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AN ELECTRON MICROGRAPHIC CYTOCHEMICAL STUDY OF CARDIAC AND RENAL PHOSPHATASES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by

Paul Thomas McCauley, B.S.

* * * * * *

The Ohio State University
1977

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Endocrinology, Dr. K. Brownell

Biological Oxidations, Drs. G. Brierly and A. Merola
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INTRODUCTION

The Problem

This dissertation tests the hypothesis that cardiac plasma membrane-bound enzymes are placed upon that membrane by epicytotic means. Epicytosis is defined as inclusion of vesicles into plasma membrane (Lindower and Marks, 1972). This epicytotic process was to be identified and traced by electron microscopic cytochemical analysis of phosphatase activity, particularly Na\(^+\)K\(^+\)Mg\(^++\) ATPase activity.

Sarcolemma and the Transverse Tubular Continuity Structure

The sarcolemma is the term commonly used to denote the plasma membrane of heart. The sarcolemma serves the dual function of containing the cell as well as regulating the inner environment of the cell (Schwartz, 1974). This regulation is achieved by first acting as barrier to high molecular weight compounds such as hemoglobin (M W 64,500), horseradish peroxidase (M W 40,000) (McNutt and Fawcett, 1974), ferritin (Huxley, 1964), albumin (Endo, 1964), and fluorescent dyes (Hill, 1964). Second, as well as acting as an osmotic barrier, this regulation is
achieved also by actively transporting ions such as Na\(^+\), K\(^+\), Ca\(^+\), and Mg\(^{2+}\) (Schwartz, 1974).

Sarcolemmal membranes differ in mammalian species from plasma membrane of most other tissues by being deeply invaginated into the cell. These sarcolemmal invaginations in mammalian muscle have been identified as transverse or "T" tubules. The continuity of "T" tubules with sarcolemma has been confirmed by freeze etching techniques in guinea pig papillary muscle (Raynes et al., 1967), observation at the electron microscopic level in rabbit and human ventricles (Nelson and Benson, 1963), sheep ventricles (Simpson and Oertelis, 1961) (Simpson and Oertelis, 1962), and electron microscopic serial section (Simpson and Rayns, 1968). The "T" tubular membrane is morphologically "identical with sarcolemma" (Simpson and Oertelis, 1962) consisting of a 60 to 80 Å thick trilaminar membrane and a 140 to 170 Å thick basement membrane. Since this "T" tubular system is an invagination of the outer membrane the lumen of the "T" tubule is extracellular space. Consequently, large molecules unable to cross plasma membrane may be able to diffuse into "T" tubule much more readily than into intracellular space. Ferritin (Huxley, 1964), albumin (Hill, 1964), and fluorescent dyes (Endo, 1964), all large molecules relatively unable to cross plasma membrane, diffuse readily into "T" tubular space.
Similarity Between the Transverse Tubular System and the Sarcolemma

Given the morphological similarity and the structural continuity of the "T" tubule and the sarcolemma, one might ask if the "T" tubule is capable of conducting an action potential as the sarcolemma does. Significantly, electrical stimulation of frog skeletal muscle with "T" tubules disrupted by osmotic shock yielded action potentials, but no muscle contraction. Similarly treated tissue with intact "T" tubules yielded both action potentials and contractions (Howell and Jenden, 1967) (Gage and Eisenberg, 1967).

Based upon resting potential and sarcolemmal surface area, muscle tissue has a much higher capacitance than similar surface areas of nerve fibers (Peachy, 1965). A relatively simple explanation for this high capacitance is that surface area of muscle cells is greater than that calculated based upon an elliptical surface. When the surface area of muscle is computed to include "T" tubular surface area, capacitance of muscle more closely approximates that of nerve (Peachy, 1965). Indeed, if "T" tubules are disrupted by osmotic shock, frog skeletal muscle capacitance falls from 6.13 μ farads/cm² to 2.25 μ farads/cm². The 2.25 μ farads/cm² value is close to the value of 1 μ farad/cm² found in similarly treated, but not disrupted nerve (The treatment does not disrupt plasma
membrane). The 2.25 μ farads/cm² capacitance also compares well with the predicted muscle capacitance (Eisenberg and Gage, 1967) of 2.45 μ farads/cm² of an elliptical cell devoid of "T" tubules.

Based upon this capacitance data, one might assume "T" tubular membrane to have a resting potential similar to the more traditional sarcolemma resting potential, as well as an ability to depolarize with a normal action potential (Peachy and Porter, 1959) (Edwards et al., 1956). This idea is further supported by Huxley's experiments with frog striated muscle. Using microelectrodes and small depolarizing current, Huxley caused localized muscle contraction (Huxley, 1957). The current density used was not sufficient to cause an action potential upon the membrane. With this small amount of current, Huxley was only able to elicit a contraction when the microelectrode was placed upon the Z-line area of the cell. The Z-line itself had sensitive and insensitive locations. Sensitive areas upon the Z-line were spaced approximately 5 μ apart. When contraction was accomplished, it was localized to a distance of one half of one sarcomere upon either side of the Z-line. Several investigators, including Huxley (Peachy, 1965) (Huxley, 1964) (Endo, 1964), have speculated that since frog "T" tubules are spaced 5 μ apart and are located upon the Z-line, Huxley was depolarizing "T" tubules selectively. If "T" tubules have a resting
potential and depolarize, then they must have a membrane bound cation active transport system, a likely candidate being the Na\(^+\)K\(^+\)Mg\(^++\) adenosinetriphosphatase.

**Basement Membrane Carbohydrate Localization in Golgi Apparatus, Plasma Membrane and Transverse Tubule**

In addition to the trilaminar nature of the sarcolemma (McNutt and Fawcett, 1974) (Rambourg et al., 1966), there is a carbohydrate rich "cell coat" on the external surface (Rambourg et al., 1966) (Simpson and Oertelis, 1962). In mammalian myocardium, this "cell coat" invaginates with the sarcolemma forming a carbohydrate sleeve inside of the lumen of the entire mammalian cardiac "T" tubular system (Fawcett and McNutt, 1969) (Simpson and Oertelis, 1962).

