemed to be composed of most of the mitochondrial fragments which had previously been in P_3. Fraction N_D contained most of the P_3 contaminating fragments of collagen, elastin and larger

mitochondrial fragments. When liver was homogenized by VirTis and teflon-glass homogenization the distribution of the mitochondria in the density gradient fractions was also similar. However, the amount of O_2 uptake in fraction N_D was greater from the teflon-glass homogenized preparations than from the VirTis homogenized ones. This difference indicated that there were more whole mitochondria than fragments in the teflon-glass homogenized preparations. Moreover, electron microscopy of N_D fractions revealed more whole mitochondria in teflon-glass homogenized preparations than in those prepared from VirTis homogenates.

The P_D fraction obtained from aorta which contained most of the ATPase and p-nitrophenyl phosphatase activity was therefore much less contaminated by mitochondrial fragments than fraction P_3. Electron microscopy showed a more uniform vesicular membrane material in the P_D fraction.

Since this work was started other reports have appeared which involve the isolation of microsomal fractions from aortic smooth muscle (Whereat, 1966; Portman and co-workers, 1967; Verity and Bevan, 1969). However, in these studies glass-glass homogenization was used followed by differential centrifugation and in each case mitochondrial fragment contamination was not completely eliminated from the microsomal fractions. Portman and co-workers (1967) applied their crude microsomal fraction of aorta onto a density gradient of 0.32-2.0 M sucrose and were able to obtain a light microsomal fraction equilibrating in a layer at about 0.9 M sucrose. This fraction was found to be composed of many smooth surfaced vesicles similar to that of fraction P_D reported here. The peak

percentages of total phospholipids as sphingomyelin were found in their light microsomal gradient fraction. They also had a pellet at the bottom of the 2.0 M sucrose layer high in protein but low in phospholipid and RNA which contained finely fragmented elastin.

CHAPTER IV

ATPase ACTIVITY OF RABBIT AORTA

CHAPTER IV. ATPase ACTIVITY OF RABBIT AORTA

A. Introduction.

Few attempts to isolate a $(Na^+ + K^+)$-ATPase from vascular smooth muscle have been reported. Some early studies revealed that both a Mg^{++} and a Ca^{++} dependent ATPase activity exist in the homogenate of vascular smooth muscle (Krantz and co-workers, 1951; Carr and co-workers, 1952, 1953). However, the effects of Na^+ plus K^+ or ouabain on ATPase activity were not tested.

In 1961 Bonting and co-workers reported Mg^{++}-ATPase activities in homogenates of various tissues which were stimulated by the addition of Na^+ plus K^+. Rabbit aorta was one of the tissues studied and under their conditions the Mg^{++}-ATPase appeared to be slightly stimulated by Na^+ plus K^+.

Recently a more detailed study of the ATPase activity in rabbit aorta has been presented by Verity and Bevan (1969), who studied various fractions of aortic homogenates prepared by differential centrifugation. Most of the Mg^{++}-ATPase activity was found in their microsomal fraction. However, they were unable to demonstrate Na^+ plus K^+ stimulation or ouabain sensitivity in this preparation.

B. Results.

i. ATPase Activity of Total Homogenate. The ATPase activity of rabbit aorta was studied first in a 5% (w/v) homogenate of the intima-media smooth muscle prepared by glass-glass homogenization.

For ATPase activity the presence of a divalent cation was found necessary (Figure 20). Mg^{++} was found to be the best activator and Ca^{++} was found to be about one-half as effective. The rate of ATP hydrolysis in the presence of Mg^{++} or Ca^{++} was found to be linear up to 60 minutes and then the rate decreased. Optimal ATPase activity was obtained with 2 mM Mg^{++} when the Mg:ATP ratio was 1:1; with 4 mM Mg^{++} the optimal activity was less dependent on the Mg:ATP ratio, and ratios of 2:1 and 1:1 were found equally effective (Figure 21). A broad pH optimum at 7.5 and 8.5 was obtained in Tris-HCl buffer for the Mg^{++}-ATPase activity (Figure 22).

TABLE IX. Effects of Na^{+} and K^{+} on the ATPase Activity.

Sodium Conc. mM/1	μM P_i/mg Protein N/hr		
	Mg^{++}	Mg^{++} + 5 mM K^{+}	Mg^{++} + 10 mM K^{+}
5	9.02 (9.12)	8.95	8.81
10	8.79	8.80	8.57
20	8.60	8.52	8.47
40	8.30 (8.31)	8.06 (8.10)	8.10 (8.09)
80	6.85	6.80	6.48

Values were obtained from a 5% homogenate preparation.

Assay Media: 2 mM Mg^{++}, 2 mM Tris-ATP, 0.1 mM EDTA, various concentrations of Na^{+} and K^{+}, Tris-HCl buffer pH 7.5. Values in parentheses from media which included 0.1 mM ouabain.

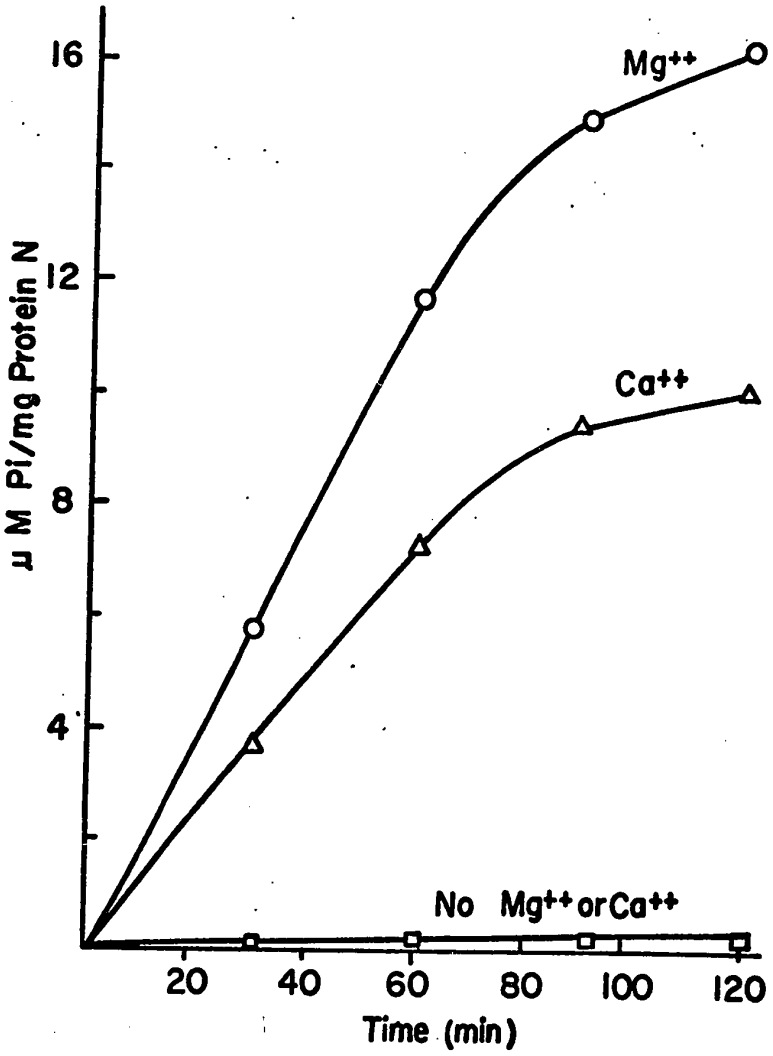


Figure 20. Divalent cation requirement for ATPase activity of aortic homogenate. Assay media: 2 mM Tris-ATP, 0.1 mM EDTA, 100 mM Tris-HCl buffer, pH 7.5; ± 2 mM Mg^{++} or ± 2 mM Ca^{++}. The enzyme activity is expressed as μ moles of inorganic phosphate hydrolyzed from ATP within the time indicated.

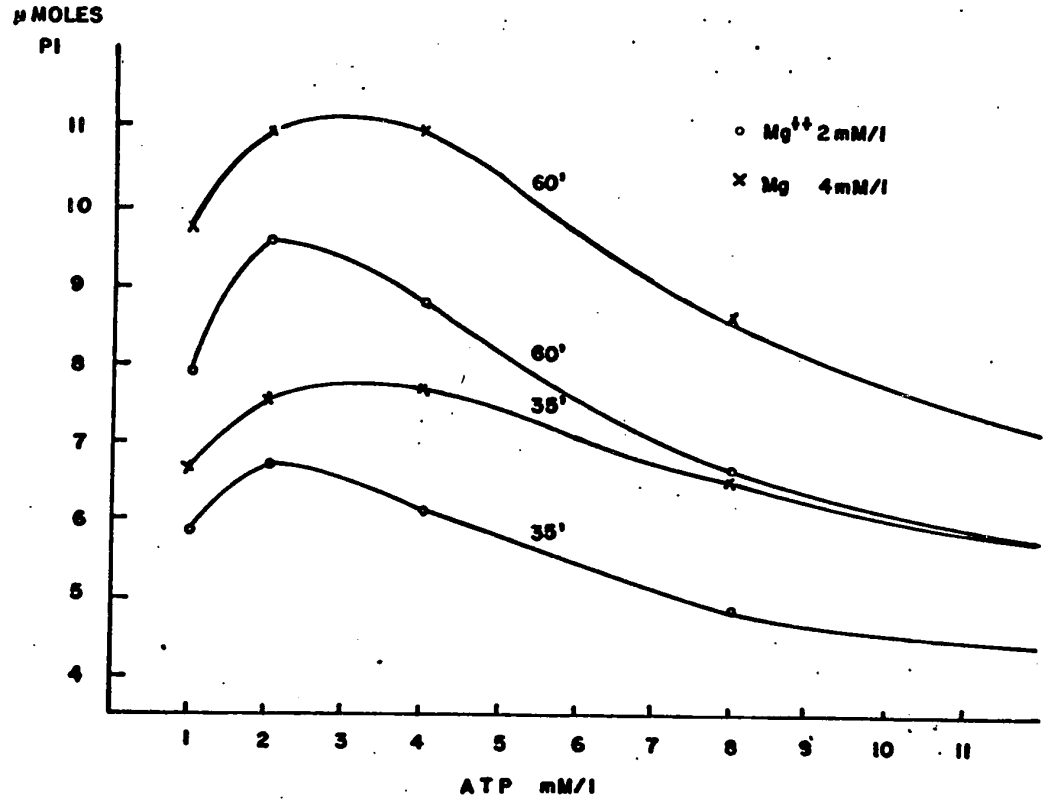


Figure 21. The effect of increasing ATP concentration on the ATPase activity of rabbit aortic homogenate. Reaction media: 2 mM or 4 mM Mg^{++}, 0.1 mM EDTA, 100 mM Tris-HCl buffer, pH 7.5; 0.92 mg protein N (homogenate); variable concentrations of Tris-ATP. Enzyme activity is expressed as µ moles inorganic phosphate hydrolyzed from ATP within the time indicated in minutes.

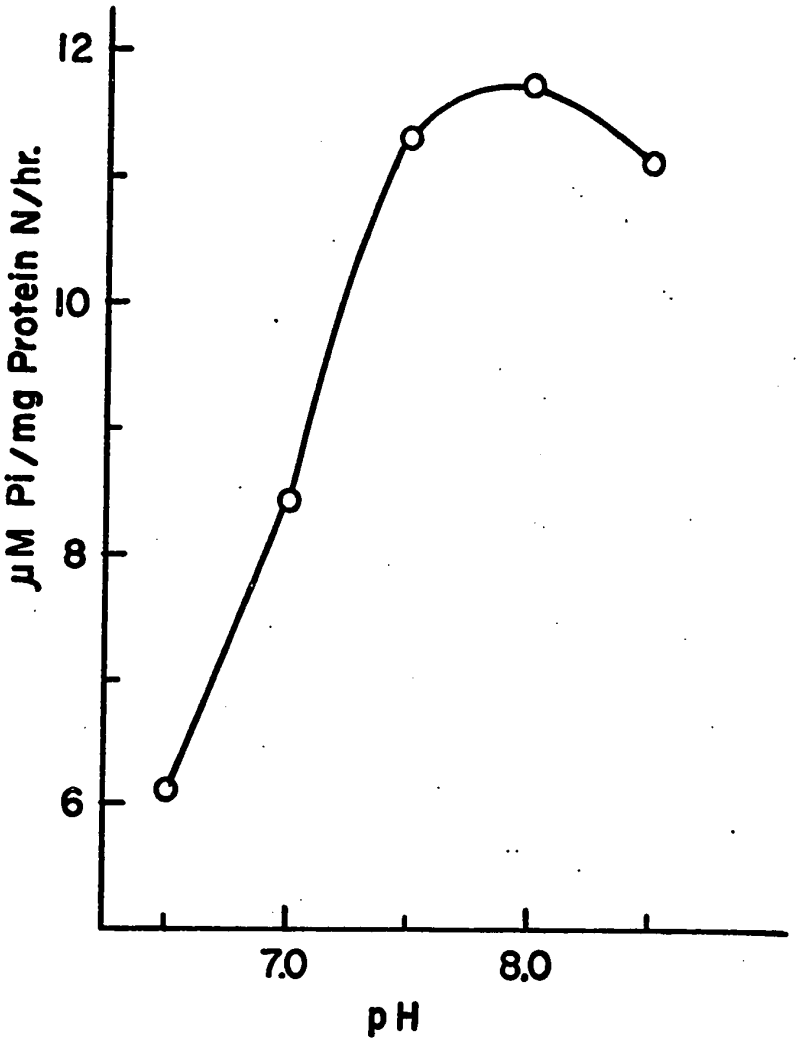


Figure 22. The effect of pH upon the ATPase activity of rabbit aortic homogenates. Assay media: 2 mM Mg^{++}, 2 mM Tris-ATP, 0.1 mM EDTA, 100 mM Tris-HCl buffer at variable pH.

Addition of Na^+ or K^+ alone or together in different concentrations inhibited the Mg^{++}-ATPase activity (Table IX). Also ouabain at a concentration of 0.1 mM was without effect on the Mg^{++}-ATPase activity with or without Na^+ and K^+.

Figure 23 shows that Ca^{++} could inhibit the Mg^{++}-ATPase activity and with increasing concentrations of Ca^{++} the enzyme activity began to approach the level usually observed with Ca^{++} alone.

Not only could ATP act as a substrate but also ITP, GTP, UTP, UMP and AMP were found to be readily hydrolyzed by the total homogenate (Figure 24). Hydrolysis of UMP and AMP was complete within 15 minutes. Also all three of the available phosphate groups on the nucleoside triphosphates were hydrolyzed, therefore implying that dinucleotides could also be hydrolyzed. Table X shows the hydrolysis of ATP, ADP and AMP and the effects of monovalent cations. None of the activity was affected by the addition of Na^+ plus K^+ nor was this activity sensitive to 1 mM ouabain.

It was found that the glass-glass homogenization technique liberated Na^+ from the glass into the homogenate; therefore different techniques to prepare a homogenate or membrane preparation were also used.

When the aortic smooth muscle was homogenized in a teflon-glass homogenizer as described in the Methods or by using a VirTis homogenizer the homogenates obtained contained Mg^{++}-ATPase activity. However, the enzyme activity could not be stimulated by the addition of Na^+ plus K^+ or inhibited by the addition of ouabain.

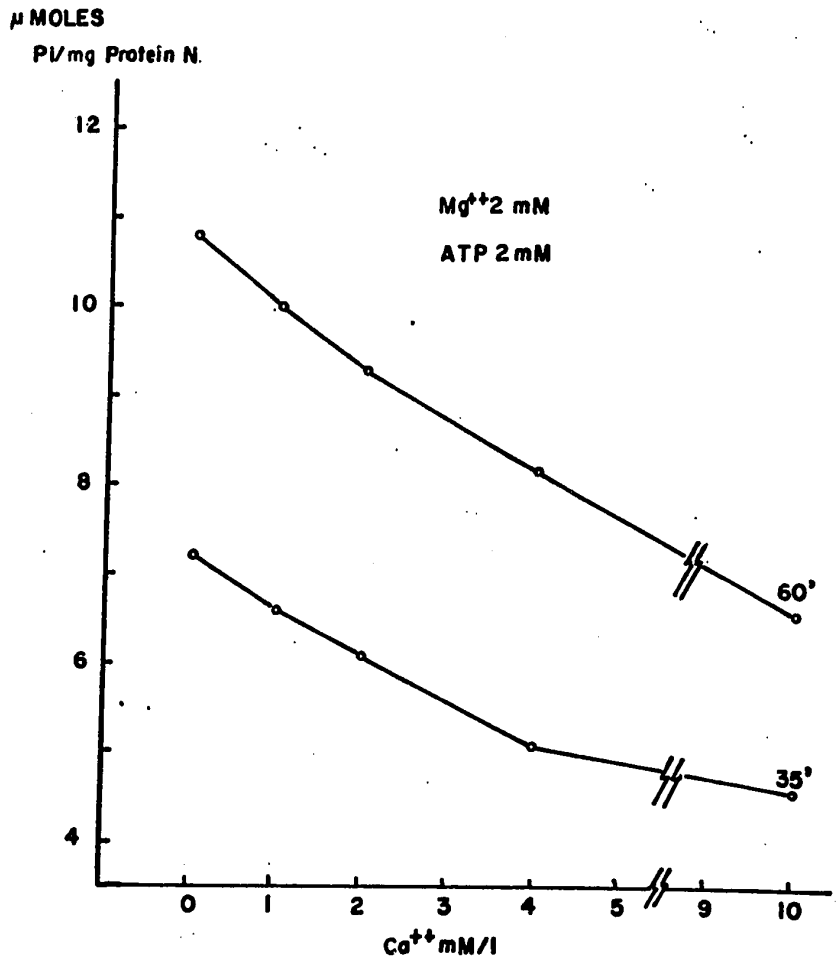


Figure 23. The effects of Ca^{++} on the Mg^{++}-ATPase activity of rabbit aortic homogenate. Assay media: 2 mM Mg^{++}, 2 mM Tris-ATP, 0.1 mM EDTA, 100 mM Tris-HCl buffer, pH 7.6; variable concentrations of Ca^{++}. Enzyme activity is epxressed as µ moles inorganic phosphate hydrolyzed from ATP within the time indicated in minutes.

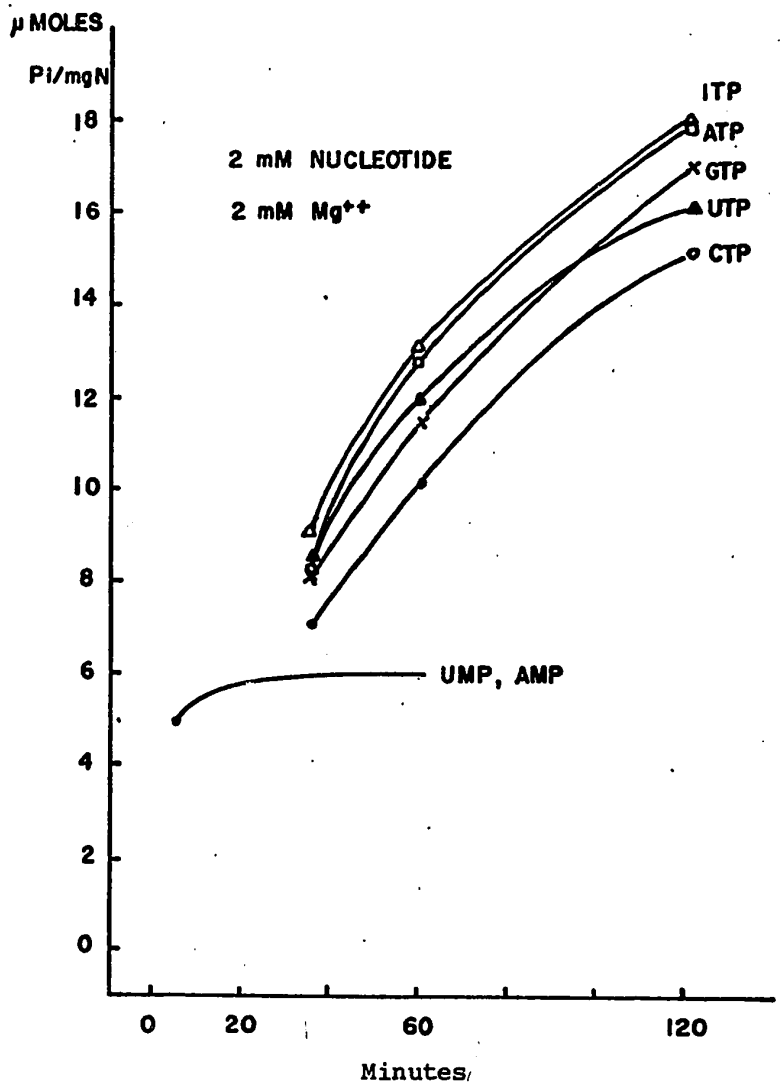


Figure 24. Hydrolysis of nucleotides by the homogenate of rabbit aorta. Assay media: 2 mM Mg^{++}, 2 mM nucleotide, 0.1 mM EDTA, and 100 mM Tris-HCl buffer, pH 7.5. Enzyme activity expressed as μ moles inorganic phosphate hydrolyzed from nucleotide within the time indicated. An activity of 6 is equivalent to the complete hydrolysis of the terminal phosphate of the nucleotide.

TABLE X. Effect of Na^+ plus K^+ and Ouabain on the Hydrolysis of Other Adenine Nucleotides.