Further consideration of this carbohydrate rich basement membrane or "greater" membrane follows. In 1964 a colloidal thorium method for staining mucopolysaccharides was devised (Revel, 1964). Staining for mucopolysaccharides in mouse, rabbit, and cat chondrocytes at the electron microscopic level demonstrated three positive sites: plasma membrane, mature face of the Golgi apparatus, and specifically apparent Golgi vesicles. The same type of localization has been seen in amoeba (Revel and Ito, 1964).

In 1966 using a periodic acid stain for mucopolysaccharides (Rambourg et al., 1966), basement membranes
were demonstrated in all tissues tested in rat. Three years later (Rambourg et al., 1969), identified mucopolysaccharides upon the mature face of Golgi apparatus, apparent Golgi vesicles, and plasma membrane of rat liver.

These findings led Wise and Flickinger (Wise and Flickinger, 1970) to speculate that carbohydrates synthesized upon Golgi apparatus are transported via Golgi vesicles to plasma membrane to form the "greater" or basement membrane.

**Autoradiographic Sequence Studies in Golgi Apparatus, Plasma Membrane and Transverse Tubular Carbohydrate Structures**

Caution must always be used when ascribing direction to static micrographs. Therefore, Wise and Flickinger (Wise and Flickinger, 1970a) investigated the possibility that the flow of "greater" membrane glycoprotein may be from membrane to Golgi apparatus. From observations on phagocytotically active amoeba in media containing ferritin and peroxidase. Further, they reported that although there was heavy pinocytotic activity, none of the ferritin or peroxidase localized in Golgi apparatus. Golgi vesicles, as well as pinocytotic vesicles appeared to, however, contribute to food vacuoles.

Strong support for the Wise and Flickinger hypothesis has been given by histochemical and autoradiographic studies. Sulfate in rat colonic goblet cells is
predominately incorporated into ester-sulfate-mucopoly-
saccharides which are eventually secreted into the colonic lumen (Lane and Caro, 1964). Time course studies show S\textsuperscript{35} first concentrating in Golgi mature face, then later Golgi vesicles and finally being secreted. Similar sequence of incorporation was shown in chondrocytes. In this case the label finally became incorporated into chondroitin sulfate which forms the cartilage matrix around the chondrocyte much like a "greater" membrane (Goodman and Lane, 1964).

The "greater" membrane of mammalian cells consists of glycoprotein. Both galactose and fucose are constituents of this cell coat in rat duodenal epithelium (Bennett and LeBlond, 1970). Autoradiographic studies with fucose and galactose demonstrate incorporation first into the mature face of Golgi apparatus, then later into Golgi vesicles and finally into the cell coat. This same progression was observed with fucose in liver, epididymis, and stratified epithelium (Bennett and LeBlond, 1970) (Bennett, 1970).

Redman and Cherian (1972) suggest that glycoprotein synthesis actually begins at rough endoplasmic reticulum with the attachment of compounds such as N-acetylglucosamine. Redman and Cherian found, however, that radiolabeled galactose was primarily incorporated in smooth membrane fragments.
Very similar results were demonstrated by Hirando (Hirando et al., 1972). Using ferritin conjugates of two plant agglutinins, Hirando was able to covalently bind saccharide residues and view the resultant binding in microsomal fractions of myeloma cells on the electron microscope. Two significant findings were (1) ferritin-concanavalin A which binds B-D-mannopyranosyl and B-D-glycopyranosyl residues bound both rough and smooth surfaced microsomes, but ferritin-ricin which binds B-D-galactopyranosyl residues bound only some smooth-surfaced microsomes. (2) In all cases the agglutinins bound only one side of the membrane. "It is proposed that membrane elements are assembled starting from rough endoplasmic reticulum. They are then transmitted and converted into smooth membrane and Golgi elements where they are packaged into vesicles. These vesicles, it is suggested, then fuse with already-existing plasma membrane..." (Hirando et al., 1972).

In the two previously mentioned studies smooth surfaced vesicles were never identified as being of Golgi apparatus, smooth endoplasmic reticulum, mitochondria, or plasma membrane. A look at the autoradiographic studies of Bennett (Bennett, 1970) and Zangury (Zangury et al., 1970) strongly suggest that the smooth galactose incorporating membranes are mature face of Golgi apparatus. Bennett found that within 2 1/2 minutes of introducing radiolabeled galactose to rat duodenum epithelium, 70
percent of the marker was located over Golgi apparatus and Golgi vesicles. After 10 minutes, much of the radioactivity was located in cytoplasmic vesicles. A specific plasma cell tumor autoradiograph is further demonstrated support for this interpretation (Zangury et al., 1970). In this study galactose label moved from Golgi apparatus to plasma membrane over a period of 90 minutes. Glucosamine moved from cytoplasm associated with rough endoplasmic reticulum and polysomes to Golgi apparatus over a similar time course.

Therefore, several radiolabel studies have shown that labeled sugars are incorporated into glycoproteins at either rough endoplasmic reticulum or Golgi apparatus. These sugars appear to migrate from rough endoplasmic reticulum to Golgi apparatus to plasma membrane. Fucose and galactose are implicated strongly (Bennett, 1970) (Bennett and LeBlond, 1970) in "greater" membrane formation.

**Marker Enzymes in Golgi Apparatus, Plasma Membrane and Transverse Tubule**

If, in fact, this migration is indicative of formation of new plasma membrane as Hirando suggests, structures such as rough and smooth endoplasmic reticulum, Golgi apparatus, and plasma membrane should progressively show greater biochemical similarity to plasma membrane. It appears that this is the case. In addition to the increase in glycoprotein evidence by sensitivity to colloidal
thorium and periodic acid stains in Golgi apparatus, vesicles, and plasma membrane, mentioned earlier, Hirando (Hirando et al., 1972) demonstrated with plant agglutinins what might be the origin of the "greater" membrane in rough endoplasmic reticulum.

Further biochemical similarities may be seen by studying the marker enzyme 5'-nucleotidase. Widnell (1972) used rat liver microsomes to demonstrate that 5'-nucleotidase, a plasma membrane marker enzyme (Touster et al., 1970) was not only found in plasma membrane, but in rough and smooth endoplasmic reticulum, as well as Golgi apparatus. Widnell's study demonstrated membranes increasing order of activity for 5'-nucleotidase as rough, smooth endoplasmic reticulum, Golgi apparatus, and finally, plasma membrane. Further more, de Duve (1971) correlated cholesterol content in membranes with 5'-nucleotidase activity and found the plasma membrane has the highest content of cholesterol and Golgi apparatus is second highest.

Farquhar (Farquhar et al., 1974) demonstrated cytochemically in rat liver cells that 5'-nucleotidase activity is highest in mature or "trans" face of Golgi apparatus and Golgi vesicles and plasma membrane surfaces most highly associated with Golgi secretory activity; although, 5'-nucleotidase activity is demonstrable on the entire plasma membrane.
Ouabain Na$^+$K$^+$Mg$^{++}$ ATPase and Golgi Apparatus

Vaughan and Cook reported in 1972 that Hela cells cultured in 1 mM ouabain for one hour, washed for 4 hours in ouabain free media, and reexposed for 1 hour with ouabain bound as much ouabain as they did originally.

Ouabain treated Hela cells, preincubated with $^{86}\text{Rb}$, released $^{86}\text{Rb}$ at pre-treatment levels three hours following cessation of ouabain treatment. Cycloheximide, a protein synthesis inhibitor, abolished this effect. These results may be explained if ouabain directly or indirectly: (1) uncovers cryptic Na$^+$K$^+$Mg$^{++}$ ATPase sites, (2) stimulates synthesis of new enzyme, (3) inhibits degradation of old enzyme.

Lindower et al. (1973) reported that one hour after intra-peritoneal injection of 300 $\mu$g/Kg ouabain, an increased appearance of glycogen and ribosomes in the nuclear polar region of guinea pig cardiac ventricle occurred. Additionally, there was a size increase in "T" tubular and Golgi apparatus. Two or four hours after treatment, ribosomal and glycogen content of nuclear polar regions had decreased, but "T" tubular Golgi apparatus and Golgi vesicles continue to increase (Lindower and Marks, 1976). Modified "T" tubular structures, suggestive of either pinocytosis or epicytosis, are apparent in increased numbers 2 and 4 hours after injection. Epicytotic mechanisms are suggested by the increased population of
Golgi vesicles and Golgi apparatus, and the increased size of "T" tubules. Further support for epicytotic, rather than pinocytotic mechanisms are found in a study by Kaye and Donn (Kaye and Donn, 1965). This investigation found that $10^{-4}$ to $10^{-6}$ M ouabain decreased pinocytotic uptake of thorium oxide by 70 to 80 percent in rabbit cornea. Some ouabain treated corneas had large apical folds of plasma membrane near the apical complex, suggestive of increased plasma membrane area.

Summary

In summary: (1) It appears transverse tubules are structurally continuous with plasma membrane and that "T" tubular systems in mammalian myocardium have a resting potential and possibly depolarize with the sarcolemma. Either one of these functions would require a cation pump, most likely Na\(^+\)K\(^+\)Mg\(^++\) ATPase. (2) Golgi apparatus has been strongly implicated in plasma membrane and possible "T" tubular biosynthesis, specifically glycoprotein synthesis. (3) Na\(^+\)K\(^+\)Mg\(^++\) ATPase is suspected of being composed of two subunits—one being a sialoglycoprotein (Kyte, 1972) (Schwartz, 1974) (Dahl and Hokin, 1974) containing among its sugars glucosamine, galactosamine, and neutral sugars (Kyte, 1972). These carbohydrates suggest sugar addition at both rough endoplasmic reticulum and Golgi apparatus. The second and larger subunit is a
simple protein. Since the enzyme, 5'-nucleotidase, may be transported to plasma membrane by Golgi vesicles, it is suggested that Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase and other phosphatases may be transported to the "T" tubules or sarcolemma by the same means. (4) Consistent with observations of Vaughan and Cook on the synthesis of new Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase in HeLa cells, the work of Lindower and Marks suggests a similar pathway for the synthesis of Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase. Their findings provide a model to study this mechanism. (5) The objective of this dissertation was to test this mechanism in cardiac tissue to determine if phosphatases, specifically Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase, can be localized cytochemically upon Golgi apparatus and/or Golgi vesicles and/or plasma membrane.

**Wachstein and Meisel Phosphatase Localization**

In 1957 Max Wachstein and Elizabeth Meisel published their neutral pH modification of the Gomori (Gomori, 1941) lead phosphate technique (Wachstein et al., 1957). The Wachstein-Meisel lead phosphate technique was designed to mark areas of phosphatase activity for visualization with the light microscope.

The basic parts of the system are:

1. tissue with at least partial phosphatase activity remaining,

2. phosphatase substrate and ions required for
phosphatase activity,

(3) a phosphate precipitating ion, in most cases lead (Pb$$^{++}$$),

(4) and for light microscopy, a coloring agent for visualization of the precipitate.

In the original article by Wachstein, both thick sectioned frozen liver and formalin fixed (6 percent for 24 hours) liver tissue from rat, guinea pig, and human autopsy were used. Substrates for the reaction included glucose-6-phosphate, glycerol phosphate, and adenosine mono, di, and tri phosphate in separate experiments.

Lead nitrate was used to precipitate phosphate, liberated presumably by enzymatic activity and ammonium sulfide was the coloring agent used.

Wachstein and Meisel's apparent success at localizing glucose-6-phosphatase, sodium magnesium adenosine triphosphatase and 5'-nucleotidase activity at pH 7.2 was followed by several other attempts to localize magnesium ($$Mg^{++}$$) requiring adenosine triphosphatases (ATPases) and magnesium and sodium ($$Mg^{++}Na^+$$) ATPases in several species and tissues. Some of the tissues examined include rat liver (Berg, 1964) (Koshiba and Oda, 1967), rat heart (Essner et al., 1965) (Rostgaard and Behnke, 1965), rat intestin (Berg et al., 1972), blow fly flight muscle (Tice and Smith, 1965) (Tice and Engel, 1966), mouse smooth muscle (Land, 1967), frog epidermis (Farquhar and Palade,
1966), frog retina (Scarpelli and Craig, 1963), and toad gastric gland (Koenig and Vial, 1973).