	μM P_i/mg Protein N/hr		
SUBSTRATE	Mg^{++}	$Mg^{++}+Na^+ +K^+$	$Mg^{++}+Na^+ +K^+ +0$
ATP	15.60	15.35	15.35
ADP	10.35	10.25	10.25
AMP	17.40	17.20	17.25

Assay Media: 2 mM Mg^{++}, 2 mM Tris-ATP, ± 5 mM K^+, ± 20 mM Na^+, ± 1.0 mM ouabain and 100 mM Tris-HCl buffer pH 7.5. Each value is the mean of two determinations obtained from a 5% homogenate of rabbit aorta.

ii. ATPase Activity in Membrane Preparations of Aorta.

TABLE XI. ATPase Activity of Aortic Membrane Preparations.

Membrane Preparation		(1)	(2)	(3)
mM Na^+	±1 mM ouabain			
0	-	1.58	1.84	1.216
20	-	1.82	1.65	1.15
40	-	1.64	1.63	1.067
100	-	1.24	1.45	0.833
20	+	1.78	1.70	1.133
40	+	1.52	1.65	1.067
100	+	1.36	1.48	0.867

Membranes were isolated by the method of Carroll and Sereda (1968).
Assay Media: 2 mM Mg^{++}, 2 mM Tris-ATP, 5 mM K^+, varying concentrations of Na^+, ± 1 mM ouabain in 100 mM Tris-HCl buffer pH 7.5.

When aortic membranes were isolated by the method of Carroll and Sereda (1968) a very low specific ATPase activity was obtained which could not be stimulated by the monovalent cations (Table XI). In the presence of 5 mM K^+ increasing the concentration of Na^+ resulted in an inhibition of ATPase activity. Ouabain had no effect.

Table XII shows the ATPase activities of heart and aorta after

TABLE XII

ATPase Activity of Salt Extracted Rabbit Heart and Aorta

Preparation		μM P_i/mg protein N/hr					Activity Ratio	
		(1) Mg^{++}	(2) + K^+	(3) + Na^+	(4) + Na^++K^+	(5) + Na^++K^+ + ouabain	(4)/(1)	(5)/(1)
Heart	a	12.54	12.54	12.50	27.28	12.03	2.175	0.958
	b	19.00	17.70	17.00	52.30	19.00	2.753	1.000
Aorta	a	24.78	24.78	26.44	25.60	26.34	1.033	1.062
	b	23.36	22.56	20.46	20.95	22.07	0.896	0.945

LiBr salt extraction of the tissue homogenates was carried out as described in the Methods. Assay media: 2 mM Mg^{++}, 2 mM Tris-ATP, 100 mM Tris-HCl buffer pH 7.6; ± 80 mM Na^+, ± 10 mM K^+, ± 0.1 mM ouabain.

extraction of the respective homogenates with 1 M LiBr for 18 hours. This method was developed for cardiac tissue by Potter and co-workers (1966) and was found to work well on rabbit heart. The ATPase of the heart preparation was not affected by Na^+ or K^+ alone but markedly stimulated by the addition of Na^+ plus K^+ together. This Na^+ plus K^+ stimulation could also be completely inhibited by ouabain. When aortic homogenates were treated and prepared in the exact same manner no Na^+ plus K^+ stimulation or ouabain inhibition could be observed.

iii. ATPase Activity of Subcellular Fraction. When VirTis homogenized aorta was fractionated by differential centrifugation three pellets and a supernatant were obtained (P_1, P_2, P_3, and S_{3A}). The distribution of Mg^{++}-ATPase activity in these fractions are listed in Table XIII.

TABLE XIII. Distribution of Mg^{++}-ATPase in Aortic Subcellular Fractions.

	Homogenate	P_1	P_2	P_3	S_{3A}
% of Total Activity	100	15	20	60	5
Specific Activity (in μM P_i/mg protein N/hr)	10.1±1.78	1.59±0.17	9.68±1.29	90.0±5.61	5.5±0.79

Assay Media: 2 mM Mg^{++}, 2 mM ATP, o.1 mM EDTA, 100 mM Tris-HCl pH 7.5. Specific activity values are means ± S.E.M. of 4 different preparations. Total activity is expressed as %/gm. equivalent of tissue wet weight.

Although fraction P_1 contained most of the total protein N it contained the least amount of Mg^{++}-ATPase activity. Most of the total Mg^{++}-ATPase activity emerged in fraction P_3 and the specific activity in this fraction was about ten times that of the original homogenate (Table XIII).

When the Na^+ plus K^+ dependence or ouabain sensitivity of the various fractions were assayed, no significant effects were observed. High concentrations of Na^+ (> 50 mM) significantly inhibited ATPase activity in all fractions. The properties of the ATPase activity of the fractions were also similar to those found in the complete homogenate with respect to the Mg^{++} or Ca^{++} activation, pH dependence and nucleotide specificity.

iv. Actomyosin ATPase and Adenylate Kinase Activity of Fractions. In order to see how much of the ATPase activity could be due to actomyosin ATPase the actomyosin of the fractions was extracted by 0.6 M KCl as described in the Methods. A control extraction was carried out with 0.25 M sucrose for comparison. The extract and extracted pellets of each fraction were assayed for Mg^{++}-ATPase and Ca^{++}-ATPase activity (Table XIV). To study the stimulation of actomyosin Ca^{++}-ATPase by high ionic strength the ionic strength of the assay media was increased by the addition of K^+.

Compared to the controls 0.6 M KCl extraction slightly reduced the ATPase activities found in the extracted fractions with the greatest reduction occurring in fraction P_1. In both the sucrose and the 0.6 M KCl extracted fractions the ATPase activity in the presence of Mg^{++} was

TABLE XIV

ATPase Activities of Subcellular Fractions and Extracts After Extraction of Actomyosin.

	µM P_i/gm Equivalent of Tissue/hour					
	Control 0.25 M Sucrose Extracted			Experimental 0.6 M KCl Extracted		
Extracted Fraction	Mg^{++}	Ca^{++}	$Ca^{++} + K^{+}$	Mg^{++}	Ca^{++}	$Ca^{++} + K^{+}$
P_1	17.37	9.46	7.27	6.67	3.41	2.02
P_2	24.63	12.79	10.39	21.58	10.65	8.99
P_3	147.62	53.93	31.21	137.00	40.79	24.01
Extract						
P_1E	0.78	0.78	0.47	3.26	3.57	16.29
P_2E	2.59	1.61	1.51	1.61	2.00	2.40
P_3E	6.60	2.64	2.57	5.98	2.00	3.41

Values $P_1 \rightarrow P_3$ represent the activities left in the fractions after extraction of pellets with 0.25 M sucrose as control and with Weber-Edsall (0.6 M KCl) for the experimental. $P_1E \rightarrow P_3E$ are the corresponding tissue extracts. Assay media: 2 mM Tris-ATP, 100 mM Tris-HCl buffer, pH 7.5; 2 mM Mg^{++} or 2 mM Ca^{++} ± 0.425 M K^{+}. (I = 0.074 without and I = 0.5 with K^{+} added.)

greater than in the presence of Ca^{++}. Also high ionic strength stimulation of the Ca^{++}-ATPase activity in the extracted fractions was not observed.

With the exception of the 0.6 M KCl extract of P_1, both the control and experimental extracts contained only a small percentage of the ATPase activity found in the original fraction. The Ca^{++}-ATPase activity of the experimental extracts could be stimulated by high ionic strength, whereas the Ca^{++}-ATPase activity in the control extracts did not have this property. If the Mg^{++}-ATPase activities in the 0.6 M KCl extracts P_1E, P_2E, and P_3E represent all the Mg^{++}-sensitive actomyosin ATPase which contaminated the original fractions then this activity would account for approximately 25-30% of the Mg^{++}-ATPase activity in P_1, 6-7% in P_2, and 4% in P_3.

ADP was hydrolyzed by the homogenate and by all the fractions with the highest activity in fraction P_3. The possibility that adenylate kinase activity followed by ATP splitting accounted for ADP hydrolysis was examined. Adenylate kinase assay was carried out using thin layer chromatography as described in the Methods. By boiling the fractions at pH 1.0 for 30 minutes all the nucleoside phosphatases were denatured and none of the fractions showed significant ADP hydrolysis. Figure 25 shows that spots for ATP and AMP could be observed on the chromatoplate after spotting and chromatographing the medium in which the complete homogenate and the subcellular fractions were incubated with ADP. In control incubations with ADP alone (no enzyme) and homogenates or fractions alone (no substrate), neither ATP nor AMP formation was found. The amount of

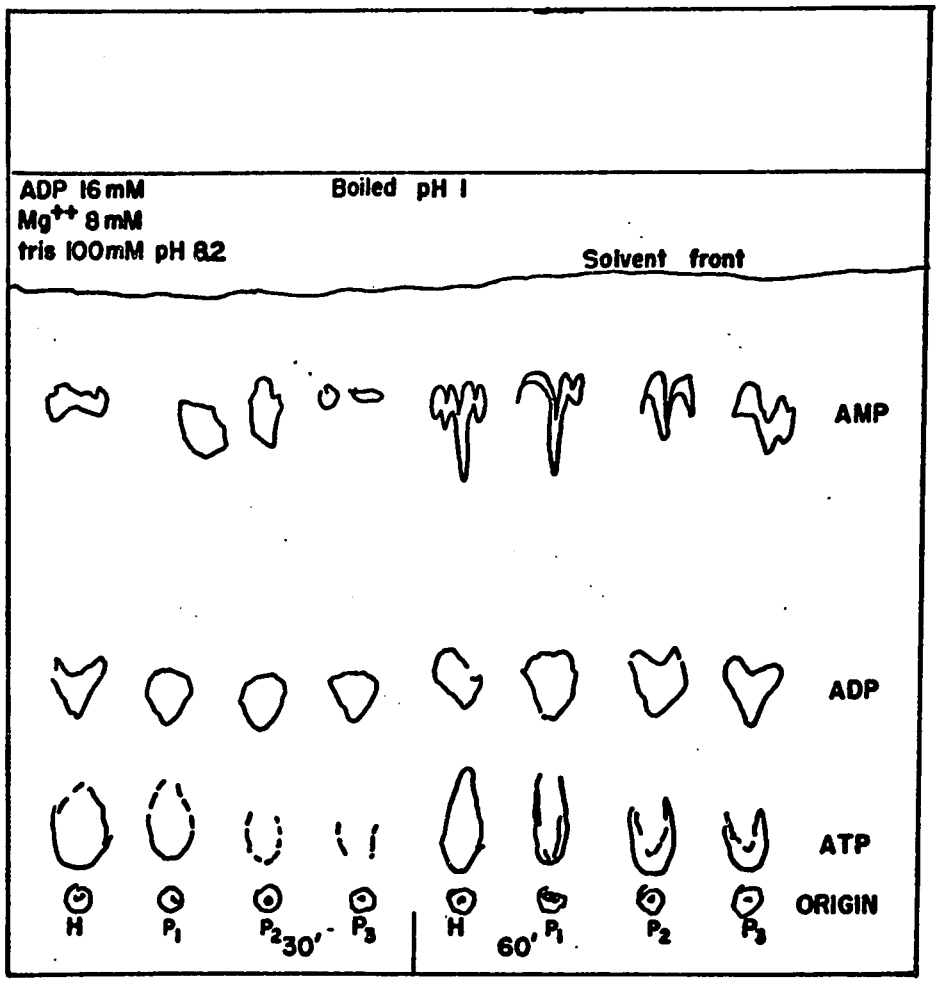


Figure 25. Thin layer chromatogram as visualized under a U.V. light. The origin was spotted with aliquots of the media in which the boiled fractions were incubated in the presence of ADP to test for adenylate kinase activity.

ATP formation by the preparations was greatest for the complete homogenate and P_1 fraction. A 60 minute incubation showed more ATP formation.

v. Use of Histidine Buffer Instead of Tris Buffer for ATPase Activity. Figure 26 shows that the rate of hydrolysis of the adenine nucleotides in the presence of histidine buffer instead of Tris buffer was reduced. The ATPase activity was reduced to about one-half, the ADPase activity was reduced to one-fifth, and the AMPase activity was almost absent.

Table XV shows the effects of Na^+ plus K^+ on the Mg^{++}-ATPase activity in the homogenate and subcellular fractions using a histidine buffered assay medium. A 26.2% Na^+ plus K^+ stimulation of the Mg^{++}-ATPase activity was observed in the total homogenate; however, when the homogenate was fractionated the resulting Mg^{++}-ATPase activity in the pellets P_1, P_2 and P_3 was not stimulated by Na^+ plus K^+ to a significant degree. In order to determine at what stage of the subcellular fractionation the monovalent cation stimulation was lost the supernatant fractions S_1, S_2 and S_{3A} were studied. The Na^+ plus K^+ stimulated activity was found in S_1 and S_2. After isolation of fraction P_3 from S_2 most of the Mg^{++}-ATPase material sedimented into fraction P_3, but this activity was not stimulated by Na^+ plus K^+ addition. The S_{3A} supernatant contained very little ATPase activity and was not significantly stimulated by cations. When the supernatant S_{3A} was added back to fraction P_3 the Na^+ plus K^+ stimulation was regained.

Figure 27 shows that by varying the Na^+ and K^+ concentration optimal stimulation was obtained when Na^+ was 50 mM and K^+ 2 mM. Figure 28

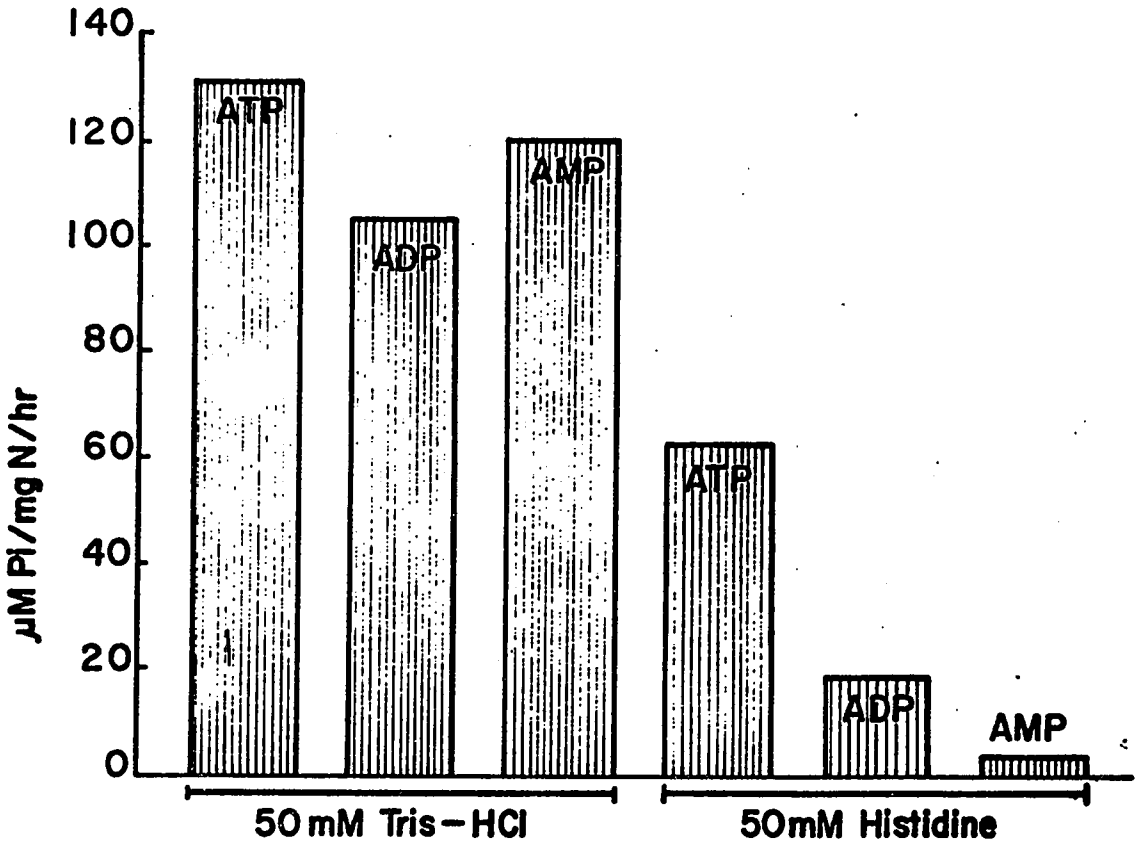


Figure 26. Effects of buffers on the rates of hydrolysis of nucleotides by fraction P_3 of rabbit aorta. Assay media: 4 mM Mg^{++}, 4 mM nucleotide and 50 mM Tris-HCl, buffer pH 7.6, or 50 mM histidine buffer, pH 7.6. Values are means from 3 preparations.

TABLE XV

Distribution of Na^+ plus K^+ Stimulated ATPase Activity in Subcellular Fractions.

Fraction	μM P_i/mg protein N/hr Mg^{++}	Mg^{++}, Na^++K^+	Activity Ratio	Paired t-test
Homogenate	5.67	7.75	1.262	$P < 0.05$
P_1	0.825	0.859	1.042	$P > 0.05$
P_2	10.17	10.83	1.065	$P > 0.05$
P_3	60.00	63.72	1.062	$P > 0.05$
S_1	16.86	22.12	1.312	$P < 0.05$
S_2	18.56	25.27	1.362	$P < 0.05$
S_{3A}	3.68	4.05	1.101	$P > 0.05$
$P_3 + S_{3A}$	20.87	27.82	1.333	$P < 0.05$

Assay media: 4 mM Mg^{++}, 4 mM Tris-ATP, 50 mM histidine-buffer pH 7.6; ± 50 mM Na^+ plus 2 mM K^+.

Activity ratio = Mg^{++}, Na^+ + K^+ activity/Mg^{++} activity.

Values are means from three different preparations.

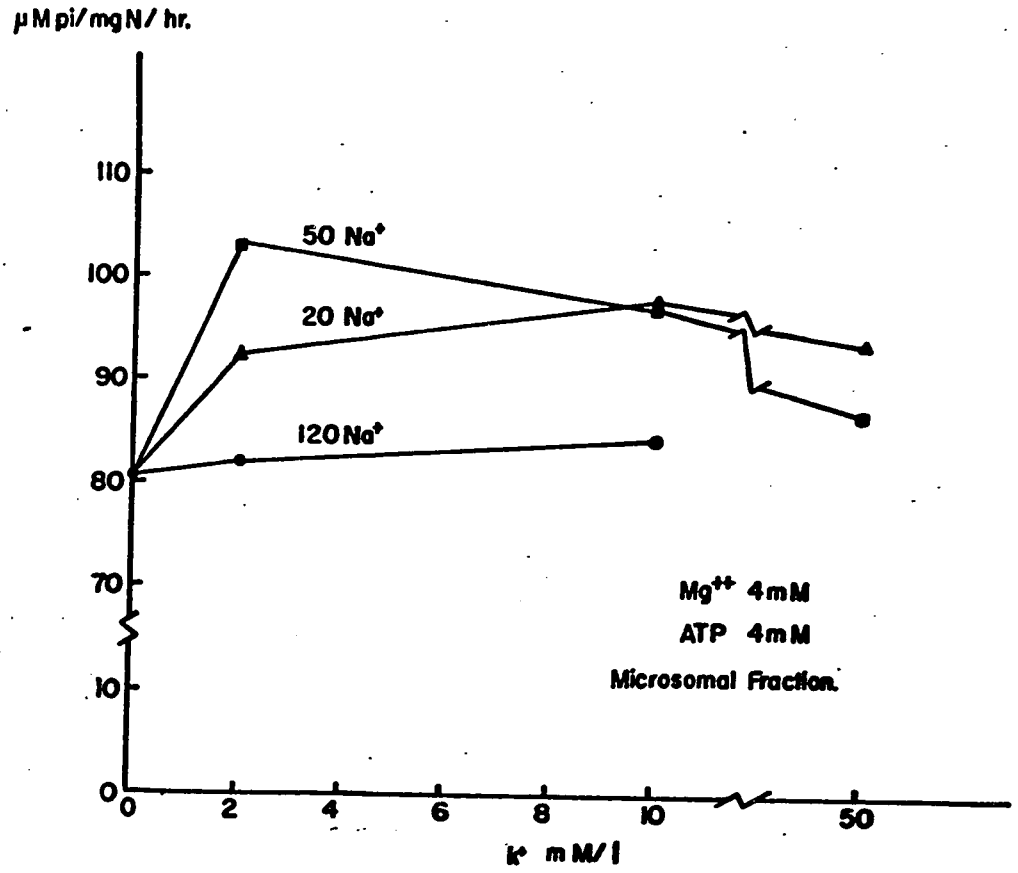


Figure 27. The effect of variable concentrations of K^+ at three constant levels of Na^+ on the ATPase activity of rabbit aortic fraction P_3. Assay media: 4 mM Mg^{++}, 4 mM Tris-ATP, 50 mM histidine buffer pH 7.6; variable concentrations of Na^+ and K^+.

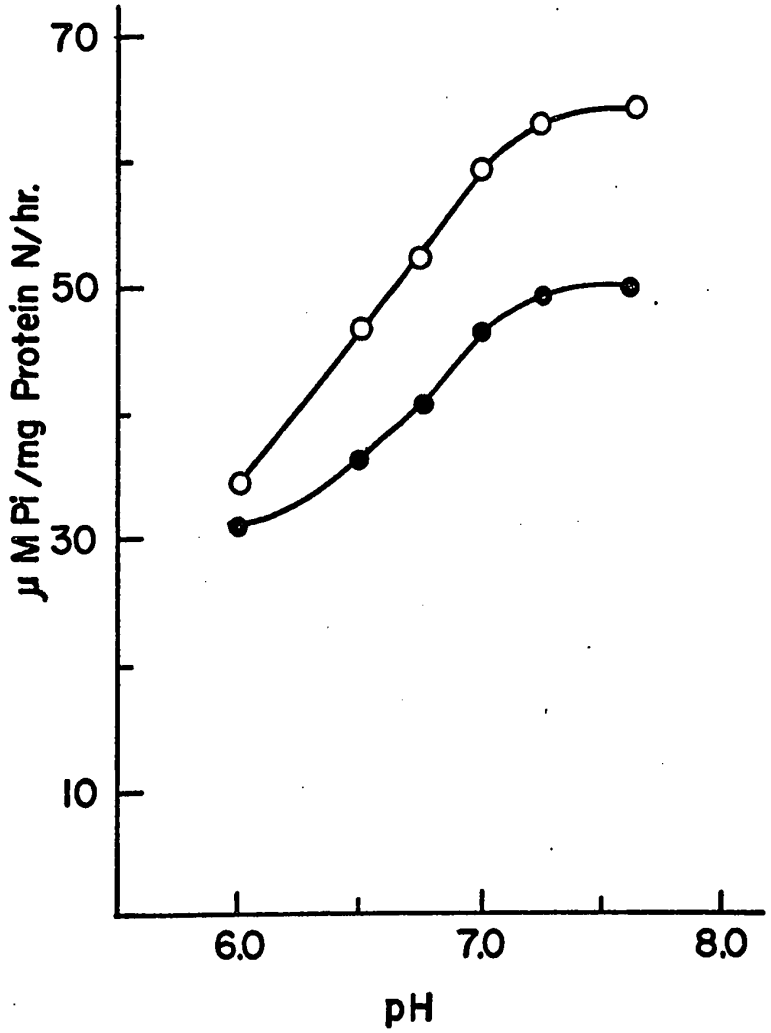


Figure 28. The effect of pH on the ATPase activity of rabbit aortic fraction $P_3 + S_{3A}$. Assay media: 4 mM Mg^{++}, 4 mM Tris-ATP; ± (50 mM Na^+ and 2 mM K^+), 50 mM histidine buffer at variable pH. Open circles with Na^+ and K^+, closed circles without Na^+ and K^+.

shows that the ATPase activity reaches an optimal level in the pH range 7.3-7.6. Also at pH 7.6 Na^+ plus K^+ stimulation was greatest. 50 mM histidine buffer was found to be adequate for the ATPase assay and after a 15 minute enzyme reaction incubation which contained enough of fraction P_3 to hydrolyze 2 µM P_i from 4 µM of ATP/ml in 15 minutes the pH was still the same. Also for most assays the reaction was stopped before one-quarter of the terminal phosphate of ATP was hydrolyzed.

vi. ATPase Activity of Density Gradient Fractions. Table XVI shows the distribution of the ATPase activity of the density gradient fractions obtained from both aortic and kidney homogenates prepared in the same manner. 73.6% of total ATPase activity from the aortic preparations was located in fraction P_D, 23.2% in fraction M_D and 3.24% in N_D. 73-80% of the total ATPase activity from the kidney preparation was in P_D. The kidney P_D fraction also contained the greatest proportion of $(Na^+ + K^+)$-ATPase which was highly stimulated by the addition of Na^+ and K^+.

The aortic density gradient fractions did not show any difference in ATPase activity in the presence of Na^+ plus K^+. Again as with the previous fractions some of the 100,000 x g supernatant material was necessary for Na^+ plus K^+ stimulated activity to be observed.

In order to concentrate the S_{3A} supernatant material it was dialyzed against cold de-ionized water. This resulted in the formation of a flocculent precipitate (S_{3B}) which was easily separated and then redissolved in 5 mM histidine buffer pH 7.6. When increasing concentrations

TABLE XVI

Distribution of ATPase in Density Gradient Prepared Fractions.

Fraction	μM P_i/mg protein N/hr		Activity Ratio
	Mg^{++}	Mg^{++}, Na^++K^+	
Aorta			
P_D	165.3	171.4	1.037
M_D	52.18	51.2	0.981
N_D	7.28	6.95	0.955
Kidney			
P_D	207.1	398.3	1.923
M_D	53.91	88.14	1.635
N_D	5.87	7.70	1.312

Assay Media: 4 mM Mg^{++}, 4 mM Tris-ATP, 50 mM histidine buffer, pH 7.6; ± 50 mM Na^+ and 2 mM K^+. Fractions were obtained by layering S_2 from the respective homogenates onto a density gradient and centrifuging as described in the Methods.

of S_{3B} material were added to the P_D fraction addition of Na^+ plus K^+ stimulated the Mg^{++}-ATPase activity (Figure 29). The best Na^+ plus K^+ stimulation was observed when about 2 parts of the S_{3B} protein mateiral was mixed with one part of the P_D protein. This combination of S_{3B} to P_D was therefore used for the remainder of the ATPase studies.

vii. Properties of P_D + S_{3B} ATPase Activity. As with the ATPase activity of fraction P_3 and the complete homogenate the ATPase activity of P_D + S_{3B} required Mg^{++} for activity in an optimal ratio of 1:1 Mg^{++} to ATP at a concentration of 2 mM each. AT a concentration of 4 mM Mg^{++} equal enzyme activity was obtained at ratios of 1:1 and 2:1 Mg^{++}:ATP. Therefore for most of the studies a 1:1 ratio of 4 mM Mg^{++} and ATP was used so that the increase in ratio with hydrolysis of ATP would not lead to a decreased rate of hydrolysis.

Figures 30 and 31 show the effects of increasing the Na^+ concentration at a constant K^+ concentration and increasing K^+ concentration at a constant Na^+ concentration respectively. For optimal monovalent cation stimulation of the Mg^{++}-ATPase activity a concentration of 2 mM K^+ plus 50 mM Na^+ was required.

Table XVII shows that not only was ATP hydrolyzed by the preparation but also other trinucleotides could act as substrate for the Mg^{++} dependent enzyme. The hydrolysis of ADP and AMP was very low. However, only the hydrolysis of ATP could be significantly stimulated by the addition of Na^+ plus K^+.

Table XVIII shows the effects of Tris-HCl buffer on the ATPase

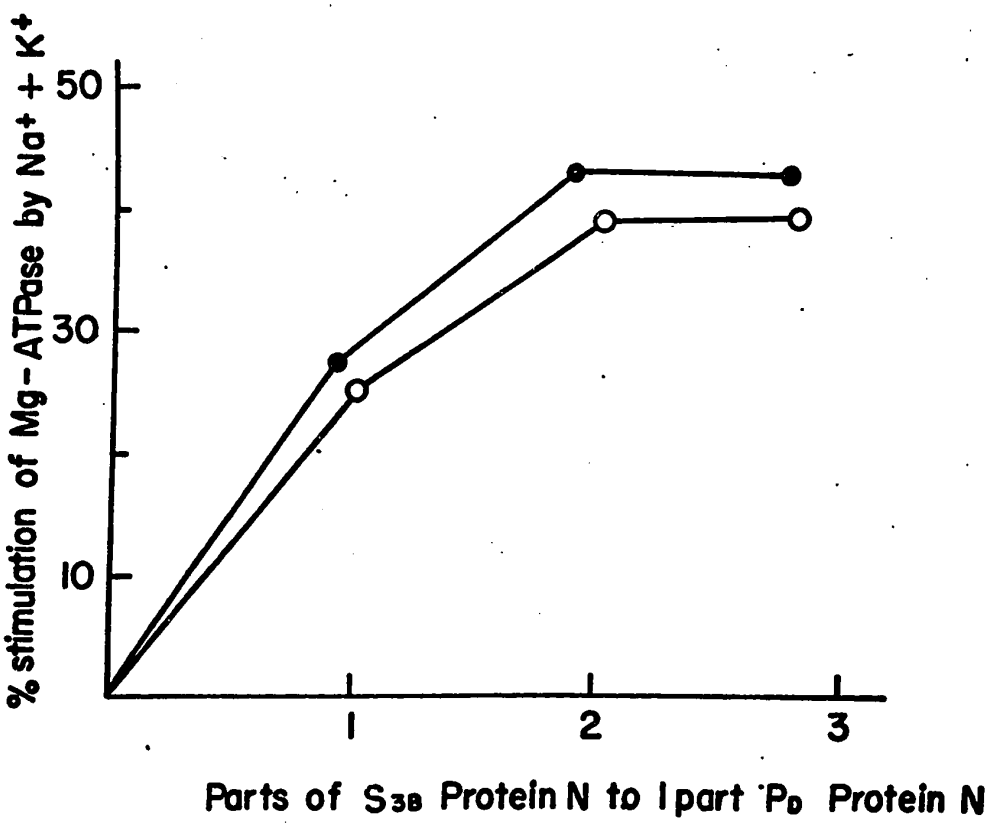


Figure 29. Effects of increasing concentration of S_{3B} material on monovalent cation stimulation of Mg^{++}-ATPase. % stimulation of Mg^{++}-ATPase activity in fraction P_D was assayed with 4 mM Mg, 4 mM Tris-ATP, 50 mM histidine buffer, pH 7.6; $\pm$ (50 mM Na^+ and 2 mM K^+). Results from two experiments are plotted.

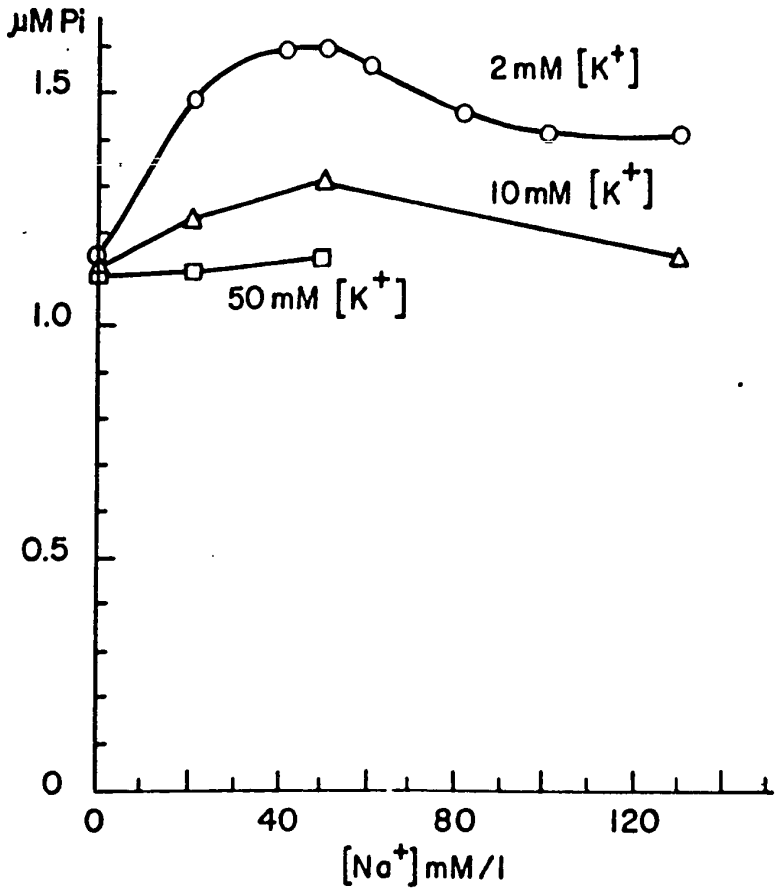


Figure 30. The effect of variable concentrations of Na^+ at three constant levels of K^+ on the ATPase activity of rabbit aortic fraction $P_D + S_{3B}$. Assay media: 4 mM Mg^{++}, 4 mM Tris-ATP, 50 mM histidine buffer, pH 7.6; variable concentrations of Na^+ and K^+. Enzyme reaction time - 15 minutes.

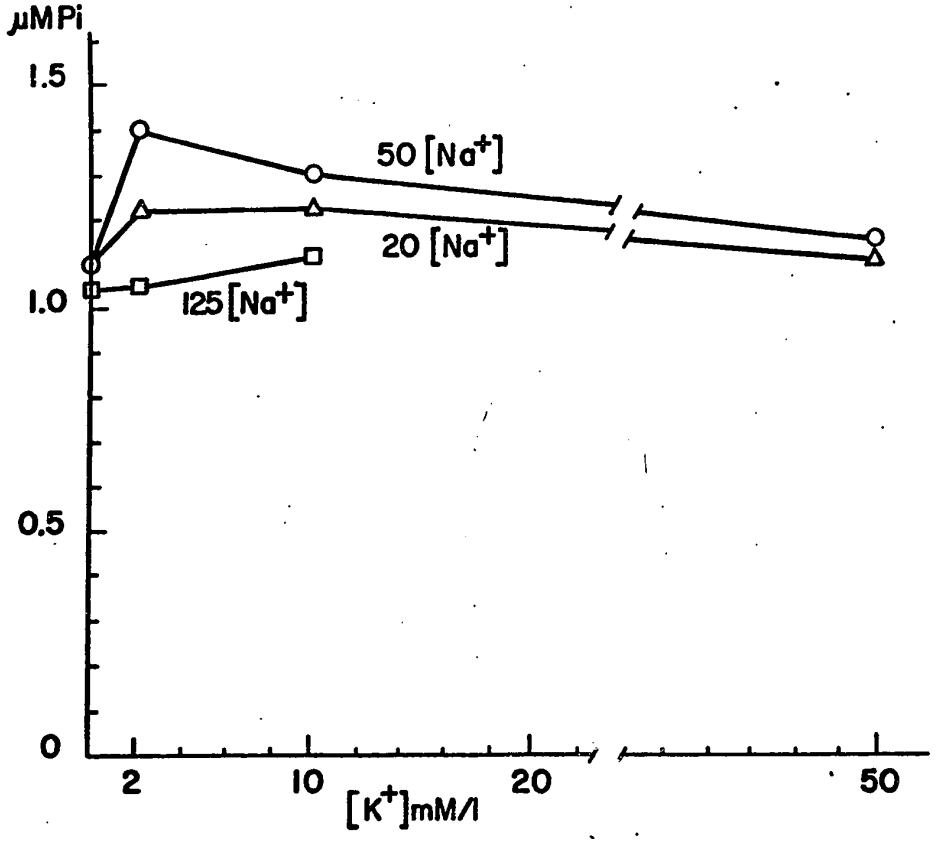


Figure 31. The effect of variable concentrations of K^+ at three constant levels of Na^+ on the ATPase activity of rabbit aortic fraction $P_D + S_{3B}$. Conditions same as in figure 30.

TABLE XVII

Hydrolysis of Other Nucleotides by $P_D + S_{3B}$ and the Effects of Na^+ plus K^+.

Substrate	µM P_i/mg protein N/hr		Activity Ratio	Paired t-test
	Mg^{++}	Mg^{++}, $Na^+ + K^+$		
GTP	112.2	120.0	1.068	N.S.
ITP	128.4	136.5	1.067	N.S.
UTP	95.4	91.5	0.959	N.S.
CTP	64.4	61.7	0.957	N.S.
ATP	67.3	96.2	1.429	$P < 0.05$
ADP	18.1	17.3	0.956	N.S.
AMP	5.5	5.6	1.018	N.S.

Assay media: 4 mM Mg^{++}, 4 mM substrate, 50 mM histidine buffer pH 7.6; ± 50 mM Na^+ and 2 mM K^+.

Values are means of activities from three experiments with each substrate.

TABLE XVIII

Effect of Tris-HCl on the ATPase Activity of $P_D + S_{3B}$.

Tris-HCl Concentration mM/l	µM P_i/mg protein N/hr		% Cation Stimulation	Paired t-test	No.
	Mg^{++}	Mg^{++}, Na^++K^+			
0	46.19	67.07	45.2	$P < 0.05$	6
4	63.29	77.01	21.0	$P < 0.05$	6
12	117.15	124.4	7.75	N.S.	5
50*	131.16	116.63	- 11.2	$P < 0.05$	4

Assay media: 4 mM Mg^{++}-ATP, 50 mM histidine buffer pH 7.6, varying concentrations of Tris-HCl pH 7.6; ± 50 mM Na^+ plus 2 mM K^+.

* 50 mM Tris-HCl was used to replace histidine. Values are means of 4 to 6 determinations.

activity of $P_D + S_{3B}$. Addition of increasing amounts of Tris-HCl pH 7.6 to the assay medium resulted in an increase in the specific activity of the enzyme. However, the rate of Mg^{++}-ATPase activity increased more than the same activity in the presence of Na^+ plus K^+, with a net loss in monovalent cation stimulation. When 50 mM Tris-HCl pH 7.6 was used to replace histidine buffer an inhibition of the Mg^{++}-ATPase activity occurred with the addition of Na^+ plus K^+. Because of the effects of Tris-HCl on the enzyme activity the Mg^{++} salt of ATP was used instead of the Tris salt in order to obtain better Na^+ plus K^+ stimulation.

The effects of Ca^{++} on the ATPase activity are presented in Table XIX. Addition of Ca^{++} to the enzyme reaction medium caused an inhibition of the ATPase-activity both in the presence and in the absence of Na^+ plus K^+, and decreased the per cent stimulation by addition of Na^+ plus K^+. The inhibition of the monovalent cation stimulated portion of the activity was significantly greater than the inhibition of the Mg^{++} activity. Also when Ca^{++} was used to replace Mg^{++} the Ca^{++}-ATPase activity obtained was not stimulated by the further addition of Na^+ plus K^+.

Table XX compares the effects of Na^+ and K^+ individually and in combination on the Mg^{++}-ATPase activity. The slope of the individual lines in the inserted figure represent the effects of Na^+ (B) on the Mg^{++}-ATPase activity in the absence (A_1) and in the presence (A_2) of K^+. Na^+ ions significantly stimulated the enzyme in both the presence and absence of K^+. In addition K^+ alone was able to stimulate the enzyme, but to a lesser degree, and this stimulation by K^+ appeared to be independent of the presence or absence of Na^+.

TABLE XIX

Effects of Ca^{++} on the ATPase Activity of $P_D + S_{3B}$.

mM Ca^{++} Added	µM P_i/mg protein N/hr		% Cation Stimulation	Paired t-test
	Mg^{++}	Mg^{++}, $Na^+ + K^+$		
0	39.8	60.5	52.0	$P < 0.05$
4	33.0	38.1	14.0	$P > 0.05$
% Inhibition by Ca^{++}	17.0	37.0		$P < 0.05$
4 mM Ca^{++} 0 Mg^{++}	44.8	43.2	- 2.1	$P > 0.05$

Assay media: 4 mM Mg^{++}-ATP, 50 mM histidine-buffer pH 7.6; ± 4 mM Ca^{++}, ± 50 mM Na^+ plus 2 mM K^+

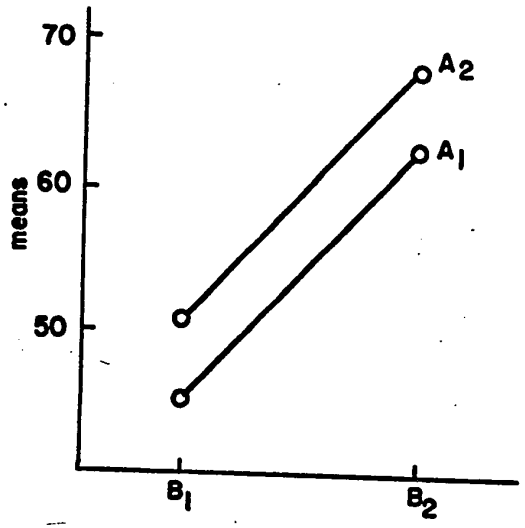
Values are means obtained from 3 enzyme preparations.

TABLE XX. The effects of Na^+ and K^+ individually and in combination on the Mg-ATPase of $P_D + S_{3B}$

	μM Pi/mg protein N/hr			
	A_1B_1	A_1B_2	A_2B_1	A_2B_2
	$0Na^+$, $0K^+$	$50Na^+$, $0K^+$	$0Na^+$, $2K^+$	$50Na^+$, $2K^+$
means	45.233	62.204	50.649	67.912

Summary of the Analysis of Variance for Data

Source of Variation	Sum of Squares	d.f.	Mean Square	F
A	123.74	1	123.74	158.19
B	1171.95	1	1171.95	1498.27
A x B	0.0851	1	0.0851	0.1087
Blocks	1.4025	3	0.4675	
Error	7.0405	9	0.7822	
TOTAL	1304.2181	15		



Assay media contained 4 mM Mg-ATP, ± 50 mM Na^+, ± 2 mM K^+ and 50 mM histidine buffer, pH 7.6. Insert plot compares "B" (the Na^+ effect) as a function of "A" (the K^+ effect). Data were analyzed by analysis of variance for a 2 x 2 factorial experiment with a block design. F values for the A effect, the B effect and the A x B interaction are significant with $\alpha = 0.05$ when F is > 5.12.

The slopes of the Na^+ effects do not deviate significantly from being parallel, and the extra stimulation observed in the presence of Na^+ and K^+ together was not due to an interaction between the individual effects. The effect of Na^+ plus K^+ together therefore appears to result from addition of the individual effects.

Table XXI shows that ouabain had no significant effect on the Mg^{++}-ATPase or the stimulation of the Mg^{++}-ATPase by Na^+ or K^+ alone. Only a small but significant fraction of the Na^+ plus K^+ stimulated activity was inhibited by ouabain. This reduced the Na^+ plus K^+ stimulated activity down to the level of stimulation observed with Na^+ alone.

Figure 32 shows the effects of different concentrations of ouabain on the Na^+ plus K^+ stimulated portion of the ATPase. Only about 20-25% of the stimulation could be inhibited by ouabain in the concentration range of 10^{-3} to 10^{-6} M. Lower concentrations of ouabain (10^{-8} and 10^{-10} M) were also tried but no significant inhibition was observed.