Cardiac Glycosides as Cytochemical Tools

In the same year that Wachstein and Meisel published their article, Skou published (Skou, 1957) his article demonstrating a Na⁺Mg⁺⁺ dependent potassium (K⁺) stimulated ATPase in crab nerve, which apparently was responsible for maintaining the resting potential. That same year Glynn (1957) demonstrated in red blood cells (RBC) that cardiac glycosides decrease resting potential by interfering with ion flux by a direct inhibition of the ion pump. Since that time cardiac glycosides have been used extensively in the determination of Na⁺K⁺Mg⁺⁺ ATPase activity that do not demonstrate cardiac glycoside inhibition (the exception being in highly purified preparations of the enzyme).

Na⁺K⁺Mg⁺⁺ ATPase Cytochemistry—Claims and Counter Claims

Since 1964 several articles have appeared that claim successful cytochemical localization of Na⁺K⁺Mg⁺⁺ ATPase in several tissues including mouse kidney and brain cortex (McClurkin, 1964), guinea pig RBC ghosts (Marchesi and Palade, 1967), rabbit ciliary body (Palkama and Uusitalo, 1968; Palkama and Uusitalo, 1970), rat optic nerve (Palkama and Uusitalo, 1972), rabbit retina (Cole,
1964), sea gull salt gland (Abel, 1969), and rat and frog skeletal muscle (Grancamella et al., 1967). All of the above authors used either the Wachstein-Meisel method or some small variation thereof. There are also those who are unable to cytochemically demonstrate Na⁺K⁺Mg++ ATPase by the Wachstein-Meisel method. The Tice group was unable to demonstrate cytochemically, ouabain inhibitable Na⁺K⁺Mg++ ATPase in rabbit cornea (Kaye and Tice, 1966), rabbit psoas muscle (Tice and Engel, 1966) (Engel and Tice, 1966) using a low lead (1 mM Pb++) Modification of Wachstein-Meisel. Lessel and Kuwabara (1964) were unable to demonstrate ouabain inhibitable enzyme in rabbit retina using the Wachstein-Meisel level of 3.6 mM lead. Novikoff (Novikoff et al., 1961) had the same difficulty in rat liver, kidney uterus and oviduct using the Wachstein-Meisel media. Sommer and Spack (Sommer and Spack, 1964) could not demonstrate ouabain inhibitable enzyme in dog heart using a Wachstein-Meisel like media, even when hearts were pre-perfused with ouabain. Novikoff (Novikoff et al., 1966) and Bosch (Bosch et al., 1967) [read McClurkin response (McClurkin, 1967)] were unsuccessful at demonstrating a Na⁺K⁺Mg++ ATPase cytochemically by either the Wachstein-Meisel or McClurkin modification in rat kidney and Tormey (Tormey, 1966) was unable to reproduce the findings of McClurkin or Cole in rabbit eye epithelium.
Problems with Wachstein-Meisel Technique

One of the major criticisms of the Wachstein-Meisel method was the high level of lead (3.6 mM) in the incubation media. Several investigators have shown in several species and diverse tissues that Na\(^+\)K\(^+\)Mg\(^++\) ATPase is strongly (85 to 95 percent) inhibited by 3.6 mM lead (Novikoff, 1958), (Bonting, 1962), (Marchesi and Palade, 1967), (Moses and Rosenthal, 1968), (Jacobsen et al., 1969), (Rosenthal et al., 1969), (Charnock et al., 1972), and (Schulze et al., 1972). In order to circumvent this problem, Schulze and Wollenberger incorporated into their incubation media a lead chelate, either Tiron (4,5-dihydroxy-m-benzenedisulfonic acid disodium salt) or Na\(^+\) and K\(^+\) tartrate. Using chelates, Schulze and Wollenberger claimed success in cytochemically localizing Na\(^+\)K\(^+\)Mg\(^++\) ATPase and adenyl cyclase in guinea pig heart and striate muscle, as well as rat striate muscle (Wollenberger and Schulze, 1966; Schulze and Wollenberger, 1967; Schulze and Wollenberger, 1971; Schulze and Wollenberger, 1971a). Schulze and Wollenberger also claimed their cytochemical stain was fully reversible by 10\(^{-3}\) M ouabain or by deletion of either Na\(^+\), K\(^+\), or Mg\(^++\) or any combination of the three cations. Somogyi (Somogyi et al., 1971) was able to reproduce these results using tartrate as a lead chelate. However, Gillis (Gillis and Page, 1967) found that most all stain localized by the Schulze modification could be
reproduced simply by soaking tissue in phosphate and then in lead. He concluded that lead chelate, while reducing levels of free lead to allow more enzymatic activity (less than 0.1 mM Pb\(^{++}\)) (Schulze et al., 1972) lead levels were so low as to allow random localization and precipitation was more dependent on diffusion boundaries than on sites of inorganic phosphate production.

In experiments with rat kidney and liver, unless levels of free lead exceeded 0.5 mM Pb\(^{++}\) (Rosenthal et al., 1969a), only 23 percent of the enzymatically liberated phosphate was precipitated. Therefore, at that level of lead, 77 percent of the inorganic phosphate was free to diffuse to the plasma membrane or extracellular space where lead levels were higher. Polyacrylamide films of 200 to 700 \(\mu\) in thickness (7 Å pore size) when wet were synthesized (Cornelissen and Duijn, 1973) and impregnated with inorganic phosphate ranging in levels from 0.5 mM to 4.0 mM. When the films were immersed in equimolar lead salt solutions, several bands of phosphate precipitate developed parallel to the surface of the polyacrylamide gel (Cornelisse and Duijn, 1974). Heaviest precipitates are noted at higher phosphate and lead concentrations, and upon thicker polyacrylamide films. Strikingly reminiscent of many histochemical studies in animal tissue is the heaviest band of precipitation, on and just inside the surface of the gel. Up to three additional bands of phosphate
precipitate are seen inside the gel.

The Ernst Method

Clearly then, there is a certain amount of controversy concerning the lead phosphate precipitate method of enzyme localization. In order to circumvent some criticism of the lead ATPase methods, Ernst (Ernst, 1972a) introduces a method using strontium as a capture ion and para-nitrophenyl phosphate (PNPP) as enzyme substrate.

The most frequent criticisms of the Ernst method are (1) the high pH necessary for strontium phosphate precipitation (pH 9.0) and (2) the substitution of para-nitrophenyl phosphate for ATP as substrate for \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase as there is a certain amount of controversy whether \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase is the same enzyme as para-nitrophenyl phosphatase.