That the ouabain inhibition could be overcome by K^+ is shown in Table XXII. At a K^+ concentration of 10 mM, ouabain inhibition was considerably reduced and 20 mM K^+ abolished it.

Ethacrynic acid was found to inhibit the Mg^{++}-ATPase activity and the monovalent cation stimulated activity (Table XXIII). The inhibition of the Na^+ and of the Na^+ plus K^+ stimulated activities were of the same relative magnitudes and greater than the inhibition of the

TABLE XXI

The Effects of Ouabain on the ATPase Activity of $P_D + S_{3B}$.

Addition	Activity Ratio of ATPase Activity		Paired t-test
	Mg^{++}	Mg^{++} + Ouabain	
None	1.000	1.002	$P > 0.05$
$Na^+ + K^+$	1.461	1.329	$P < 0.05$
Na^+	1.349	1.351	$P > 0.05$
K^+	1.065	1.063	$P > 0.05$

Assay Media: 4 mM Mg-ATP, 50 mM histidine buffer, pH 7.6; ± 50 mM Na^+, ± 2 mM K^+, ± 0.1 mM ouabain. Values are means from 3 experiments. Activity ratio is the Mg^{++} + other addition ATPase activity/Mg^{++}-ATPase activity.

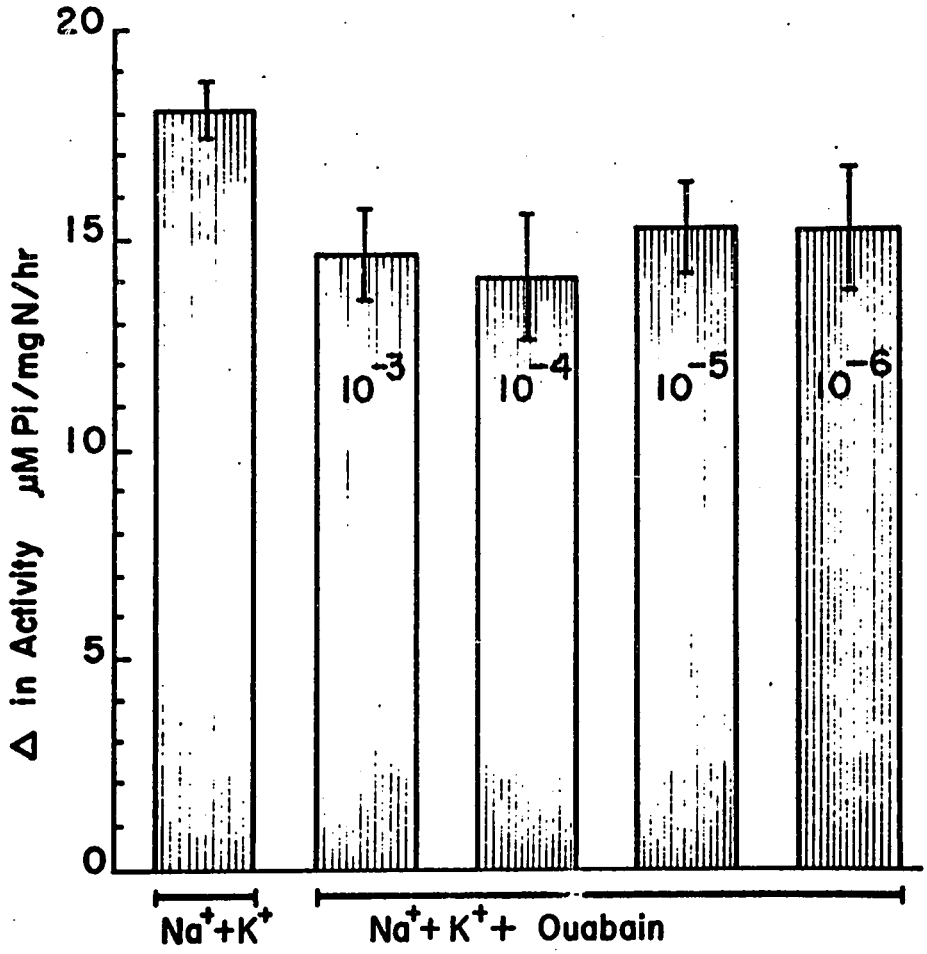


Figure 32. Effect of ouabain on the monovalent cation stimulated Mg^{++}-ATPase activity of rabbit aortic fraction $P_D + S_{3B}$. Assay media: 4 mM Mg-ATP, 50 mM Na^+, 2 mM K^+, 50 mM histidine buffer, pH 7.6; variable concentrations of ouabain in moles. Values are means ± S.E.M. from four different preparations.

TABLE XXII

Effect of K^+ on Ouabain Inhibition.

% Inhibition of Na^+ plus K^+ Stimulation by Ouabain	K^+ mM/1
19.13	2
16.71	4
3.04*	10
0.00*	20

Assay Media: 4 mM Mg^{++}-ATP, 50 mM Na^+, 0.1 mM ouabain, 50 mM histidine buffer pH 7.6; and varying concentrations of K^+. Values are means from 4 experiments.

* $P < 0.05$ when compared to the 2 mM K^+ value (Paired t-test).

TABLE XXIII

Effect of Ethacrynic Acid on ATPase Activity of P_D and S_{3B}.

	$\mu M\ P_i$/mg protein N/hr		
	Mg^{++}	$Mg^{++} + Na^+$	Mg^{++}, $Na^+ + K^+$
Control	48.76	65.67	68.75
+ 5×10^{-4} M Ethacrynic Acid	39.32	48.98	51.40
% Inhibition by Ethacrynic Acid	19.4	25.4	25.20

Assay media: 4 mM Mg^{++}-ATP, 50 mM histidine buffer pH 7.6; ± 50 mM Na^+, ± 2 mM K^+, ± 5×10^{-4}M ethacrynic acid.

Values are means from two experiments.

Mg^{++}-ATPase activity.

viii. Effects of Other Treatments and Agents. Table XXIV shows the effects of aging on the ATPase activity. Aging did not increase the stimulation by Na^+ and Na^+ plus K^+. The extra stimulation usually observed with Na^+ plus K^+ together was almost completely lost after aging for one day at -15°C. The Na^+ stimulated activity was always less after aging than in the fresh preparation, and the specific enzyme activity decreased with increasing time of aging.

In an attempt to increase the sensitivity to ouabain the enzyme was allowed to pre-incubate with ouabain at 37°C in the reaction flask before the reaction was started by the addition of Mg^{++}-ATP. This did not change the degree of ouabain inhibition. Also pre-incubation in the presence of ouabain and Na_2ATP for 30 minutes prior to initiation of reaction by addition of Mg^{++} did not increase ouabain sensitivity.

Azide was found to inhibit the Mg^{++}-ATPase activity more than the monovalent cation stimulated activity (Table XXV). This effect of azide resulted in an apparent increased per cent stimulation of the Mg^{++}-ATPase by the monovalent cations individually or together.

Attempts to deplete the fractions of possible K^+ contamination did not alter the K^+ dependence or ouabain sensitivity of the ATPase activity of $P_D + S_{3B}$. Neither depletion of tissue K^+ by soaking overnight in K^+ free Krebs-Ringer at 5°C prior to homogenization nor washing the P_D material with a K^+ chelating agent (tetraphenylboron) changed the

TABLE XXIV

Effects of Aging on ATPase Activity.

Days Aged	μM P_i/mg protein N/hr			
	(1) Mg^{++}	(2) + Na^+	(3) + Na^++K^+	(3) - (2)
Fresh	58.39	78.25	83.51	5.26
1	47.78	64.26	64.50	0.24
3	42.16	51.86	51.86	0.0
7	37.48	43.17	43.09	- 0.08
14	34.35	38.82	38.98	- 0.16

Suspensions of P_D plus S_{3B} in 5 mM histidine buffer pH 7.6 and kept frozen at -15°C for the number of days indicated, and then thawed at 37°C.

Assay media: 4 mM Mg^{++}-ATP, 50 mM histidine buffer pH 7.6; ± 50 mM Na^+, ± 2 mM K^+.

TABLE XXV

Effect of Azide on ATPase Activity of $P_D + S_{3B}$.

	μM P_i/mg protein N/hr			
	Mg^{++}	+ Na^+	+ Na^++K^+	+ Na^+, K^+ + ouabain
Control	48.24	62.74	66.36	63.10
+ 0.2 mM Azide	28.36	41.13	44.43	41.07
% Inhibition by Azide	41.21	34.44	33.05	34.91
	% Stimulation of Mg^{++}-ATPase			
		+ Na^+	+ Na^++K^+	+ Na^+, K^+ + ouabain
Control		30.10	37.56	30.81
+ 0.2 mM Azide		45.03	56.66	44.82

Assay media: 4 mM Tris-ATP, 4 mM Mg^{++}, 50 mM histidine buffer pH 7.6; ± 50 mM Na^+, ± 2 mM K^+, ± 0.1 mM ouabain and ± 0.2mM azide.

Values are means obtained from three preparations.

relative stimulation of the Mg^{++}-ATPase by Na^{+} or Na^{+} plus K^{+}.

ix. Studies on the S_{3B} Material. Table XXVI shows that the increase in the per cent cation stimulation of the Mg^{++}-ATPase activity of P_D with S_{3B} addition was not due to an increase in the hydrolysis of ATP in the presence of Mg^{++}, Na^{+} plus K^{+} ($P > 0.05$) which was the same before and after addition of S_{3B}. However, the hydrolysis of ATP in the presence of Mg^{++} alone was significantly decreased ($P < 0.05$). The S_{3B} material itself contained very little ATPase activity.

Table XXVII shows that when the S_{3B} material was extracted with a chloroform-methanol mixture most of the active material was found in the non-aqueous (lipid) phase of the extract. Also when increasing amounts of lecithin were added to P_D instead of S_{3B} a similar effect on the P_D ATPase activity was observed. Lecithin addition increased the per cent stimulation of the Mg^{++}-ATPase activity by decreasing the amount of ATP hydrolyzed in the presence of Mg^{++} alone as did S_{3B}. Both the S_{3B} material and phospholipid addition, therefore, seemed to convert the Mg^{++}-ATPase activity to a form which also required monovalent cations for full activity. This property of the S_{3B} material was also found to be heat stable; it was not destroyed by heating at 100°C for 30 minutes.

Some work was also carried out to characterize further or purify the S_{3B} material. When the S_{3B} material was introduced into an electrofocusing apparatus which separates compounds in a pH gradient according to their isoelectric points the results in Figure 33 were

TABLE XXVI

Effects of S_{3B} Material on ATPase Activity of P_D.

Fraction	mg Protein N/ml	μM P_i hydrolyzed/15 minutes		% Cation Stimulation
		Mg^{++}	Mg^{++}, $Na^+ + K^+$	
S_{3B}	0.072 ± 0.008	0.066 ± 0.03	0.073 ± 0.04	8.0 ± 4.0
P_D	0.039 ± 0.01	1.469 ± 0.14	1.681 ± 0.15	14 ± 2.0
$P_D + S_{3B}$	0.108 ± 0.007	1.193 ± 0.15	1.675 ± 0.17	43 ± 5.0

Assay Media: 4 mM Mg-ATP, histidine buffer pH 7.6; ± 50 mM Na^+, ± 2 mM K^+.

Values given represent the mean ± S.E.M. with No.= 4 for experiments S_{3B}, and No.= 6 for experiments P_D and $P_D + S_{3B}$.

TABLE XXVII

Effects of Lipid Extracts of S_{3B} and Lecithin on the Per Cent Na^+ plus K^+ Stimulation of the P_D ATPase.

		μM P_i liberated/15 minutes		% Stimulation
		Mg^{++}	Mg^{++}, Na^++K^+	of Mg-ATPase
A.	P_D Control	1.321	1.533	16.1
	P_D + Aqueous Lipid Extract of S_{3B}	1.233	1.529	24.0
	P_D + Non-aqueous Lipid Extract of S_{3B}	1.158	1.531	32.0
B.	P_D Control	1.540	1.698	10.2
	P_D + 0.5 mg Lecithin	1.510	1.723	14.1
	P_D + 1.0 mg Lecithin	1.426	1.679	17.8
	P_D + 1.5 mg Lecithin	1.369	1.696	24.0

Assay media: 4 mM Mg-ATP, 50 mM histidine buffer pH 7.6; ± 50 mM Na^+, ± 2 mM K^+. In preparation A all of the lipid extract from 5 ml of S_{3B} (0.035 mg protein N/ml) was resuspended in 5 ml of H_2O and 1 ml was mixed with 1 ml of P_D (0.0162 mg protein N /ml). In preparation B the amount of lecithin was in 1 ml of final assay medium.

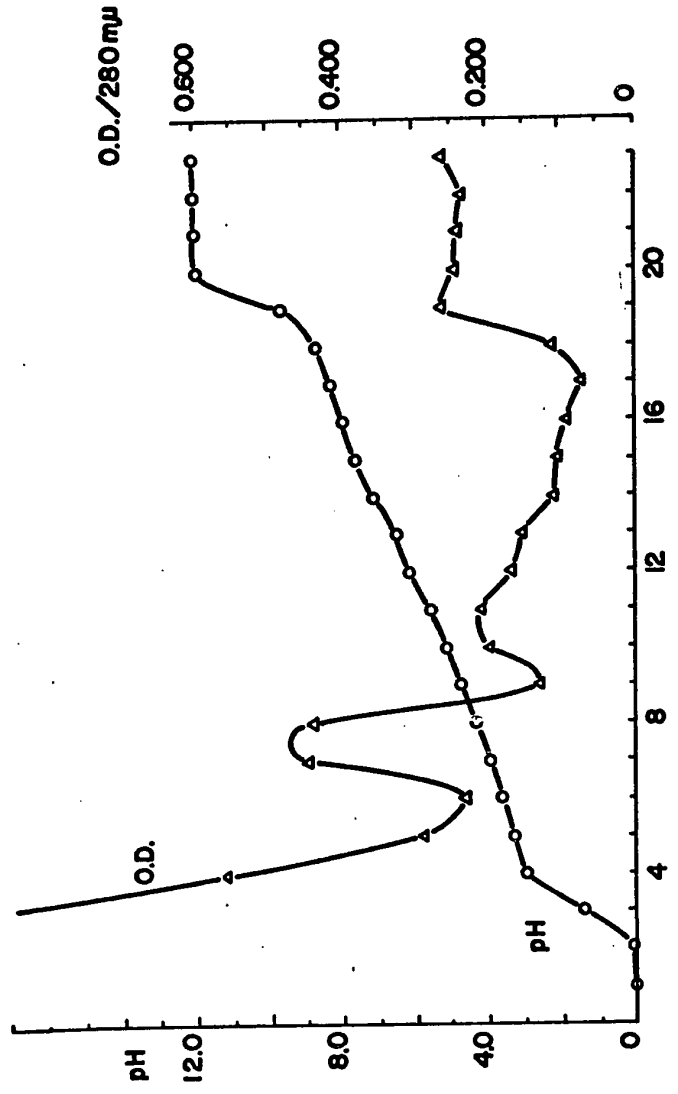


Figure 33. Distribution of S_{3B} material after electrofocusing for 48 hours. Triangles indicate the protein distribution in the collected fractions and the circles indicate the pH of the fractions. Volume of each fraction was 4 ml.

obtained. Two distinct peaks of material were obtained and separated, one in the pH range 3.89 to 4.3 (fraction 7 + 8) and another from pH 5.1 to 5.5 (fraction 10 + 11). The fractions in this area were then collected, dialyzed and assayed for their effects on the P_D ATPase activity.

TABLE XXVIII. Effects of Fractions of the S_{3B} Material Obtained After Electrofocusing on P_D ATPase.

	mg protein N/ml added to P_D (0.027 mg protein N/ml)	% Stimulation of P_D Mg^{++}-ATPase by Na^+ plus K^+
Control	--	11.06
S_{3B}	0.049	44.1
Fraction 7 + 8	0.0151	13.37
Fraction 10 + 11	0.0075	39.1

Per cent stimulation was calculated as described in the Methods.

Assay Media: 4 mM Mg-ATP, 50 mM histidine pH 7.6; ± 50 mM Na^+ ± 2 mM K^+.

Equal volumes of S_{3B} and fractions were mixed with P_D, H_2O was used for the control.

Table XXVIII shows that material obtained from fractions 10-11 possessed the properties of S_{3B} which were required for Na^+ plus K^+ stimulation of the P_D Mg^{++}-ATPase. Since this activating material from S_{3B} equilibrated in the pH range of 5.1-5.5, the material must be weakly acidic with its isoelectric point in this pH range.

By treating the S_{3B} material with anion (DEAE) and cation (CMC) exchange resins the activating material of S_{3B} was found to bind to the anion exchange resin DEAE (Table XXIX). DEAE treatment removed 31.5% of the available protein in S_{3B} and CMC removed 62.0%. However, only the unbound material remaining after CMC treatment possessed the essential Na^+ plus K^+ activating properties of S_{3B}.

When some of the CMC treated S_{3B} material was passed through a sephadex G-100 column a single peak followed by a tail of lower molecular weight compounds was obtained (Figure 34). The three fractions composing the major portion of the peak (fraction at 24 ml, 28 ml, and 32 ml) were tested for their ability to induce the Na^+ plus K^+ stimulation of P_D ATPase. Each of the fractions were found to work equally well and yielded results not different from those obtained with the CMC treated S_{3B} material.

C. Discussion.

i. The ATPase Activities in the Presence of Tris-HCl Buffer.

When the ATPase activities in 5% homogenates of rabbit aorta were assayed in a Tris buffered assay medium significant amounts of Mg^{++} activated and Ca^{++} activated ATPase were found. However, unlike other tissue homogenates the Mg^{++}-ATPase activity of aortic homogenates could not be stimulated by Na^+ plus K^+ or inhibited by ouabain. The Mg^{++} dependent enzyme had very low substrate specificity and could be generally defined as a nucleotide phosphatase activity. If only a small portion of $(Na^+ + K^+)$-ATPase activity actually existed in this preparation, high

TABLE XXIX

Protein N Distribution of Ion Exchange Resin Treated S_{3B} Material and Effect on P_D ATPase.

	P_D	S_{3B}	DEAE Supernatant	CMC Supernatant
Protein N/ml	0.046	0.086	0.0576	0.0320
% protein N Bound by Resin			31.5%	62.0%

Per Cent Na^+ plus K^+ Stimulation of P_D Mg-ATPase Activity After Addition of 1 ml of S_{3B} Preparations to 1 ml of P_D Suspensions.

Control (H_2O)	S_{3B}	DEAE Supernatant	CMC Supernatant
4.33%	55.89%	8.65%	50.82%

S_{3B} material was treated with the ion exchange resins as described in the Methods. The resulting unbound material was then used for ATPase assay with P_D as described in Table XXVIII.

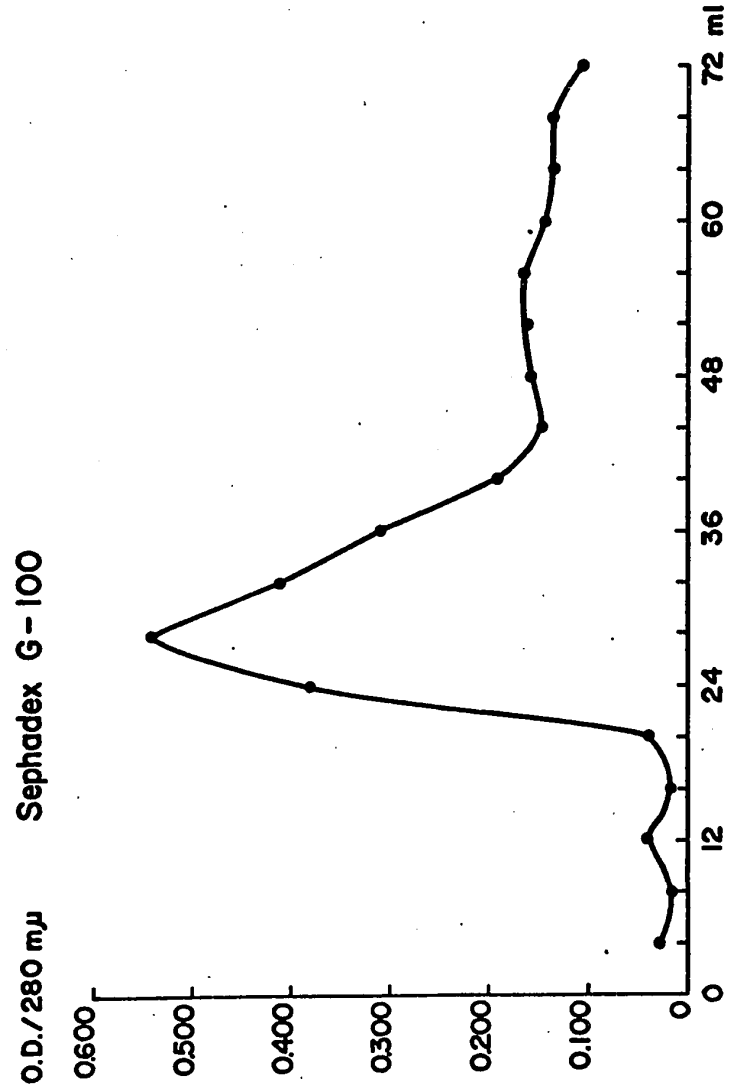


Figure 34. Distribution of CMC-treated S_{3B} material after elution through a Sephadex G-100 column. Material was eluted with 5 mM histidine buffer, pH 7.6.

nucleotide phosphatase activity from other cell components could have easily masked such activity. Also if the homogenate contained sufficient Na^+ plus K^+ contamination to stimulate an existing $(Na^+ + K^+)$-ATPase fully then further addition of these ions could result in the inhibitory effects obtained. This would not, however, explain the lack of ouabain inhibition.