Aldehyde Fixation Verses Enzymatic Activity

There is also considerable controversy over the best method of tissue preparation prior to incubation. Briefly, let us review some of these techniques. The tissue to be tested cytochemically is usually either frozen or fixed prior to cytochemical analysis, although some investigators have prepared unfixed and unfrozen cell fractions or ghost preparations for cytochemistry. The tissue is then placed in a medium containing a buffer (usually collidine or cacodylate at pH 7.4) \( \text{Na}^+ \), \( \text{K}^+ \), \( \text{Mg}^{++} \), \( \text{Pb}^{++} \), and ATP.
In the case of PNPPase cytochemistry the medium contains a buffer (Tris-base) pH 9.0, K\+, Sr\++, Mg\++ and PNPP. It was assumed that the enzyme that survived pretreatment would be activated by the appropriate ion(s) and cleave ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pi). The phosphate would form a lead or strontium phosphate salt and precipitate out of solution at or very near the site of the enzyme.

Glutaraldehyde fixation has been shown in various tissues to inhibit ATPase activity (Koshiba and Oda, 1967; Koenig and Vial, 1973; Sabatini and Bensch, 1963; Goldfischer et al., 1964; Essner et al., 1965). Specifically, glutaraldehyde has been shown to inhibit Na\+K\+Mg\++ ATPase in guinea pig erythrocyte ghosts. At concentrations of 0.5 percent it inactivates ouabain inhibitable ATPase by 90 percent; 2 percent inhibits 98 percent (Marchesi and Palade, 1967). In avian salt gland 0.5 percent glutaraldehyde inhibits 90 percent of the Na\+K\+Mg\++ ATPase after 20 minutes and 100 percent after 60 minutes (Ernst and Philpott, 1970a). However, when 3 percent formaldehyde was used, 95 percent of the Na\+K\+Mg\++ ATPase activity remained after 30 minutes and 70 percent remained after 60 and 90 minutes of fixation (Ernst and Philpott, 1970a). Palkama and Uusitalo (1968) reported Na\+K\+Mg\++ ATPase in rabbit epithelium was inhibited by formal, but was resistant to glutaraldehyde. It is apparent that the
selection of the proper fixation time and concentration of fixative for the tissue under study is essential.

Cell Fractionation as an Alternative to Aldehyde Fixation

The use of unfixed cell fractions present a way to circumvent the fixation inhibition problem. Since Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase is thought to be a membrane bound enzyme (Schwartz, 1974; Dahl and Hokin, 1974), enzymatic activity should be found in the microsomal pellet. However, microsomes may be composed of membranes from any one of many structures (McIlwain and Bachelard, 1971). In most cases, the microsomes formed by homogenization of tissue look identical regardless of their structure of origin. This makes localization of stain on a microsome of little value in many cases. Farquhar and Palade (1966) localized Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase upon guinea pig erythrocyte ghosts; but, the usefulness of this technique is limited to cells amenable to ghost preparation.

Cryostatic Sections as an Alternative to Aldehyde Fixation

Another alternative is to freeze the tissue. The tissue is thick sectioned while still frozen, and then incubated in the appropriate medium (Charnock et al., 1972) (Uusitalo and Palkama, 1970). A problem associated with this approach is the poor state of preservation resulting from autolysis and ice crystal formation. Water-miscible solvents such as dimethyl sulfoxide (DMSO) have been used
to minimize ice crystal formation (Guth and Albers, 1974). However, these solvents do not alter autocatalysis and, because of the high concentrations used, cause osmotic artifacts in heart tissue (Schlafer and Karow, 1971) (Feuvray and DeLeiris, 1973). The use of unfixed frozen tissue for cytochemical localization is usually limited to light microscopy; details usually observed at the electron microscope level are badly altered or missing.

In this dissertation primary importance was placed upon using minimal fixation (that required to preserve Golgi apparatus and plasma membrane) followed by incubation in one of the three types of incubation media.

The Ernst, para-nitrophenyl phosphate method as well as the Schulze and Wollenberger, Tiron modification of the lead-ATP method and simple modification of the Wachstein-Meisel method are described in this dissertation. A further discussion of the advantages and disadvantages of these techniques is found in the Discussion section.
METHODS

Treatment of Animals

Except where noted, male guinea pigs weighing between 450 and 650 grams were used throughout. The animals were housed in a constant temperature and humidity vivarium with 12 hour day-12 hour night cycles. All animals were allowed free access to food and water until the morning they were used. Unless otherwise noted, the guinea pigs were injected IP with 20 mg/Kg sodium pentobarbital and 300 µg/Kg ouabain at the concentration of 20 µg/ml. Ouabain control animals were injected with an equivalent volume of distilled water. The injections, unless otherwise noted, were administered one hour prior to sacrifice.

Preparation of Hearts

Guinea pigs were sacrificed by a blow on the head. A lateral incision was made immediately below the rib cage in the abdominal region in order to gain access to the rib cage. The skin ventral and lateral to the rib cage was teased away with blunt scissors. At the midpoint of the lateral incision a second incision was started running rostral and parallel with the neck region. The diaphragm was
punctured promptly with sharp scissors and cut away from the rib cage. The heart was then freed from the anterior mediastinal tissues and the entire ventral and lateral rib cage was cut away and removed. Fine "mouse tooth" forceps were used to secure muscular and fatty tissue immediately rostral to the heart in the mediastinum region. With fine scissors a cut was made dorsal to the forceps in a rostral to caudal direction, freeing large parts of the mediastinum, heart and lungs. This freshly excised tissue was immersed in either cold 0.9 M sodium chloride or Krebs Henseleit solution for further dissection.

After the remainder of the pericardium was removed with fine blunt scissors, the fatty tissue was teased away from the aorta and a nick was made high in the aorta. The lower lip of the nick was secured with a fine hemostate and the upper part of the aorta was removed. The aorta was then cannulated with a 3 mm inside diameter stainless steel cannula fitted with a Luerlock and secured with surgical thread. During the cannulation, one of two isotonic salt solutions was continually pumped through the cannula tube.

**Perfusion Apparatus**

The center of the perfusion apparatus was a Buchler polystaltic pump capable of pumping four different solutions simultaneously without mixing. However, never
were more than two solutions pumped simultaneously during the course of one experiment. The perfusion apparatus consisted of tygon tubing with an inside diameter of 4 mm arranged such that when one end of the tubing was drawing solution from a reservoir the other end of the tubing was returning an equal volume of solution back to the same reservoir. This was duplicated with a second set of tubing and reservoir. A bubble trap was placed at the highest point consisting of a "y" shaped glass tube with one leg of the glass tube pointing up 90 degrees from horizontal. At the end of this tube, a three way valve was placed which allowed for filling and when closed, the maintainance of the solution in the 90 degree leg. Interrupting these two independent circular pathways were two additional three way valves which when properly adjusted shunted the solution independently into the cannula. This apparatus allowed switching of perfusion solutions with minimal loss of time, no mixing of solutions, and without introduction of air bubbles. After each experiment, the apparatus was washed with 250 ml of distilled water. Prior to reuse, a fresh 100 ml of the desired perfusion solution was pumped through the tubing and discarded.

**Electron Microscopy**

Unless otherwise stated the first solution perfused into the cannulated heart was Krebs Henseleit solution
(27.2 mM NaHCO₃, 118.0 mM NaCl, 4.8 mM KCl, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, and 11.1 mM glucose) (Palay et al., 1962), at 0 to 4 degrees centigrade or at room temperature as noted in the Results section. The pH of Krebs Henseleit solution was maintained constantly at pH 7.4 by bubbling 95 percent O₂-5 percent CO₂ through the solution during the entire experiment and for at least 30 minutes prior to use. Heparin was added to the Krebs Henseleit in the ratio of 0.2 ml (165 mg/ml stock solution) of heparin solution per liter of Krebs Henseleit. The heart was perfused at room temperature until this solution was only slightly colored by blood.

Following saline perfusion, the order of the following three steps was varied as follows.

Variation 1: Varying concentrations of glutaraldehyde and/or paraformaldehyde buffered with one of several buffers was perfused. This was followed by excising and dicing the left ventricle into approximately 1 mm cubes. Next, the cubes were washed in a buffered solution and immersed in one of several solutions designed to cause the deposition of some electron dense ion upon or very close to Na⁺K⁺Mg²⁺ adenosine triphosphatase. Ouabain 10⁻² M or cysteine 10⁻² M, both, or neither, were included in these solutions; this will be discussed later in the Methods section under the topic Cytochemical Solutions.
Variation 2: Perfusion with a fixative solution followed by perfusion with a cytochemical entrapment solution. Then the tissue was excised, diced and fixed.

Variation 3: Perfusion first with a cytochemical entrapment agent, then fixation and excision. Excised tissue was then diced and fixed.

Variation 4: Perfusion of the heart with a buffered fixative followed rapidly by excision of the left ventricle and rapid freezing by immersion in liquid nitrogen. Sections were cut frozen on a Porter-Blum cryostatic microtome. Sections were floated on a phosphate free buffered solution and transferred to a cytochemical entrapment solution. Further variations in times and temperatures of the above steps will be discussed in the Results section. In all cases, after the tissue was diced, it was transferred to polyethylene topped glass 3.5 ml vials. Solution changes were achieved by aspirating the initial solution with a Pasteur pipette and replacing it with a second solution to this nearly dry vial.

In some cases tissue blocks were post fixed in 6 percent glutaraldehyde in 100 mM phosphate buffered to pH 7.4 for one hour at 0 to 4 degrees centigrade.

In all cases, the tissue was immersed in 1 percent osmium tetroxide. The buffer in the osmium solution was either 100 mM cacodylate or 100 mM phosphate pH 7.4. The duration of this step was one hour and the temperature of
the solution was 0 to 4 degrees centigrade.

The dehydration procedure that followed consisted of exposing the tissue to fifty percent ethanol-distilled water solution at 0 to 4 degrees centigrade with two washes of 15 minutes duration as the first step, followed by one 70 percent ethanol wash overnight at 0 to 4 degrees centigrade or for 30 minutes at room temperature. Tissue immersion in 95 percent ethanol for a half hour at room temperature and then two fifteen minute immersions in 100 percent ethanol at room temperature completed the dehydration procedure.

The tissue was then prepared for embedding. As in the dehydration procedure, uniformity between all experiments in these final steps was sought. Preparation included two 15 minute immersions in propylene oxide. The embedding media, Spurr low viscosity embedding media (SEM) (Spurr, 1969), consisted of 10 gm vinylcyclohexane dioxide, 6 gm Dow epoxy resin 736, 26 gm nonenyl succinic anhydride, and 0.4 gm of dimethylaminoethanol. The reagents for the preparation of SEM were added in that order and mixed for at least 60 minutes with a motor driven teflon worm gear mixer. A small portion of the SEM was mixed 1:1 with propylene oxide and tissue specimens were immersed one hour at room temperature in this mixture following the final propylene oxide wash. The last step before the actual embedding procedure was a one hour room temperature
For each group of tissue cubes, 5 polyethylene embedding vials were filled with SEK. Most of the resin was aspirated from the tissue cubes while still in the glass vials. The remaining resin and tissue was poured out onto several thicknesses of 10 cm Wattman No. 1 filter paper. Tissue specimens were then selected for embedding by size and shape. Smaller rectangular shaped specimens were taken up with hand wood splinters and placed upon the surface of the Spurr resin in the embedding vials. Being more dense, the specimens found their lowest level in the inverted shortened four sided pyramid at the bottom of the embedding vial.

The resin was "seasoned" for at least 16 hours at 70 degrees centigrade. Removal of the tissue blocks from the embedding capsules required waiting until the blocks cooled to approximately room temperature. Care was taken to free the block from the capsule by gently squeezing with a pair of common slip joint pliers. Then the capsules were gently squeezed at the tip, forcing the hardened Spurr with embedded tissue out of the open end of the embedding capsule.

Tissue blocks were mounted under a Baush and Lomb dissecting microscope and all excess resin was dissected away with acetone cleaned razor blades. The face of the trimmed block was always square or trapezoidal in shape in immersion in 100 percent SEK.
order to facilitate thin sectioning. Forty five degree glass knives were prepared on an LKB knife maker type 7801B. Flotation troughs were made from masking tape sealed with pie seal and filled with distilled water.