Varying the homogenization technique or attempts at isolation of membrane preparations did not reveal $(Na^+ + K^+)$-ATPase activity in aorta, although the method for cardiac muscle worked very well for rabbit heart muscle. When aortic homogenates were fractionated by differential centrifugation most of the total ATPase activity was in the microsomal P_3 fraction. However, the properties of the ATPase activities of the fractions obtained were not unlike those observed in the original homogenate and the presence of a $(Na^+ + K^+)$-ATPase activity could not be demonstrated.

Thus the results obtained using Tris-HCl buffer for ATPase assay of aortic homogenates or subcellular fractions were in agreement with those recently published by Verity and Bevan (1969), and would tend to support their conclusion that aortic smooth muscle does not possess the classical $(Na^+ + K^+)$-ATPase. However, such an assumption is only true under the conditions of ATPase isolation and assay used. The possibility of the $(Na^+ + K^+)$-ATPase activity being destroyed or transformed by the isolation technique or masked by the method of ATPase assay must also be considered.

ii. ATPase Activities in the Presence of Histidine Buffer. In 1964 Skou reported that the buffer system used can influence the activating

effect of Na^+ on the $(Na^+ + K^+)$-ATPase and that the stimulation was higher in a histidine buffer than in a Tris-buffer at the same pH. When aortic homogenates were assayed for ATPase activity in media containing histidine buffer instead of Tris-buffer, a 26% stimulation of the Mg^{++}-ATPase activity in the homogenate was obtained by the addition of Na^+ plus K^+ together. After isolation of fractions P_1 and P_2 most of the monovalent cation stimulated Mg^{++}-ATPase remained in the resulting S_2 supernatant. However, when fraction S_2 was further centrifuged to obtain P_3 and S_{3A}, almost all of the Mg^{++}-ATPase activity in S_2 sedimented in the microsomal fraction P_3; however, this activity no longer possessed the monovalent cation stimulated properties. A factor remained in the 100,000 x g supernatant material which when added back to fraction P_3 restored the Na^+ plus K^+ stimulation of the Mg^{++}-ATPase activity in this fraction. Optimal stimulation of the Mg^{++}-ATPase of the $P_3 + S_{3A}$ mixture was obtained in the presence of 50 mM Na^+ and 2 mM K^+.

In Tris buffer the rates of ATP, ADP and AMP hydrolysis by $P_D + S_{3B}$ mixtures were higher than in histidine-buffer of the same concentration and pH. In histidine buffer the relative rates of hydrolysis of each of the adenosine nucleotides also differed more than in Tris. The rate of hydrolysis of ADP in histidine was much lower than that of ATP and AMP hydrolysis was almost absent. Moreover, only the hydrolysis of ATP could be stimulated by addition of Na^+ plus K^+ in histidine buffer.

When increasing concentrations of Tris buffer were added to monovalent cation stimulated ATPase preparations in histidine two things happened. Increasing concentrations of Tris buffer resulted in a gradual

loss in the Na^+ plus K^+ stimulated activity with a concomitant increase in the Mg^{++}-ATPase activity. Even small concentrations of Tris associated with Tris-ATP could limit the amount of actual monovalent cation stimulation observed in a histidine buffered assay medium. Therefore Mg^{++}-ATP was used to avoid this problem.

The Tris may be acting as a cation itself and thus activating the enzyme in place of Na^+ and K^+, or it may be directly involved in the mechanism of enzymatic hydrolysis of ATP. Dayan and Wilson (1964) have reported that the presence of Tris can increase alkaline phosphatase activity by catalyzing a transphosphorylation of Tris itself which occurs at a faster rate than that of the intermediate normally involved. Tris could also be interfering with the enzyme reaction by causing a physical change in ATPase enzyme, similar to that reported for tryptophanase by Gopinathan and De Moss (1968).

iii. Involvement of Other Enzymes in the ATPase Activity of Aortic Fractions. Aortic smooth muscle cells also contain actomyosin contractile proteins and since these proteins possess ATPase activities, the effect of their possible contamination of the ATPase activities of the aortic fraction were investigated. Actomyosin has been successfully isolated by Filo and co-workers (1963) and by Mallin (1966) from vascular smooth muscle by extraction of homogenates with a high KCl medium. The properties of the actomyosin ATPase activity they isolated were similar to those of skeletal muscle preparations with the exception that the preparations from vascular smooth muscle contained much lower activities.

The actomyosin ATPase activity in the presence of Ca^{++} was stimulated by increasing the ionic strength.

When rabbit aortic fractions were extracted similarly with a high KCl medium most of the actomyosin ATPase activity was found to be located in the P_1 fraction. The Ca^{++}-ATPase activity extracted from P_1 was also stimulated by high ionic strength. The Ca^{++}-ATPase activity of the extracted fractions or the control fractions always was inhibited by high ionic strength. Since the P_3 fraction contained most of the total Mg^{++}-ATPase activity the amount of possible actomyosin contamination was calculated to be less than 5%.

Since ADP was hydrolyzed by the aortic ATPase preparations the possibility existed that the hydrolysis of ADP resulted from the formation of ATP and AMP by the action of adenylate kinase. The results presented indicate that adenylate kinase activity can be demonstrated in the subcellular fractions. Although the amount of ATP formed may have been low, it is possible that in the unboiled preparations the kinase activity is of greater magnitude. If in such a sequence of reactions the adenylate kinase reaction is not the rate limiting step, then the ADP hydrolysis should also possess the monovalent cation stimulated and ouabain inhibited properties of the ATPase hydrolysis in histidine buffer. However, if the adenylate kinase reaction is rate limiting, then the stimulation by cations would depend on the amount of substrate available. Also in Tris buffer neither the ATPase nor the ADPase is Na^+ plus K^+ dependent so the above argument cannot be used in that situation. Therefore the possibility of adenylate kinase participation cannot be ruled out.

The possibility of mitochondrial ATPase contamination of the aortic ATPase preparation will be discussed when the properties of the $P_D + S_{3B}$ ATPase preparation are considered.

iv. ATPase Activity of Density Gradient Fractions and the Requirement of S_{3B}. Density gradient centrifugation of fraction P_3 resulted in most of the ATPase activity being localized to P_D with about a 2 to 3 fold increase in specific activity. As indicated in the first chapter this separation technique removed some of the fragments of mitochondria and other structures which were previously in P_3. To further support that the isolated P_D fraction arises mainly from the cell membrane kidney cortex homogenized and isolated in the same manner yielded the highest $(Na^+ + K^+)$-ATPase activity in the same fraction.

The ATPase of density gradient fractions from aorta, like the P_3 fraction, required addition of the 100,000 x g supernatant material in order to reveal a Na^+ plus K^+ stimulated activity. Maximum Na^+ plus K^+ stimulation was reached when two parts of the S_{3B} protein were mixed with one part of P_D protein. However, the increase in per cent cation stimulation by addition of S_{3B} was not due to an increase in the rate of ATP hydrolysis in the presence of Mg^{++}, Na^+ plus K^+, but due to less ATP hydrolysis in the presence of Mg^{++} alone. It therefore seems that the S_{3B} material converts some of the Mg^{++}-ATPase activity to a form which is inactive unless the monovalent cations are also present.

A similar situation has been reported in cardiac muscle, brain and parotid gland (Schwartz and Laseter, 1964). They found a heat stable

factor, which they believed to be a basic protein histone, that could increase the Na^+ plus K^+ stimulation of their preparations by inhibiting the Mg^{++}-ATPase activity. They also showed that their material and histones caused the same effects as those which were observed in aging of their fractions.

The S_{3B} material from vascular smooth muscle was found to be heat stable. However, unlike cardiac muscle when the aortic fractions were aged an increase in the proportion of cation stimulated enzyme was not observed. When aortic S_{3B} material was subjected to electrofocusing and treatment with ion exchange resins, the activating factor in the S_{3B} material was found to be acidic with its isoelectric point in the range of pH 5.1-5.5. The anionic form of the factor was active, since it could selectively be bound by anion exchange resin and not by a cation exchange resin. The factor isolated by Schwartz and Laseter was a basic protein and thus the S_{3B} material probably is not the same as their factor.

Tanaka and Abood (1964) have reported that addition of lecithin to their ATPase preparation increased the $(Na^+ + K^+)$-ATPase portion of the activity without affecting the Mg^{++}-ATPase activity. Lecithin addition to the aortic P_D fraction instead of S_{3B} also resulted in an increase in the cation stimulation; however, this effect was obtained by decreasing the Mg^{++}-ATPase activity. Also when the S_{3B} material was subjected to a lipid extraction the lipid portion of the extract was capable of inducing the cation stimulated effect.

Although the studies on the S_{3B} material presented cannot identify the factor, its properties would indicate that it is a

non-enzymatic (heat stable) high molecular weight component, possibly a lipoprotein. The lipid portion of this complex may be the necessary factor and the protein portion supplies it with the physical properties demonstrated.

v. Properties of the $P_D + S_{3B}$ ATPase Activity. Most of the ATPase properties of the $P_D + S_{3B}$ mixture were similar to those found in other membrane $(Na^+ + K^+)$-ATPase preparations. The aortic Mg^{++}-ATPase preparation was found to be maximally stimulated when, in the presence of 4 mM ATP, the concentration of Na^+ was 50 mM, and K^+ was 2 mM. The monovalent cation stimulation was found to be specific for ATP. The cation sensitive ATPase activity was inhibited by the presence of Ca^{++} and when Ca^{++} was used to replace Mg^{++} no cation stimulation was observed. However, the ATPase activity was found to differ from the classical membrane enzyme in two properties.

The aortic Mg^{++}-ATPase was slightly stimulated by K^+ alone and highly stimulated by Na^+ alone, but neither of these activities was affected by ouabain. When Na^+ plus K^+ were added the enzyme stimulation was greater than that with Na^+ alone but not significantly different from an additive effect of Na^+ alone and K^+ alone. This extra stimulation observed with Na^+ plus K^+ could, however, be inhibited by ouabain down to the level of stimulation observed with Na^+ alone.

The Na^+ stimulation of the Mg^{++}-ATPase activity could result from: (1) Contamination of the ATPase preparation by K^+ so that its activity actually is Na^+ plus K^+ dependent; (2) ATPase activity which

was originally Na^+ plus K^+ dependent, but which was damaged and lost this property during isolation; and (3) Existence of a Na^+ stimulated ATPase in intact cells. If K^+ contamination was the reason, then ouabain should have completely inhibited the activity. Ouabain had no effect on the Na^+ stimulation; also treatments intended to remove or reduce potassium contamination were unable to abolish the Na^+ stimulated effect.

Askari and Fratantoni (1964) while using a preparation of broken red cell membrane which contained the classical ATPase, stimulated only by the presence of Na^+ and K^+ together and inhibited by ouabain, were able to show that further disruption of these membranes by sonication changed their ATPase activity. After such treatment their enzyme preparation could be significantly stimulated by Na^+ or K^+ alone and this stimulation was not ouabain sensitive. Also when Na^+ and K^+ were added together an extra ouabain sensitive stimulation was obtained. The properties of the ATPase in their sonicated membrane preparation was therefore very similar to that found in aorta and would suggest that the homogenization and isolation technique employed resulted in such a change. However, when kidney membrane ATPase was homogenized and isolated in the same manner the ATPase in a kidney P_D fraction was not stimulated much by the addition of Na^+ alone but could show a high stimulation with Na^+ and K^+ together and furthermore ouabain could completely inhibit this stimulation. Also a similar Na^+ stimulated Mg^{++}-ATPase and a small $(Na^+ + K^+)$-ATPase activity were found to exist in a microsomal preparation of uterine smooth muscle prepared by a homogenization procedure using a loosely fitting teflon pestle (Daniel and co-workers, 1969).

The possibility therefore remains that the Na^+ stimulated

Mg^{++}-ATPase activity is an enzyme present in undamaged cells.

The extra stimulation of the Mg^{++}-ATPase above that seen with Na^{+} alone in aortic preparation was completely inhibited by ouabain. Also this ouabain sensitivity was K^{+} dependent and could be overcome by increasing the K^{+} concentration in the media. The results obtained with ouabain lead to the conclusion that although the extra stimulation by Na^{+} plus K^{+} appears to be additive, an interaction must have occurred resulting in a Na^{+} plus K^{+} dependent ouabain sensitive enzyme activity.

Aging of the aortic $P_D + S_{3B}$ enzyme preparation could not increase the amount of $(Na^{+} + K^{+})$-ATPase activity but actually inhibited this portion of the activity after one day at 15°C, leaving only a Na^{+}-stimulated activity. When azide was added to the enzyme assay an increase in the per cent monovalent cation stimulation was obtained. This was due to a preferential inhibition of the Mg^{++}-ATPase activity by azide. The results are similar to those reporting the effects of azide on other $(Na^{+} + K^{+})$-ATPase preparations (Schwartz and Laseter, 1963; Samaha and Gergely, 1965). Although azide can act as an inhibitor of mitochondrial ATPase (Lindberg, 1961), it also has been reported to stimulate this enzyme (Robertson and Boyer, 1955). Therefore it is not certain that the Mg^{++}-ATPase inhibition observed was due to mitochondrial contamination. Also if it can be assumed from the O_2 consumption data that the decrease in contamination of fraction P_D by mitochondrial fragments also meant removal of a proportional amount of mitochondrial Mg^{++}-ATPase, then the contribution of the latter ATPase activity to the $P_D + S_{3B}$ fraction is probably minimal since the per cent cation stimulation in this fraction is not much

different from that observed in fraction $P_3 + S_{3B}$. Ethacrynic acid was found to inhibit the activities of both the Mg^{++}-ATPase and the activity sensitive to monovalent cations. The inhibition of the cation-sensitive activity was more marked than that of the Mg^{++}-ATPase and consequently the enzyme showed a decrease in sensitivity to cations after inhibition with ethacrynic acid. Irrespective of the inhibitor or treatment used, any decrease in the Na^+ stimulated activity was invariably accompanied by a decrease in the Na^+ plus K^+ stimulated, ouabain-sensitive activity.

In summary a monovalent cation stimulated Mg^{++}-ATPase activity could be demonstrated in a density gradient isolated microsomal membrane fraction of rabbit aorta, provided histidine buffer was used in the assay media instead of Tris buffer. Addition of a factor in the 100,000 x g supernatant material was necessary to demonstrate Na^+ plus K^+ stimulated activity. The greater portion of the monovalent cation stimulated ATPase activity was due to a ouabain insensitive Na^+ stimulation of the Mg^{++}-ATPase. However, a small part of this activity resembled a ouabain sensitive $(Na^+ + K^+)$-ATPase.

CHAPTER V

SODIUM EXCHANGE IN THE RABBIT AORTA

CHAPTER V. SODIUM EXCHANGE IN RABBIT AORTA

A. Introduction.

Previous studies of the efflux of ^{22}Na and ^{42}K from rat and dog vascular smooth muscle suggest a very high permeability to Na^+ in this tissue (Garrahan and co-workers, 1965). These studies showed that the efflux of ^{22}Na had to be described by at least three exponential terms (a three compartment system). The first and fastest phase of exchange was shown to be extracellular in origin. The second phase was found to have a temperature and K^+ dependency and a ouabain sensitivity consistent with an intracellular location. The third phase, with the slowest exchange, was assumed to be bound at some undetermined site. Along with the efflux studies other work also supports the existence of an active Na^+-pump in vascular smooth muscle (Barr and co-workers, 1962; Vidlakova and Kleinzeller, 1963; Daniel, 1965). The evidence thus suggests that active Na^+ transport occurs by a mechanism similar to that in other tissues and hence that a $(Na^+ + K^+)$-ATPase exists in this tissue.

In the smooth muscle of rat uterus the second cellular phase of ^{22}Na efflux was shown to be ouabain sensitive, however a further inhibition of efflux from this compartment was observed by ATP depletion, suggesting the existence of a ouabain insensitive ATP dependent Na^+-pump (Daniel and co-workers, 1969; Daniel and Robinson, 1969a, b, and c). The ATPase activity in this tissue was also found to be similar to that reported in the previous chapter for rabbit aorta.

Because of this similarity the efflux of ^{22}Na in rabbit aortic smooth muscle was also studied.

B. Results.

i. Analysis of Efflux Data. Efflux of ^{22}Na was plotted as the logarithm (log) of the counts per minute (c/m) remaining in the tissue against time and then resolved into its exponential components by curve peeling, as described in figure 35. Resolving the data by such a technique could at best be described by a minimum of three exponential components. The limitations and usefulness of such an analysis have been discussed by Robertson (1957) and more recently by Daniel and Robinson (1969a).

The curve peeling technique is only valid if the last linear portion of the efflux curve represents efflux from a single homogeneous compartment. A test for efflux from more than a single compartment, involving log-log plots of the rate of efflux (c/m^2) against the corresponding c/m of the last few points (2-4 hours) has been described (Keynes and Swan, 1959; Persoff, 1960). If the slope of such a plot is not equal to one, efflux from more than one compartment or the presence of a very slowly exchanging or bound fraction is indicated. In aortic strips, presence of a bound fraction (D) was shown by extrapolating arithmatic plots of the c/m^2 against the corresponding c/m by the method of Dick and Lea (1964) (insert of figure 35).

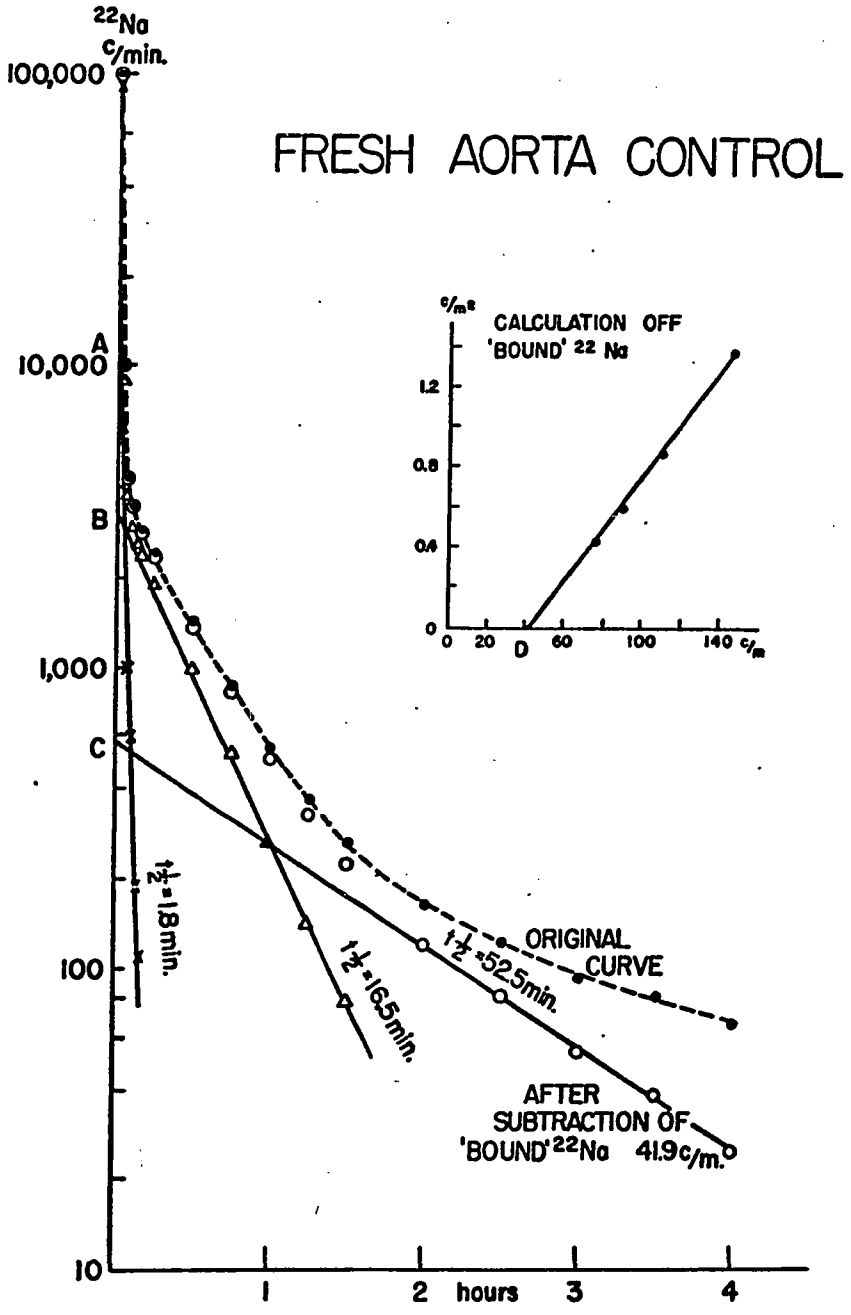


Figure 35. Illustration of the method used to analyze efflux data; efflux of ^{22}Na from fresh aorta. Inset shows determination of 'bound' ^{22}Na from plot of logarithmic average of c/min. against c/min^2. 'Bound' ^{22}Na was then subtracted from the original c/min. in the tissue and the resultant curve peeled by backward subtraction.

This bound fraction (D) was then subtracted from the total tissue c/m to obtain a new efflux curve, the last part of which now behaved like efflux from a homogeneous compartment. Extrapolation of the linear part of this curve to the ordinate (zero time) resulted in a slope with a half-time of 52.5 minutes and a rate constant of 0.0132 minutes^{-1}. The extrapolation at the ordinate (C) showed that less than one percent of the total tissue counts was in this compartment.

The subtraction of this extrapolated line from the remainder of the curve yielded a third curve which straightened after 15 minutes. The straight part of this curve extrapolated at zero time at about 3.54% (B) of the initial counts, having a half-time of 16.5 minutes and a rate constant of 0.042 minutes^{-1}.