Thin sectioning was performed with a Porter-Blum Ultra microtome type MT-2. Sections displaying interference colors of silver or light gold representing thicknesses of 600-1000 Å were accepted. These sections were transferred to distilled water filled petri dishes warmed for the purpose of flattening and mounted upon 300 mesh copper grids for viewing in a Philips model 300 Electron Microscope. Sections were normally viewed at an electron accelerating voltage of 60 Kv.

When sections were to be stained with lead citrate and uranyl acetate, the method of Reynolds (Reynolds, 1963) was used. Grids were submerged in a 1:1 ethanol:distilled water solution saturated with uranyl acetate and filtered. The whole of the uranyl acetate procedure was carried out in total darkness. Grids were washed vigorously and profusely with 1:1 ethanol:distilled water solution and then with distilled water.

Following this procedure the lead citrate solution was made as follows: 1.76 gm of sodium citrate monohydrate was dissolved into 30 ml distilled water. To this 1.33 gm lead nitrate was added with vigorous stirring. The solution then became turbid. Vigorous shaking
intermittently for 30 minutes followed by the addition of 0.8 ml of 10 N sodium hydroxide, resulted in the solution becoming crystal clear. The solution was then made up to 50 ml volume with distilled water.

As with the uranyl acetate procedure, the grids were immersed in the lead citrate solution for 30 minutes. In this case, however, the procedure was carried out in the light but the container was air tight. Grids were washed vigorously and profusely with 0.2 N sodium hydroxide followed by distilled water.

Cell Homogenate Experiments

Guinea pig hearts were treated as previously described up through and including perfusion with Krebs Henseleit solution and fixative (Fixatives in these cases were 1 percent paraformaldehyde 0.25 percent glutaraldehyde and 100 mM potassium cacodylate pH 7.4 (Ernst, 1973). Perfusion of fixatives were continued for 20 minutes at room temperature). Following fixation, the heart was perfused by syringe with 20 ml of 300 mM sucrose. Both ventricles were immersed in sufficient 100 mM Tris buffer, 250 mM sucrose solution, pH 9.0, to make a 13 percent homogenate. The ventricles were then homogenized with a tissumizer until no large pieces of heart could be seen. The homogenate was then more finely homogenized with a motor driven teflon to glass Potter-Elvejehm tissue
homogenizer for fifteen up and down passes. The entire homogenization procedure was carried out on ice and the buffered sucrose media was ice cold at the time of addition. Two tenths of a milliliter of homogenate was diluted to a final volume of 2.0 ml with a final ion concentration of 5 mM magnesium chloride, 10 mM potassium chloride, and 5 mM PNPP. In addition to the above, a second tube contained 10^-2 M ouabain, another contained 10^-2 M cysteine, and the fourth tube contained neither ouabain nor cysteine. Phosphate capture metals were varied according to the experiment at hand as was the pH. Incubation time was constant at 30 minutes and room temperature. The incubation was ended by the addition of 0.2 ml of 10 N sodium hydroxide. The homogenate was then centrifuged in a table top centrifuge for 3 minutes at 2000x gravity and the optical density of the supernatant fraction was measure at 420 mu on a Gilford U.V. spectrophotometer to quantitate para-nitrophenyl concentration.
RESULTS

Para-nitrophenyl Phosphatase Methods

Parallel Heart and Kidney Cytochemistry

In 1974 Firth (Firth, 1974) published an article demonstrating ouabain inhibitable PNPPase activity in distal tubule of rat kidney. It was decided to run parallel experiments in guinea pig heart and kidney, it, hopefully, would also work in heart.

The heart and a kidney from a guinea pig were excised. The heart was posted and briefly perfused with Krebs Henseleit solution which was followed by perfusion in a 3 percent paraformaldehyde cacodylate buffer (pH 7.4) at room temperature for 30 minutes. The kidney was first perfused briefly with Krebs Henseleit solution and then with the 3 percent paraformaldehyde solution by syringe. The kidney cortex was then sliced into thin slices with a razor and superfused in the 3 percent paraformaldehyde solution for 30 minutes. The kidney slices were transferred to 100 mM cacodylate buffer (pH 7.4), 350 mM sucrose solution and washed 3 times for 5 minutes. At the same
time left ventricular tissue was diced and washed 3 times in the same buffered sucrose solution. Tissues were then washed 3 times in 350 mM sucrose buffered to pH 9.0 with Tris·base and embedded in agarose block containing 350 mM sucrose and 100 mM Tris·base pH 9.0. The agarose blocks were sliced with a McIlwain tissue chopper and incubated 30 minutes at room temperature in a new PNPP incubation media. This media, described by Firth, included 10 mM magnesium chloride, 10 mM potassium chloride, 5 mM PNPP, 20 mM strontium chloride and 100 mM Tris·base pH 9.0. Also added, but not mentioned in the Firth article, was 350 mM sucrose. A second media included 10^-2 M ouabain. Firth also used a third media including 10^-3 M tetramisole, an alkaline phosphatase inhibitor. Tetramisole was not available to the author at the time of this experiment; consequently, it was not used. Following incubation, tissue was washed three times in 100 mM Tris buffered, 350 mM sucrose immersion for 5 minutes at room temperature. Cacodylate buffered osmium tetroxide dehydration, embedding and sectioning followed. In kidney, proximal tubules (PT) were identified by heavily staining brush borders in the lumen and distal tubules (DT) by the absence of such staining in the entire lumen.