By again subtracting this second line from its curve a line was drawn with a half-time of 1.8 minutes and a rate constant of 0.385 minutes^{-1}. This line extrapolated on most occasions at about 50% less than the remaining initial counts. This discrepancy is possibly due to the non-exponential early phase of diffusion (only 81% of radioactivity behaves exponentially), as well as limited diffusion of Na^+ associated with mucopolysaccharides and collagen present in aortae (Headings and co-workers, 1960).

The efflux of Na^+ from the fast cellular fraction was assumed to be in series with the efflux from the extracellular fluid. In such a situation extrapolation of the second compartment of efflux to zero time leads to an over-estimation of this fraction, as has been pointed out by Huxley (1960). Huxley proposed a method of calculation for the

series case which was applied to the data presented here. The corrected values for the Na^+ in the fast phase (A) and first cellular phase (B) were then referred to as A corr. and B corr. respectively.

ii. Efflux of ^{22}Na from Fresh Rabbit Aortae. The amount of Na^+ in the fast phase (A corr.) for control fresh tissue was calculated to be 76.9 m Eq Na^+/kg wet weight. Assuming an extracellular space equal to an inulin space of 62.06% of wet weight (Bevan, 1960) for rabbit aorta, the concentration of Na^+ in this compartment was found to be 123.97 $\pm$ 3.74 m Eq/l and close to that in the normal Krebs-Ringer medium (138.55 m Eq/l).

Figures 36 and 37 compare the efflux of ^{22}Na from fresh aortae in the presence of 10^{-3} M ouabain, or 10^{-3} M of both IAA and DNP, or the absence of K^+, or cooling to 5°C to that from control aortae. The analyses of these curves are summarized in Table XXX.

Assuming that 10^{-3} M ouabain or efflux into K^+-free media inhibited the transport ATPase, inhibition of the enzyme only slightly reduced the efflux from the fastest cellular compartment. However IAA and DNP markedly inhibited the efflux from the fastest cellular compartment, reduced the amount of Na^+ emerging from this compartment (B corr.), and increased the amount in the slower phase (C). The effects of IAA and DNP when added alone were more variable, and generally either of these agents inhibited efflux less than when in combination. Cooling to 5°C inhibited ^{22}Na efflux much more than inhibition of metabolism or transport ATPase. At 5°C most of the

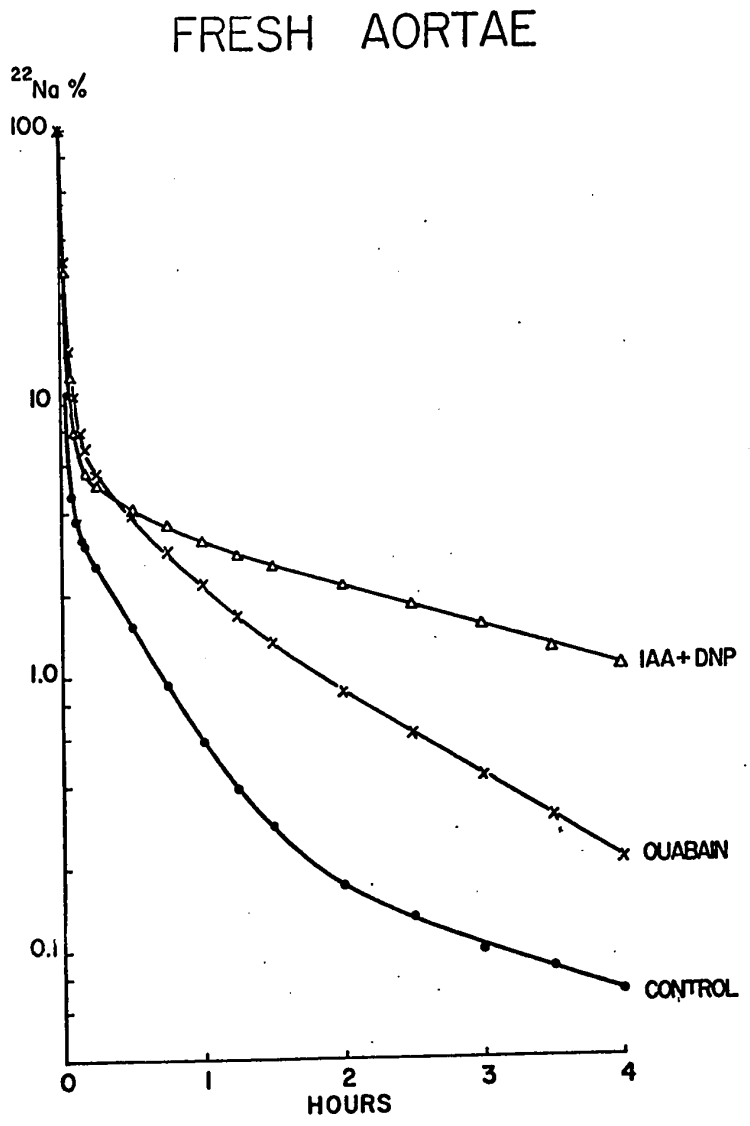


Figure 36. Efflux of ^{22}Na at 25°C from fresh arotae loaded with ^{22}Na for 2 hours at 37°C. Typical data for control tissue and for tissues treated with 10^{-3} M IAA + DNP and 10^{-3} M ouabain.

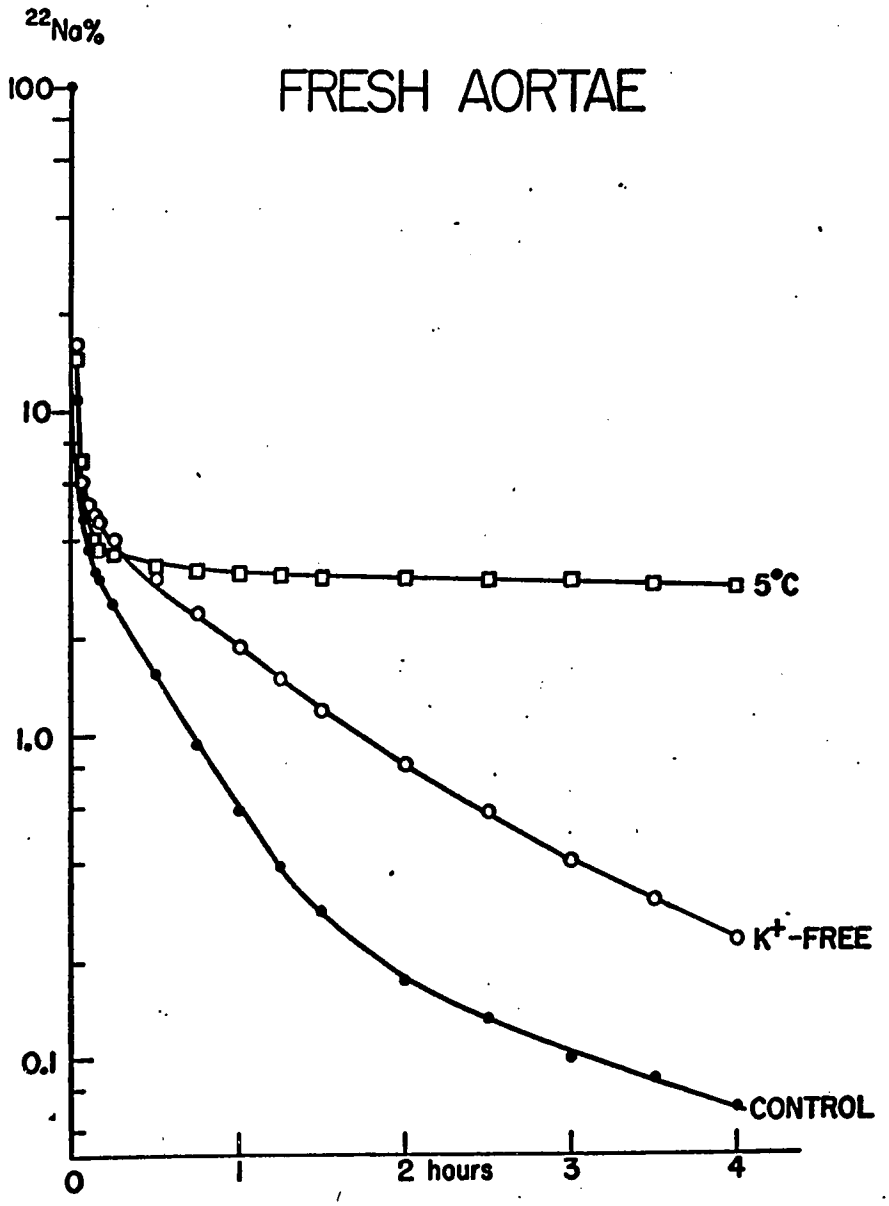


Figure 37. Efflux of ^{22}Na from fresh aortae loaded with ^{22}Na for 2 hours at 37°C. Typical data for control tissue and tissue in K^+-free Krebs-Ringer solution at 25°C and in Krebs-Ringer solution at 5°C.

TABLE XXX

Analysis of ^{22}Na Efflux from Fresh Rabbit Aortae.

Experiment	No.	Minutes^{-1}			mEq Na^+/kg Wet Weight					Total Efflux mEq/kg/min	
		K_1	K_2	K_3	$A_{corr.}$	$B_{corr.}$	C	D	Y_o	$B_{corr.} \times K_2$	C x K_3
Control	4	0.401	0.044	0.0138	76.94	1.51	2.45	0.245	81.14	0.0664	0.0338
10^{-3}M IAA	2	0.533	0.048	0.0578	91.87	1.93	2.81	0.0	96.61	0.0925	0.163
10^{-3}M DNP	2	0.462	0.031	0.010	79.62	1.07	2.16	0.542	83.4	0.0332	0 .0216
10^{-3}M IAA + DNP	2	0.385	0.037	0.006	76.59	0.846	3.13	0.152	80.7	0.0313	0.0182
10^{-3}M Ouabain	2	0.396	0.042	0.013	79.92	1.506	2.75	0.287	84.5	0.0625	0.0358
K^+-Free	1	0.310	0.046	0.014	78.52	0.970	2.92	0.095	82.5	0.0045	0.0397
5°C	2	0.533	0.035	0.005	105.5	0.775	1.29	2.72	110.4	0.0271	0.00581

Pretreatment with IAA, DNP or Ouabain was for 30 minutes; with IAA and DNP together--none. $B_{corr.}$ are the calculated values for Na^+ in the second compartment corrected for a series arrangement from the values of A, B, K_1 and K_2 (Huxley, 1960). $A_{corr.}$, the rapidly exchanging Na^+, was obtained by subtracting $B_{corr.}$ + C + D from the initial total Na^+ (Y_o). Total efflux from the fractions was taken to be the product of the rate coefficient and the Na^+ in the corresponding compartment.

cellular Na^+ appeared to be in the bound fraction (D).

iii. The Effect of Various Procedures on Net Ion Movement in Fresh Rabbit Aortae. The water content of fresh aortae, incubated at 37°C for two hours, averaged 702.7 gm/kg wet weight (Table XXXI). On incubation in Krebs-Ringer solution at 25°C it rose slightly to 753.4 gm/kg wet weight. The Na^+ content rose from 84.3 to a mean of 103.9 m Eq/kg wet weight, and this was accompanied by a decrease in the K^+ content from 27.2 to 17.9 m Eq/kg wet weight.

Incubation of fresh rabbit aortae at 25°C in the presence of ouabain or IAA and DNP alone or together always resulted in a further gain of Na^+ and loss of K^+, with the greatest change occurring in the presence of both IAA and DNP. A significant gain in water content was only found after incubation in IAA and DNP. Incubation in K^+-free Krebs-Ringer at 25°C or Krebs-Ringer at 5°C also caused net downhill ion movements and water gain.

iv. Efflux of ^{22}Na from Na^+-Rich Rabbit Aortae. Figures 38 and 39 show the efflux curves of ^{22}Na from rabbit aortae made Na^+-rich by incubation overnight in K^+-free Krebs-Ringer solution at 5°C. The curves were analyzed as described for the fresh tissues and summarized in Table XXXII.

As found with fresh aortae, assuming that the extracellular space is equal to an inulin space of 62.06% of the wet weight (Bevan, 1960), the concentration of Na^+ in the fastest compartment (A corr.)

TABLE XXXI

Effects of Various Inhibitors on Ion and Water Content in Fresh Rabbit Aortae.

Treatment	Time Hours	No.	mEq/kg Wet Weight Na^+	mEq/kg Wet Weight K^+	gm/kg Wet Weight H_2O
Control 37°C	2	7	84.3 ± 3.9	27.21 ± 1.8	702.7 ± 6.5
Control 25°C	4	7	103.96 ± 4.1	17.97 ± 3.9	753.4 ± 9.9
10^{-3}M IAA 25°C	4	2	111.7 ±15.0	13.62 ± 6.1	763.5 ± 6.3
10^{-3}M DNP 25°C	4	2	117.9 ± 6.0	15.42 ± 7.3	758.6 ± 5.2
10^{-3}M IAA + DNP 25°C	4	4	124.6 ± 8.2	5.23 ± 1.0	769.7 ± 3.9
10^{-3}M Ouabain 25°C	4	4	112.8 ± 3.7	7.08 ± 1.1	752.8 ± 4.2
K^+-Free 25°C	4	2	116.5 ± 8.2	1.63 ± 0.11	763.8 ± 1.65
Control 5°C	4	2	134.9 ± 4.3	5.13 ± 1.5	777.8 ± 15.1

Values are means ± S.E.M. for the No. of determinations listed.

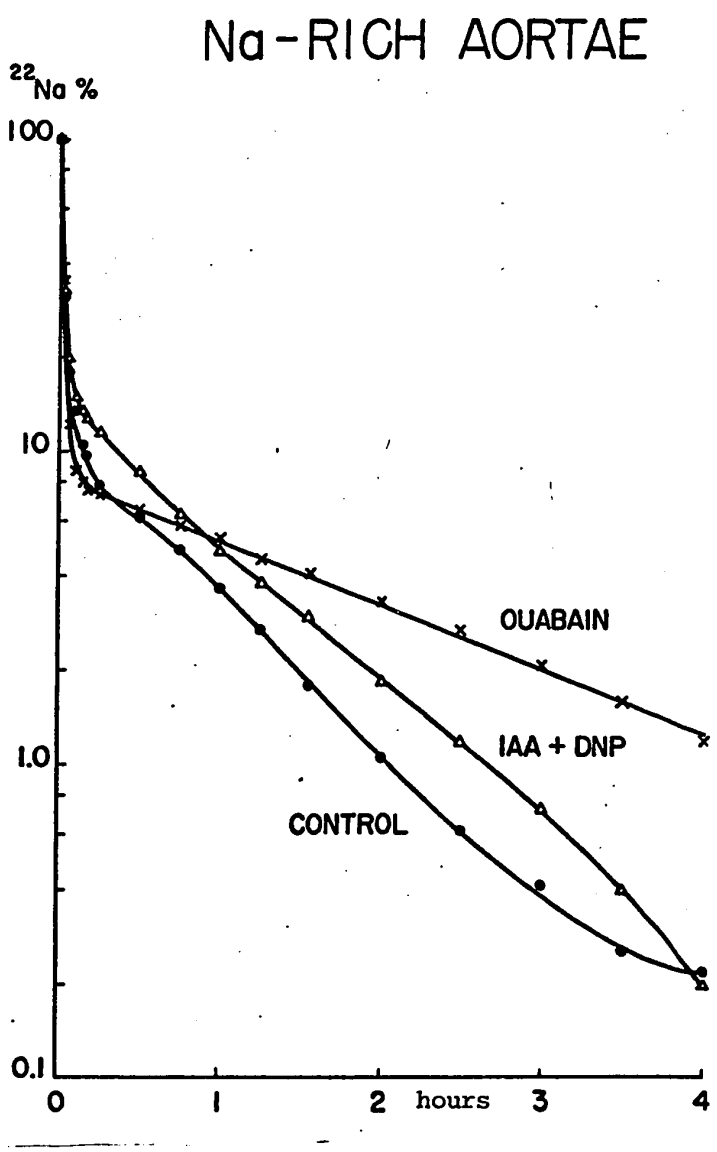


Figure 38. Efflux of ^{22}Na at 25°C from tissues made Na^+-rich and loaded with ^{22}Na overnight at 5°C. Typical data for control tissue and for tissues treated with 10^{-3} M IAA + DNP and 10^{-3} M ouabain.

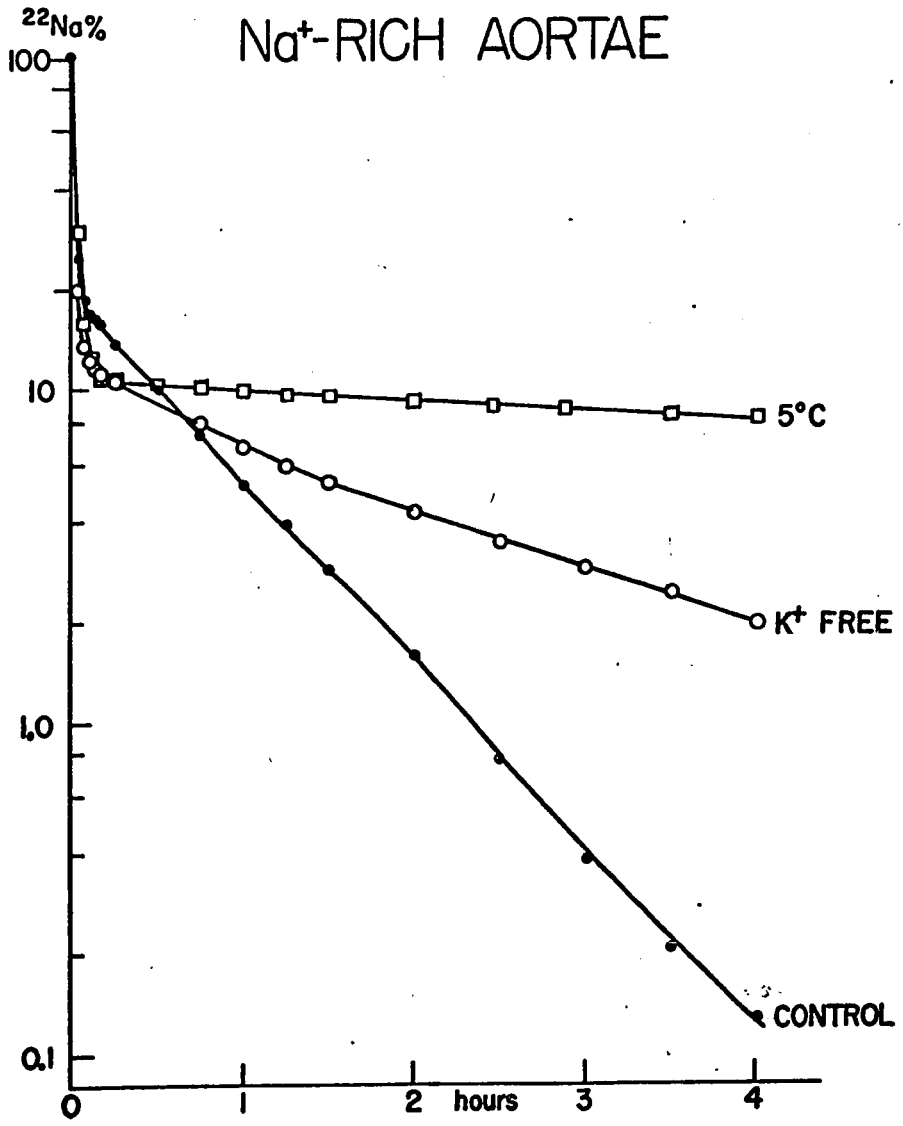


Figure 39. Efflux of ^{22}Na from tissues made Na$^+$-rich and loaded with ^{22}Na overnight at 5°C. Typical data for control tissue and tissues in K$^+$-free Krebs-Ringer solution at 25°C and in Krebs-Ringer solution at 5°C.

TABLE XXXII

Analysis of ^{22}Na Efflux from Na^+-Rich Rabbit Aortae.

Experiment	No.	Minutes^{-1}			mEq Na^+/kg Wet Weight					Total Efflux mEq/kg/min	
		K_1	K_2	K_3	$A_{corr.}$	$B_{corr.}$	C	D	Y_o	$B_{corr.} \times K_2$	C x K_3
Control	4	0.532	0.063	0.0133	90.58	9.56	6.05	0.105	106.3	0.6034	0.0805
10^{-3}M IAA	1	0.729	0.077	0.0057	85.13	2.75	15.82	0.0	103.7	0.212	0.0905
10^{-3}M DNP	1	0.693	0.069	0.0078	81.52	8.09	10.10	0.0	99.7	0.558	0.079
10^{-3}M IAA + DNP	2	0.668	0.036	0.0170	84.11	4.28	13.49	0.0	101.9	0.154	0.229
10^{-3}M Ouabain	2	0.468	0.018	0.0073	86.72	0.541	8.41	0.0	95.7	0.0095	0.061
K^+-Free	2	0.408	0.036	0.0091	109.6	4.244	12.62	1.18	127.7	0.151	0.115
5°C	2	0.529	0.046	0.0035	120.4	0.824	6.93	6.07	134.2	0.0379	0.024

Pretreatment with IAA, DNP or Ouabain was for 30 minutes; with IAA and DNP together--none. $B_{corr.}$ are the calculated values for Na^+ in the second compartment corrected for a series arrangement by the method of Huxley (1960). $A_{corr.}$, the rapidly exchanging Na^+, was obtained by subtracting $B_{corr.}$ + C + D from the initial total Na^+ (Y_o). Total efflux from the fractions was taken to be the product of the rate coefficient and the Na^+ in the corresponding compartment.

was calculated to be 145.95 m Eq/l and close to that in the K^+-free Krebs-Ringer solution used to make the tissue Na^+-rich (143.17 m Eq/l).

The rate (K_2) and the total amount of ^{22}Na efflux (B corr. x K_2) from the fast cellular compartment was much greater than that observed in fresh tissues. The amount of Na^+ in compartments B corr. and C were increased in Na^+-rich tissues; however the bound fraction (D) was small or negligible.

In contrast to fresh tissues, ouabain greatly inhibited the total efflux of Na^+ from the fast cellular compartment of Na^+-rich tissues by inhibiting the rate (K_2) and the amount of Na^+ efflux from this compartment (B corr.). Efflux into K^+-free Krebs-Ringer solution decreased K_2 to the same extent as ouabain, this was also accompanied by an increase in the Na^+ in compartment C and the bound fraction (D).

The metabolic inhibitors IAA and DNP when added together caused a decrease in the rate K_2 and an increase in K_3 and C. IAA or DNP were less effective when added alone, and primarily decreased rate K_3 and increased the concentration of Na^+ in compartment C. The effect of efflux at 5°C was marked inhibition of efflux from all cellular compartments associated with a large bound fraction (D).

v. Effect of Various Procedures on Net Ion Movement in Na^+-Rich Rabbit Aortae. Incubation of Na^+-rich aortae at 25°C in Krebs-Ringer solution caused a net loss of Na^+ and tissue water, and a gain of K^+ (Table XXXIII). Recovery of the ion content to the level found in fresh tissue at 25°C, took 2 to 3 hours. Presence of the metabolic

TABLE XXXIII

Effects of Various Inhibitors on Ion and Water Content in Na^+-Rich Rabbit Aortae.

Treatment	Time Hours	No.	mEq/kg Wet Weight Na^+	mEq/kg Wet Weight K^+	gm/kg Wet Weight H_2O
Control	0	10	107.8 ± 4.7	2.29 ± 0.56	798.6 ± 9.9
Control	4	11	94.1 ± 2.6	20.87 ± 1.7	736.5 ± 8.7
10^{-3}M IAA	4	2	103.69 ± 3.3	4.62 ± 0.75	738.6 ± 7.2
10^{-3}M DNP	4	2	100.38 ± 1.2	9.09 ± 0.34	753.8 ± 6.3
10^{-3}M IAA + DNP	4	4	107.6 ± 6.2	4.40 ± 0.36	789.0 ± 4.6
10^{-3}M Ouabain	4	4	101.6 ± 1.4	6.38 ± 0.02	738.2 ± 4.0
K^+-Free	4	2	115.1 ± 8.7	0.58 ± 0.1	775.6 ± 7.3
5°C	4	2	121.65 ± 9.8	4.01 ± 0.95	770.95 ± 10.6

Values are means ± S.E.M. Na^+-rich tissues were prepared by incubation overnight in K^+-free Krebs-Ringer solution at 5°C. The tissues were then incubated at 25°C for the times indicated.

inhibitors IAA and DNP alone or together prevented Na^+ extrusion and K^+ accumulation. This effect was most prominent with IAA and DNP together, which also prevented water extrusion from Na^+-rich tissues.

Incubation of Na^+-rich aortae in K^+-free Krebs-Ringer at 25°C or Krebs-Ringer at 5°C had the same effect as IAA and DNP together on net ion and water movement.

Ouabain inhibited recovery of Na^+ and K^+ in Na^+-rich aortae but did not prevent loss of tissue water.

vi. Effects of Inhibitors on ^{42}K Efflux from Rabbit Aortae.

Figures 40 and 41 compare the efflux of ^{42}K from fresh aortae and the effects of various inhibitors on the efflux. The analyses of these curves are summarized in Table XXXIV.

The efflux of K^+ from fresh rabbit aortae was best described by three compartments and a small bound fraction. Most of the tissue K^+ was found in the fast compartment (A corr.). Using Bevan's (1960) value of 62.06% of the wet weight for the extracellular space, then the concentration of K^+ in this compartment was calculated to be 39.9 ± 1.5 m Eq/l, i.e. much more than the amount in the Krebs-Ringer solution (4.63 m Eq/l). Much superficial, rapidly exchanging K^+ as well as extracellular K^+ must have been in this compartment.

One effect of metabolic inhibitors and ouabain was to decrease the total efflux from the second cellular compartment, by reducing the amount of K^+ emerging from this compartment (B corr.) without changing the rate (K_2) appreciably. The rate of efflux from

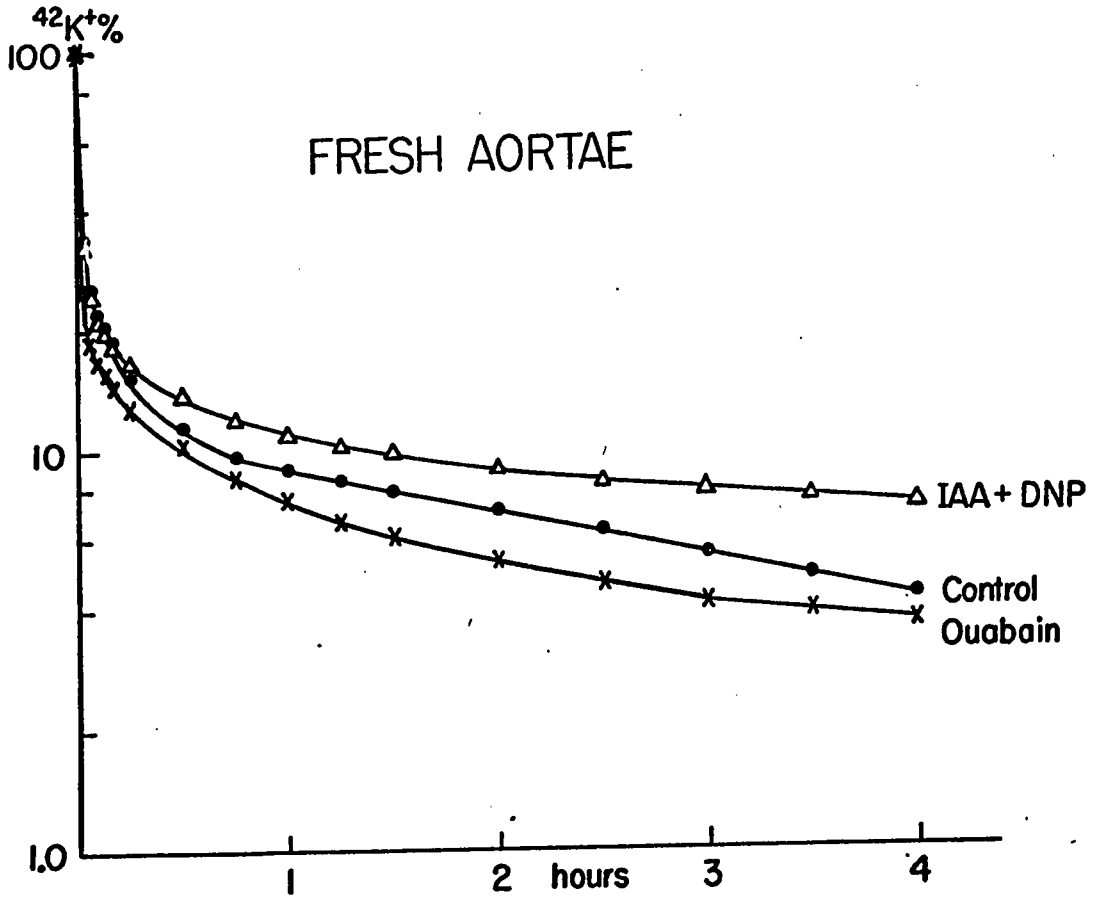


Figure 40. Efflux of ^{42}K at 25°C from fresh aortae loaded with ^{42}K for 2 hours at 37°C. Data for control tissue and for tissues treated with 10^{-3} M IAA + DNP and 10^{-3} M ouabain are plotted.

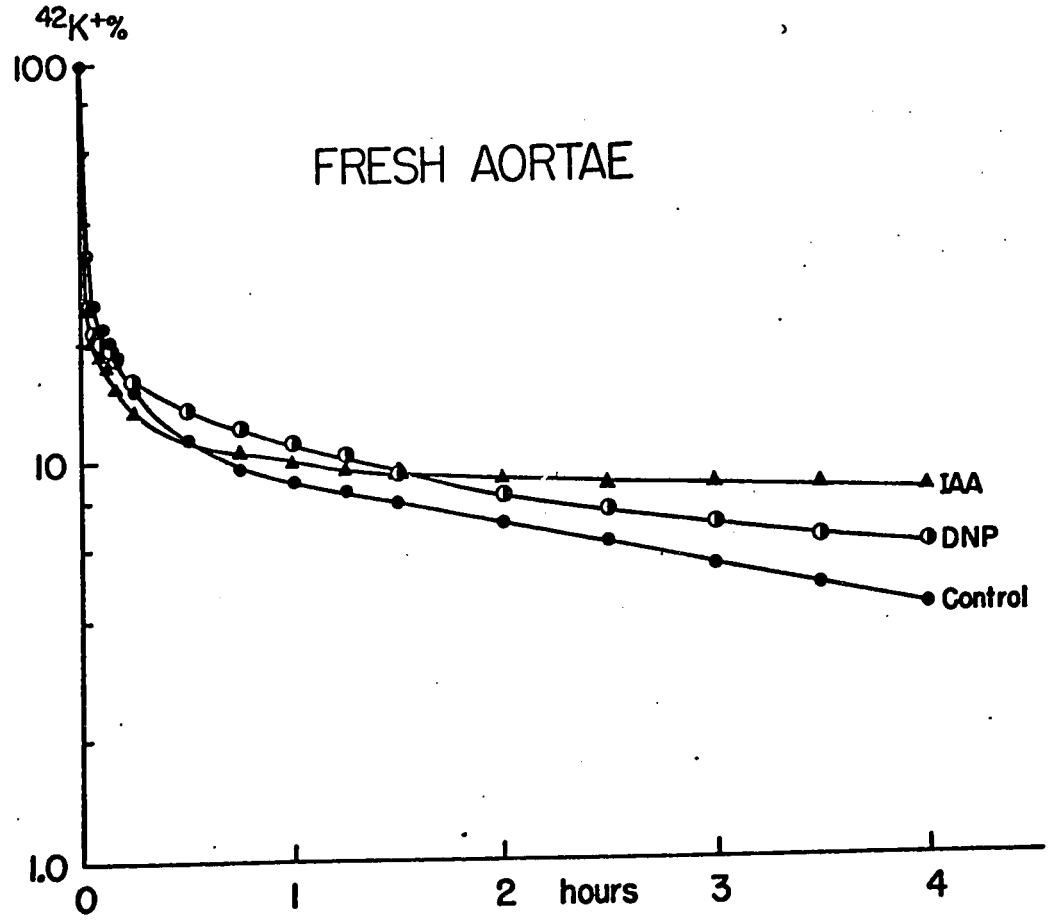


Figure 41. Efflux of ^{42}K at 25°C from fresh aortae loaded with ^{42}K for 2 hours at 37°C. Data for control tissue and for tissues treated with 10^{-3} M IAA or 10^{-3} M DNP are plotted.

TABLE XXXIV

Analysis of ^{42}K Efflux from Fresh Rabbit Aortae.

Experiment	No.	Minutes^{-1}			mEq K^+/kg Wet Weight					Total Efflux mEq/kg/min	
		K_1	K_2	K_3	$A_{corr.}$	$B_{corr.}$	C	D	Y_o	$B_{corr.} \times K_2$	C x K_3
Control	2	0.818	0.0715	0.0055	21.05	6.024	2.315	0.413	29.8	0.431	0.0127
10^{-3}M IAA	1	1.54	0.0763	0.0160	20.23	2.427	2.150	0.969	25.78	0.185	0.0344
10^{-3}M DNP	1	1.733	0.0720	0.0097	19.66	1.843	2.576	1.297	25.38	0.133	0.0250
10^{-3}M IAA + DNP	1	0.815	0.070	0.0061	23.66	2.150	1.746	1.734	29.29	0.151	0.0107
10^{-3}M Ouabain	2	1.008	0.0735	0.0144	24.28	1.640	2.43	1.014	29.36	0.121	0.0350

Pretreatment with IAA, DNP or Ouabain was for 30 minutes; with IAA and DNP together--none. $B_{corr.}$ are calculated values for K^+ in the second compartment correeted for a series arrangement by the method of Huxley (1960). $A_{corr.}$, the rapidly exchanging K^+, was obtained by subtracting $B_{corr.}$ + C + D from the initial K^+ (Y_o). Total efflux from the compartments was taken to be the product of the rate coefficient and the K^+ in the corresponding compartment.

the third compartment (K_3) was tripled by IAA or ouabain, and doubled by DNP. IAA, DNP or ouabain also increased the total efflux from the third compartment ($C \times K_3$) with ouabain being most effective. IAA and DNP had less effect on the rate (K_3) and total efflux ($C \times K_3$) from the third compartment and increased the amount of K^+ in the bound fraction (D). These effects of the inhibitors were also associated with a net downhill movement of Na^+ and K^+. The limited number of experiments on ^{22}Na and ^{42}K efflux precludes complete explanation of all the results presented. Areas requiring further investigation are presented in the discussion.

C. Discussion.

1. Compartments of ^{22}Na in Rabbit Aortae. The exchange of Na^+ from fresh and Na^+-rich rabbit aortae could be described by a three compartment system plus a very slowly exchanging bound fraction. The first and more rapid phase of exchange was not affected by temperature, K^+ concentration, ouabain or metabolic inhibitors, and was assumed to be due to diffusion from the extracellular phase.

The second phase of ^{22}Na exchange was found to be sensitive to procedures which inhibit active transport and was assumed to be of a cellular origin. These effects were most pronounced in Na^+-rich aortae in which active transport of Na^+ is increased.

^{22}Na efflux from the slower third phase was not affected as much by ouabain and K^+-free solution as the second phase. However in inhibited tissues, the Na^+ content of the third phase often

increased at the expense of Na^+ in the faster second phase, which suggests that the third compartment may also be of cellular origin. When the faster cellular phase is inhibited most of the cellular Na^+ is then exchanged via the slower cellular phase. A similar situation has recently been reported for uterine smooth muscle by Daniel and co-workers (1969), and has led them to postulate that the efflux from the slower phase is controlled by a passive mechanism while the rate of efflux from the faster fraction is determined by an active efflux.

The last component of ^{22}Na exchange was from a very slowly exchanging bound fraction. Cooling to 5°C markedly increased the Na^+ apparently in this fraction. In uterine smooth muscle these workers suggested that this fraction is Na^+ complexed to membranes and macromolecules, or sequestered in the membrane vesicles which may be involved in active Na^+ efflux and/or a passive Na^+ exchange (Daniel, 1969c; Daniel and co-workers, 1969). It is thought that cooling closes the vesicles and thus increases the apparently bound fraction. The location of the apparently bound fraction in aorta is uncertain, however since aortic smooth muscle also contains many pinocytotic vesicles a similar situation may exist to that reported for uterine smooth muscle.

ii. Cellular ^{22}Na Efflux by More Than One Na^+-Pump.

The presence of IAA and DNP together was found to inhibit Na^+ efflux from the fast cellular compartment more than ouabain in fresh tissues,

and this effect was also associated with a gain of water. In swollen Na^+-rich aortae, IAA and DNP, prevented water extrusion but this effect was not found with ouabain. Daniel and Robinson (1969b) have shown that IAA and DNP together rapidly deplete the ATP and ADP levels in the smooth muscle of the uterus, and also inhibit Na^+ efflux and water loss.

It therefore seems that besides the ouabain sensitive Na^+-pump, another Na^+-pump which is insensitive to ouabain but which also requires ATP to function may exist in rabbit aorta. It is reasonable to postulate that this latter pump controls cell volume. However this postulate is based on the assumption that the observed inhibition by ouabain was the maximum obtainable and also that IAA and DNP depleted the ATP supply in rabbit aorta.

Since exposure to K^+-free Krebs-Ringer solution caused some swelling (water gain) in fresh tissues, and prevented water loss from Na^+-rich tissues, it is suggested that the Na^+-pump controlling cell volume may also require external K^+. Whether this second Na^+-pump is a Na-Cl pump similar to those found in kidney (Kleinzeller and Knotkova, 1964, 1967) or in gall bladder (Diamond, 1962a, b, and c) is uncertain. Further studies on ^{22}Na and ^{42}K influx, and the net movement of Cl^- would be necessary to show that this is an active Na-Cl pump. Also, if the volume-pump requires external K^+ but is not coupled to K^+ influx, then a K^+-free Krebs-Ringer solution would not be expected to alter K^+ fluxes more than ouabain.

Results obtained by analysis of the data for ^{22}Na efflux,

suggests that cellular K^+ was also distributed in two fractions. Of these the second slower cellular phase of efflux was affected by inhibition of active transport (efflux increased), and thus may be associated with the ouabain sensitive Na^+-pump. If the efflux of Na^+ by the second pump (volume regulating pump) is not linked to a concommitant influx of K^+ then ATP depletion by metabolic inhibitors should increase K^+ efflux to the same degree as ouabain. However the metabolic inhibitors were found to be less effective than ouabain in altering the efflux of K^+. Since IAA and DNP also had different effects on Na^+ efflux in fresh and Na^+-rich aortae it seems that in this tissue these inhibitors may have other effects besides the inhibition of ATP supplies. Insufficient data on the effects of IAA and DNP precludes definite conclusions on their effects.

The data presented supports the involvement of a classical transport ATPase, operationally involved in a Na^+-pump in smooth muscle of rabbit aorta. The lack of ouabain effect on cell volume suggests that this pump does not control cell volume. However, there is also a component of Na^+ efflux which persists after ouabain inhibition of this pump and therefore there probably is a second ATP dependent Na^+-pump present which is unaffected by ouabain but which controls cell volume in rabbit aorta.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER VI. GENERAL DISCUSSION AND CONCLUSIONS

A. General Discussion.

The phenomenon of active Na^+ transport has been demonstrated to exist in cells of a wide variety of mammalian tissues, including vascular smooth muscle. This active Na^+ extrusion operates at the expense of cellular energy usually obtained by hydrolysis of ATP. The involvement of a membrane $(Na^+ + K^+)$-ATPase in this process has been well established. This enzyme has been found in most tissues, however to date there are no reports clearly demonstrating its presence in vascular smooth muscle. Thus the prime purpose of this thesis was to determine whether a $(Na^+ + K^+)$-ATPase exists in this tissue.

The present study was begun with the assumption that this enzyme does exist in aortic smooth muscle and careful consideration was given to the possible reasons why $(Na^+ + K^+)$-ATPase activity could not be demonstrated with the enzyme isolation and assay techniques employed by others.

Aortic smooth muscle is not the only tissue in which difficulty in demonstrating $(Na^+ + K^+)$-ATPase activity has been encountered. A classical example is cardiac muscle, in which it was found that high levels of actomyosin ATPase were contaminating the homogenate or subcellular fractions of this tissue and thus masked detection of the membrane $(Na^+ + K^+)$-ATPase. In view of this problem, Potter and co-workers (1966), developed an isolation procedure which

extracted the contaminating ATPase activity and yielded a membrane preparation in which $(Na^+ + K^+)$-ATPase activity could clearly be demonstrated. Hence the possibility of a similar situation occurring in aortic smooth muscle homogenates and subcellular fractions was considered.

Another point for consideration is that the stimulation of the membrane ATPase by the addition of Na^+ plus K^+ is an important property which is frequently used to demonstrate the presence of the enzyme. Thus contamination of the tissue homogenate either by Na^+ or K^+ could limit the effects observed upon addition of these ions. Furthermore the conditions used to determine ATPase activity may not be optimal for a demonstration of $(Na^+ + K^+)$-ATPase activity under some experimental circumstances.

Vascular smooth muscle in comparison to other tissues does not lend itself to mild homogenization techniques, therefore other problems were also anticipated in demonstrating $(Na^+ + K^+)$-ATPase activity.

Since the differential centrifugation techniques employed to isolate particulate membrane fractions (microsomes) from other tissues, depends on a separation according to particles size, the distribution of the membrane fragments in the fractions obtained have to be determined along with a similar study of the degree of contamination of the membrane fractions by fragments of other cell organelles. For instance $(Na^+ + K^+)$-ATPase activity could be masked by the presence of mitochondrial ATPase activity. Since particle

size depends to a large extent on the homogenization techniques used, and since the homogenization of aortic smooth muscle cannot be varied much, attempts at separating contaminating cell fragments from the membrane preparation, by methods which separate according to both size and density should also be considered. The possibility of destroying or changing the properties of the $(Na^+ + K^+)$-ATPase by the homogenization or isolation techniques used must be considered in studies of this nature.

Recently Verity and Bevan (1969) studied the distribution and properties of ATPase activities in homogenates and subcellular fractions of rabbit aortae. Using a Tris buffered assay media, they were able to demonstrate both Mg^{++}-ATPase and Ca^{++}-ATPase activities, in their preparations, with the level of Mg^{++}-ATPase being highest. Most of the Mg^{++}-ATPase activity was found in the microsomal fraction of their preparations. However, they were unable to demonstrate Na^+ plus K^+ stimulation or ouabain sensitivity in this fraction. Without a detailed consideration of the possibilities mentioned above, which may well have prevented detection of the $(Na^+ + K^+)$-ATPase activity, these investigators concluded that this enzyme may not exist in vascular smooth muscle.

In the present study of the rabbit aortic ATPase activities, the first consideration was in the homogenization technique used. It has been pointed out by Allen (1967) that glass-glass homogenization of tissues can lead to contamination of such homogenates with Na^+ from the glass. If Na^+ contamination from this source were present in

addition to small amounts of cellular K^+, in concentrations sufficient to stimulate ($Na^+ + K^+$)-ATPase activity then addition of further Na^+ plus K^+ in the assay system may not result in further stimulation. However if this were the case the enzyme should still be detectable as ouabain would be expected to inhibit the apparent ATPase activity. Nevertheless, to avoid possible Na^+ contamination different homogenization techniques were attempted. Unlike softer tissues such as liver, it was difficult to homogenize rabbit aorta with a teflon-pestle homogenizer, a VirTis blade type homogenization was found more suitable and therefor used.

When VirTis prepared rabbit aortic homogenates were assayed in Tris-buffered media, ATP was readily hydrolyzed in the presence of Mg^{++} and, to a less degree in the presence of Ca^{++}. However neither of these activities could be further stimulated by the addition of Na^+ plus K^+ nor affected by addition of ouabain, nor was this hydrolysis specific for ATP as other nucleotides could also be readily hydrolyzed. The homogenate therefore contained general nucleotide phosphatase activity. Thus the ATPase activities in homogenates of rabbit aortae prepared by VirTis homogenization and assayed in Tris-buffered media, were not unlike those which have been previously reported for this tissue.

Because of the existing possibility that the ($Na^+ + K^+$)-ATPase activity could easily have been masked by the presence of other ATPase activities such as myosin ATPase or mitochondrial ATPase as well as nonspecific nucleotide phosphatase activity, attempts were

made to isolate a membrane preparation from rabbit aortae which would contain mainly ($Na^+ + K^+$)-ATPase activity free of other ATP hydrolysing activity.

Differential centrifugation of tissue homogenates has often been used to obtain microsomal preparations composed primarily of endoplasmic reticulum and plasma membrane fragments (Hanzon and Toshi, 1959; Siekevitz, 1959; Hokin and Hokin, 1960; Charnock and Post, 1963). Because both the time and type of homogenization, as well as the nature of the tissue can greatly influence the composition of various fractions obtained by differential centrifugation, it was necessary to determine the nature of the subcellular fractions of rabbit aorta which were obtained by various procedures.

The nature of these fractions was determined by combining both morphological data obtained by electron microscopy and biochemical data from enzyme marker studies. Morphologically the fraction obtained by low-speed centrifugation (P_1) was found to be composed primarily of cell debris, nuclei and connective tissue. The next fraction (P_2) contained material of smaller fragments and a few mitochondria. There were few whole mitochondria in this fraction and the significance of this was not immediately recognized since whole tissue sections showed that mitochondria were not in great abundance in aortic smooth muscle. However the significance of this observation will become more apparent later when discussing the O_2 consumption of the fractions as this property was used as an indication of mitochondrial distribution. Fraction P_3, sedimenting at 100,000 x g, contained many membranous

structures and fragments but whether these were derived from the cell plasma membrane was not known.

By studying the distribution of the nucleic acids RNA and DNA most of the DNA was found in fraction P_1 while most of the RNA was in fraction P_3. This result could be anticipated if fraction P_1 contained most of the nuclear material and fraction P_3 contained ribosomalmaterial derived from the cellular endoplasmic reticulum. The distribution of enzymes reported to be localized in the plasma membrane of other tissues were also studied. These plasma membrane enzyme markers were found to be of highest specific activity in fraction P_3, with much lower activities in fraction P_2 and P_1.

Because the isolation technique used to separate the fractions depends on the size of the particles, some membrane material would be expected in all the fractions. Since most of the membrane material was in the slowest sedimenting microsomal fraction, it is apparent that the homogenization procedure must have disrupted the membranes into small particles. For this reason it was very important to study the distribution of mitochondria, since fragments of mitochondria could easily be contaminating the microsomal P_3 fraction and escape recognition by morphological examination.

The distribution of mitochondria in the aortic fractions was determined by measuring the O_2 uptake by these fractions. Surprizingly, most of the O_2 uptake was found in fraction P_3. Since morphological examination did not reveal any whole mitochondria in this fraction, contamination by mitochondrial fragments was assumed. This assumption

was further substantiated by showing that less disruptive homogenization techniques increased the amount of O_2 uptake in fraction P_2 while decreasing O_2 uptake by mitochondrial fragments in fraction P_3. Also mild glass-teflon homogenization of rabbit liver, a tissue which is abundant in mitochondria, resulted in the major O_2 uptake in fraction P_2, whereas when this tissue was VirTis homogenized the O_2 uptake of fraction P_3 increased considerably without morphological evidence of a redistribution of whole mitochondria. The results leave little doubt that VirTis, and to some extent the commonly used glass-glass homogenization techniques, can contribute mitochondrial fragments to so called microsomal fractions and thus complicate interpretation of results. A corollary of this observation is that if isolation of whole mitochondria from a tissue is desired, then the mildest possible homogenization techniques would be indicated.

Since mitochondrial fragments would contain ATPase activity of their own, a sucrose density gradient centrifugation technique was developed to separate some of this contamination from the membrane microsomal material. Here again O_2 uptake and morphologic studies of the aortic and comparative liver fractions were found useful in improving the isolation procedure. The final gradient used in this study separated the P_3 fraction into three components, (P_D, M_D and N_D). It was found that the microsomal fraction P_D obtained by this procedure now was much less contaminated by mitochondrial fragments than the original fraction P_3. Most of the mitochondrial contamination was now localized in the M_D fraction, with other fragments of collagen

and cell debris found in fraction N_D.

Thus it can be seen that accompanying characterization studies were indispensable to an interpretation of results of subcellular fractionation procedures of tissue homogenates, for which such a fractionation has not been established. The method of initial homogenization has also been shown to play an essential role in determining the final composition of the fractions obtained.

When the ATPase activities of the subcellular fractions were assayed in Tris-buffered media, most of the activity originally in the homogenate was found in the P_D fraction. However none of the Mg^{++}-ATPase activities in the subcellular fractions could be stimulated by the addition of Na^+ plus K^+, moreover the properties of the enzyme activities were not unlike those in the complete tissue homogenates. The possibility that the composition of the assay media used was not optimal for demonstrating $(Na^+ + K^+)$-ATPase activity, was therefore considered. Since it had been reported by Skou (1964) that the stimulation of the $(Na^+ + K^+)$-ATPase by monovalent cations was higher in histidine buffered assay media than in those with Tris-buffer at the same pH, the Tris-buffer in the assay media was replaced by a histidine buffer.

When assayed in the presence of histidine buffer the specific activity of the Mg^{++}-ATPase activity of the total homogenate of rabbit aorta was lower than that in Tris-buffer, but most significantly the addition of Na^+ plus K^+ resulted in some further stimulation of this activity. On the other hand, Mg^{++}-ATPase activities of any of the

subcellular fractions were not stimulated by the addition of monovalent cations. It was found that after subcellular fractionation, a non enzymatic factor, remaining in the 100,000 x g supernatant fraction (S_{3B}), had to be added back to any of these subcellular fractions in order to demonstrate Na^+ plus K^+ stimulation of the Mg^{++}-ATPase activity. This factor was not identified but a preliminary study of its properties suggest it to be a weakly acidic lipoprotein. The S_{3B} factor was heat stable, but in other respects differed from the activating factor isolated by Schwartz and Laseter (1964).

An interpretation of the effect of the supernatant factor is that it seems to induce Na^+ plus K^+ stimulation by converting some of the Mg^{++}-ATPase activity to a form which required the addition of monovalent cations for full activity. It is proposed that the S_{3B} material is very loosely bound to the enzyme and that isolation of the fractions physically separated the material. It is also possible that the VirTis homogenization could have been responsible for weakening or dislodging the factor from the enzyme protein. This hypothesis is supported by the following observation.

When assayed in a histidine-buffered assay media a mixture of $P_D + S_{3B}$ was found to contain the highest monovalent cation stimulated Mg^{++}-ATPase activity. Addition of increasing concentrations of Tris to such an enzyme preparation, resulted in a gradual loss of the Na^+ plus K^+ stimulated activity along with an increase in the Mg^{++}-ATPase activity. Tris may be acting as a monovalent cation itself in competition with Na^+ and K^+ for their activation sites on

the enzyme. Since Tris has been shown to alter the physical properties of tryptophanase (Gopinathan and DeMoss, 1968) the possibility also exists, that Tris could be interfering with the action of the S_{3B} activating factor which had to be added to the enzyme preparation. Tris also decreased the specificity for ATP hydrolysis and other nucleotides could also be readily hydrolyzed. For example in Tris-buffer, the rates of ATP, ADP or AMP hydrolysis by the aortic microsomal preparation did not differ much from each other. Although the rates of adenine nucleotide hydrolysis by these enzyme preparations in histidine assay media were lower than in Tris, there was a marked difference between the rates of hydrolysis of each of these compounds. AMP hydrolysis was very low to nonexistent in histidine buffer and also only the hydrolysis of ATP could be stimulated by addition of monovalent cations. Since the enzyme activity in Tris buffered media is not specific for ATP and behaves more like a general nucleotidase activity, it is suggested that Tris may be interfering with the mechanism of ATP hydrolysis. Dayan and Wilson (1964) have reported that the presence of Tris can increase alkaline phosphatase activity by catalyzing a transphosphorylation of Tris itself which occurs at a faster rate than that of the intermediate normally involved.

More than one type of ATPase activity was found in the P_D + S_{3B} enzyme preparation. Most of the ATPase activity was found to be a Mg^{++}-ATPase. Mg^{++}-ATPase activities are found in (Na^+ + K^+)-ATPase preparations but usually are not responsible for most of the ATPase activity. Some of this Mg^{++}-ATPase activity in the aortic preparation

could be due to the presence of mitochondrial ATPase since the isolation procedure could not completely remove all mitochondrial contamination of the P_D fraction. Azide, which is thought to be a specific inhibitor of mitochondrial Mg^{++}-ATPase under some circumstances, was found to be an effective inhibitor of the Mg^{++}-ATPase in the aortic fractions.

Ca^{++}-ATPase activity was also found in the $P_D + S_{3B}$ enzyme preparation. By employing an extraction technique reported to solubilize actomyosin, most of the Ca^{++}-ATPase activity characteristic of actomyosin was found to be localized in fraction P_1, with little contamination of the microsomal fraction. Work by other investigators has also shown that Ca^{++}-ATPase activities are often found in $(Na^+ + K^+)$-ATPase preparations from nonmuscular tissues. Whether the Ca^{++}-ATPase activity found is a separate enzyme contaminating the $P_D + S_{3B}$ ATPase preparation is not known, but this activity could be due to a Ca^{++} activation of the same ATPase that is Mg^{++} activated.

Among these ATPase activities, monovalent cation stimulated ATPase activity was also demonstrated in the aortic $P_D + S_{3B}$ preparation. Since such activity has never been demonstrated in vascular smooth muscle before, a study of the properties of this enzyme was made to see if these properties were similar to those reported for the $(Na^+ + K^+)$-ATPase from other tissues. In many respects the properties of the enzyme in vascular smooth muscle clearly resembled those of a typical $(Na^+ + K^+)$-ATPase preparation from soft tissue or red cell membranes. It required the presence of Mg^{++} for stimulation by the

addition of Na^+ plus K^+. Ca^{++} could not replace Mg^{++} for this property, and furthermore Ca^{++} could inhibit the monovalent cation stimulated activity in the presence of Mg^{++}. In addition, the hydrolysis of nucleotides other than ATP was not stimulated by monovalent cation addition.

Specific inhibition of the monovalent cation stimulated portion of the $(Na^+ + K^+)$-ATPase activity by ouabain is a unique property of this enzyme, and is therefore often used as a critical test for the presence of this enzyme. In the aortic preparation only a small portion of the total monovalent cation stimulated ATPase activity was inhibited by ouabain. However inhibition of the ouabain sensitive portion of the ATPase activity could be overcome by increasing K^+ concentration indicating a similarity to typical $(Na^+ + K^+)$-ATPase preparations. The incomplete inhibition of the monovalent cation stimulation by ouabain was found to be due to the presence of a ouabain insensitive Na^+ stimulated ATPase activity. $(Na^+ + K^+)$-ATPase activity which has been found in most tissues requires the addition of both Na^+ and K^+ together for full activation, and the stimulation obtained by adding the ions individually is negligible. It is possible that the Na^+-ATPase activity could have been due to K^+ contamination of the aortic preparation although this would not explain the lack of ouabain sensitivity. However this possibility was tested by depleting the tissue of its K^+ by soaking it in K^+ free media prior to homogenization and/or by treatment of the microsomal fraction with a K^+ chelating agent (tetraphenylboron). These attempts

at K^+ removal did not reduce or remove the Na^+ stimulation of the enzyme.

Since it has been shown by Askari and Fratantoni (1967) that ouabain insensitive Na^+-ATPase activity could be demonstrated after severe disruption of $(Na^+ + K^+)$-ATPase containing red cell membranes, the possibility that the Na^+-ATPase activity found in aorta may simply be an artifact of the homogenization procedure was considered. In order to test this possibility rabbit kidney cortex (a soft tissue, and a good source of $(Na^+ + K^+)$-ATPase) was homogenized with the VirTis and the microsomal fraction was isolated, to see if this would result in a poor $(Na^+ + K^+)$-ATPase preparation and also a high Na^+-ATPase activity. However, when assayed for these enzymes the VirTis homogenized kidney preparation contained $(Na^+ + K^+)$-ATPase activity which was very sensitive to ouabain, with no indication of Na^+-ATPase activity. This result suggests that the Na^+-ATPase activity of aortic smooth muscle is probably a real entity and not an artifact resulting from the homogenization and isolation technique used. Moreover in a membrane microsomal fraction of uterine smooth muscle prepared by loose teflon homogenization, a similar ouabain insensitive Na^+-ATPase activity has been demonstrated (Daniel and co-workers, 1969).

When various treatments and procedures were used to try and selectively inhibit the Na^+-ATPase activity, any change in the Na^+-ATPase activity was invariably accompanied by an equivalent change in Na^+ plus K^+ stimulated activity. It is therefore suggested that in intact rabbit

aortae the Na^+-ATPase function is in close proximity to the $(Na^+ + K^+)$-ATPase in the membrane of this tissue.

The evidence presented on the ATPase activities of rabbit aortic subcellular fractions indicate that in the presence of histidine buffer and a soluble co-factor, Mg^{++}-ATPase and Ca^{++}-ATPase activities exist, and in the presence of Mg^{++}, ouabain insensitive Na^+-stimulated and ouabain sensitive Na^+ plus K^+ stimulated ATPase activities can be demonstrated.

The possibility that more than one discrete ATPase activity is located in the membrane is not an unusual situation. In fact most of the biochemical studies on $(Na^+ + K^+)$-ATPase activities of purified membrane preparations also report the presence of Mg^{++}-ATPase and Ca^{++}-ATPase activities. Usually special denaturation or solubilization procedures can reduce or remove the Mg^{++} or Ca^{++} activated ATPase activities from $(Na^+ + K^+)$-ATPase activity, but this does not mean that these other enzymes were not discrete enzymes themselves. Histochemical studies have also supported the existence of more than one type of ATPase activity localized in membranes, however, controversy as to whether the histochemical lead staining methods used are valid makes interpretation of such data difficult.

Although more than one type of ATPase activity seems to exist in the cell membrane, only the $(Na^+ + K^+)$-ATPase has been assigned a physiological role in ion transport.

Recent studies have indicated that in some tissues not all the active transport of Na^+ can be inhibited by ouabain, and that other

energy dependent Na^+-pumps may exist. Since a ouabain insensitive Na^+-ATPase might be involved with one of these Na^+-pumps, some preliminary studies on the efflux of ^{22}Na and ^{42}K were carried out.

The efflux studies showed that in fresh rabbit aortae ouabain or K^+-free solutions had some inhibitory effects on efflux from the main cellular Na^+ compartment. This inhibition was more pronounced in tissues which were made Na^+-rich, and in which active Na^+ transport is increased. The results were not unlike those previously reported for ^{22}Na exchange in vascular smooth muscle of other species, and thus support the existence of a ouabain sensitive Na^+-pump in this tissue.

When ^{22}Na efflux from rabbit aortae was measured in Krebs-Ringer at 5°C, or media containing the metabolic inhibitors IAA and DNP, cellular ^{22}Na efflux was inhibited more than by ouabain. Assuming that IAA and DNP inhibited metabolism and decreased the ATP supply in this tissue, a second Na^+-pump insensitive to ouabain and possibly ATP dependent, may exist in this tissue.

By following the net movement of Na^+, K^+ and water in rabbit aortae it was found that cooling to 5°C, addition of ouabain or the metabolic inhibitors caused net downhill ion movements in fresh tissues. Cooling and metabolic inhibition also caused a gain of tissue water. The uphill movement of ions and loss of water observed when Na^+-rich tissues are incubated in Krebs-Ringer at 25°C, could be prevented by cooling to 5°C or by addition of the metabolic inhibitors. Ouabain however only prevented recovery of Na^+ and K^+ in these tissues,

and did not prevent water loss. In view of these results it was therefore further postulated that the ouabain insensitive Na^+-pump may be involved with regulating cell volume.

The exchange of ^{22}Na and net ion movements yielded results very similar to those recently reported for uterine smooth muscle (Daniel and Robinson, 1969a, b, and c), and since a ouabain insensitive Na^+-ATPase has also been found in a membrane preparation of this tissue, it is tempting to postulate that this Na^+-ATPase is in some way involved with the ouabain insensitive Na^+-pump found in these tissues. However such an association must be viewed with caution since not enough evidence is available to relate the two systems.

The results obtained from the experiments reported here suggest several further areas of research which could profitably be undertaken.

Since the homogenization technique used in this study increases the difficulty in obtaining a membrane preparation of aortic smooth muscle free of contamination from fragments of other cell organelles a less disruptive homogenization of this tissue may prove useful. Pretreatment of aortic tissues with collagenase or elastase might be useful in softening the tissue so that it could be suitable for homogenization with a teflon-glass type of homogenizer.

The use of solubilizing agents such a deoxycholate might also be helpful in obtaining a $(Na^+ + K^+)$-ATPase of higher specific activity and possibly some separation from the other ATPases that were found. Separation and isolation of the ATPases, especially the Na^+-

ATPase, from the membrane preparation would be desirable so that the individual properties of these enzymes could be studied more thoroughly. Such separation would be most desirable in any investigation of the mechanism of action of drugs on these enzymes.

Further purification and characterization of the S_{3B} factor would be essential in determining its nature, and also allow further investigation of the role this factor has in the monovalent cation stimulated ATPase reaction sequence.

In order to establish that the Na^+-ATPase described here is actually involved with a volume regulating Na^+-pump, demonstration of such an enzyme activity in other tissues which possess such a Na^+-pump should be attempted. Also a specific inhibitor of this pump and the ATPase would be useful in establishing their relation.

Further studies on ^{22}Na and ^{42}K influx and the movement of Cl^- would be necessary to establish whether the postulated volume regulating Na^+-pump is active and also its relation to the Na-Cl pumps described in other tissues.

Moreover the effects of the inhibitors of ^{22}Na efflux on the levels of nucleotides in rabbit aortae would be desirable, along with consideration of other effects the inhibitors may have (especially IAA and DNP) on the rabbit aortic smooth muscle cell. Morphological examination of the ultrastructure of these cells after treatment with these inhibitors might also provide additional information.

B. Conclusions.

1. Subcellular fractionation of VirTis homogenized rabbit aortae by differential centrifugation provided a microsomal fraction (P_3) containing primarily plasma membrane material but was highly contaminated with mitochondrial fragments. A sucrose density gradient technique was developed which allowed further separation of the mitochondrial fragments and other cell debris from the membrane microsomal fraction. The specific activity of the ATPase in this final microsomal fraction (P_D) was also higher suggesting some purification of the ATPase from other protein material.

2. In order to demonstrate a monovalent cation stimulated ATPase activity in the microsomal membrane fraction (P_D) it was found necessary to include some of the 100,000 x g supernatant material (S_{3B}). This (S_{3B}) material was heat stable and without ATPase activity itself, but when added to the microsomal enzyme preparation seemed to convert some of the Mg^{++}-ATPase activity to a form also requiring monovalent cations for full activity. Studies on the properties of the S_{3B} material indicate the material to be of a lipoprotein nature.

3. The presence of Tris buffer was found to interfere with the monovalent cation stimulation of the ATPase activity. Tris increased ATP hydrolysis by the Mg^{++}-ATPase and at the same time prevented further stimulation of the enzyme by Na^+ plus K^+ addition. Also the rate of hydrolysis of other nucleotides was increased by Tris.

4. A mixture of ATPase activities was found in the combined microsomal plus supernatant fraction ($P_D + S_{3B}$). One of these activities was found to be a Na^+ plus K^+ stimulated ouabain sensitive ATPase activity. In the presence of histidine buffer, the properties of this enzyme were found to be very similar to those reported for the typical membrane ($Na^+ + K^+$)-ATPase. Most of the monovalent cation stimulated activity in this preparation consisted of a ouabain insensitive Na^+-ATPase activity.

5. Inhibitors of active transport were found to affect part of the cellular efflux of ^{22}Na or ^{42}K in rabbit aortae supporting the existence of a ouabain sensitive Na^+-pump in this tissue. In addition, a ouabain insensitive Na^+-pump, dependent on metabolic energy and involved in the control of cell volume was postulated. The possibility therefore remains that this second Na^+-pump may involve a Na^+-ATPase activity in this tissue.

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