Figures 1 and 2 are poorly fixed proximal and distal tubules, respectively. Note the equal intensity of stain in both. Cells in Figures 1 and 2 are adjacent and a sum
Figure 1
Phosphatase Localization in Proximal Tubule of Kidney

The kidney was perfused and superfused in 3 percent paraformaldehyde buffered with cacodylate to pH 7.4. This agarose section of kidney was incubated in a media containing 10 mM magnesium chloride, 10 mM potassium chloride, 5 mM PNPP, 20 mM strontium chloride and 100 mM Tris·base pH 9.0. Notice the stain upon the plasma membrane (PM) and infolds of that membrane (16,000x).
Figure 2

Phosphatase Localization in Distal Tubule of Kidney

This tissue was treated as the tissue in Figure 1. Note the stain in essentially the same areas of distal tubule (DT) as that seen in proximal tubule in Figure 1 (16,000x).
of two different cells are shown in these two figures. Neither cell was treated with ouabain. Figures 3 and 4 are proximal tubules and distal tubules, respectively, which have been treated with $10^{-2}$ M ouabain in the incubation media. Notice the absence of heavy precipitate in Figure 4. Figure 5 is an over view of these two cells and is almost identical to Figure 8 in Firth's paper. "Dr. Ernst scanned them (Figures 1-5) and felt that they indeed showed the presence of ATPase in kidney sections". It was judged that a stain had been successfully inhibited in these cells by ouabain. Ouabain sensitivity was found only on outer wall rather than the lumenal wall of distal tubules.

Figure 6 is of left ventricle tissue treated as the kidney was treated. There was no stain in any myocardial tissue.

The Ernst technique has been used successfully to localize a ouabain sensitive stain upon two morphologically similar tissue which have been shown to contain ouabain sensitive Na\(^+\)K\(^+\)Mg\(^++\) ATPase, the kidney (Whittman and Wheeler, 1961) and avian salt gland (Hokin, 1963). In both tissues the stain was localized on invaginated plasma membrane (Ernst, 1972a; Firth, 1974). In this laboratory, parallel experiments with heart and kidney using the Firth

\(^{1}\text{Dr. J.O. O'Neill, personal letter.}\)
This tissue was treated as the tissue in Figure 1 with the exception that 10 mM ouabain was included in the incubation media. Note that the stain apparent in Figure 1 is not abated (16,000x).
Figure 4

Lack of Phosphatase Activity in Ouabain Treated Distal Tubule of Kidney

This tissue was treated as the tissue in Figure 2; except, 10 mM ouabain was included in the incubation media. Note the absence of stain that was present in Figure 2 (16,000x).
This tissue was treated as the tissue in Figures 1 and 2 except that 10 mM ouabain was added to the incubation media. Note the lack of stain in the distal tubules (DT) and the stain in proximal tubules (PT) (4,100x).
Figure 6

Lack of Phosphatase Activity in Myocardium

This left ventricular tissue was treated as the kidney tissue in Figures 1 and 2 (16,000x).
technique demonstrated \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase in kidney but not heart tissue.

**The Effect of Cysteine upon Cardiac Para-nitrophenyl Phosphatase Activity**

When Ernst (1973) published an abstract in the Journal of Cell Biology demonstrating a modified technique for staining kidney, this procedure was attempted in heart. The protocol is as follows. The normal perfusion of Krebs Henseleit solution by perfusion pump in heart was preformed just prior to similar perfusion with 0.25 percent glutaraldehyde and 1 percent paraformaldehyde-cacodylate buffer (pH 7.4) for 10 minutes at room temperature. The left ventricular heart tissue was then excised and diced into 1 mM cubes in warm fixative, then washed twice for 10 minutes each time in cold Tris pH 9.4 with 250 mM sucrose. The wash media also contained \( 10^{-2} \) M ouabain if the tissue was to be incubated in a ouabain containing media which followed the wash step. The incubation media was the same as that used by Firth and the next two experiments also used this same media (The Ernst abstract did not detail the incubation media used). The basic media contained 100 mM Tris-base pH 9.0, 250 mM sucrose, 10 mM potassium chloride, 10 mM magnesium chloride, 10 mM strontium chloride and 5 mM PNPP. There are four different treatments involved at this time and they are as follows. The control group was incubated in the basic
media. The group designated as the cysteine group was incubated in this media plus $10^{-2}$ M cysteine. Those tissues that were washed in $10^{-2}$ M ouabain containing-media were incubated in control media plus $10^{-2}$ M cysteine. This makes a total of four different treatments. The incubation was run at room temperature for one hour. Following incubation the tissues were washed 2 times for five minutes in cold 300 mM sucrose. The tissue was immersed in 2 percent lead nitrate and 300 mM sucrose for one hour. This was followed by a wash in 300 mM sucrose and 1 mM Tiron for 15 minutes at zero to four degrees centigrade (the lead chelate was included to increase the effectiveness of this wash). This was followed by a pH 7.4 cacodylate-sucrose wash and finally a pH 7.4 cacodylate-sucrose wash the next day preceding osmication, dehydration and embedding.

Cysteine was used in the incubation media because Ernst (1973) used $10^{-2}$ M cysteine as an alkaline phosphatase inhibitor. Padykula and Herman (1955) found the sulfhydryl protecting agent 2,3, dimercapto-1-propanol (BAL) inhibited brush border staining in rat kidney when ATP was used as a substrate and BAL was at 5 mM concentration. Padykula and Herman also reported that 5 mM BAL stimulated rat myocardial ATPase activity. Padykula and Herman as well as Skou (1963) found that ATPase activity inhibited by the sulfhydryl binding agent
p-chloromercuribenzoate (PCMB) could be reversed by 5 mM or 10 mM cysteine.

Two more experiments followed with identical protocol except the fixative used was either 1 percent or 2 percent lead nitrate step but did not contain Tiron and the tissue samples were not preincubated in ouabain. Figures 7 and 8 are from tissue fixed in 0.25 percent glutaraldehyde and 1 percent paraformaldehyde and are tissues treated in the basic media with $10^{-2}$ M ouabain or the basic media with $10^{-2}$ cysteine, respectively. The control preparation shows heavy globular precipitates with apparent concentrations upon "T" tubules. Tissue incubated in ouabain containing media is similar to other micrographs of tissue incubated in non-ouabain containing media. Heavy globular precipitates upon "T" tubules and plasma membrane are pronounced. Figure 8 is typical of tissue incubated in $10^{-2}$ M cysteine containing media. Figures 9 and 10 are photographs of tissue fixed in 1 percent glutaraldehyde. Figure 9 had $10^{-2}$ M ouabain in the incubation media; Figure 10 had $10^{-2}$ M cysteine in the incubation media. Myocardium incubated without inhibitors look similar to the tissue in the presence of ouabain (Figure 9). Notice the similarity of stain in tissues previously treated with the same inhibitors (Fig. 7 and 8). The tissue fixed in 1 percent glutaraldehyde had a lateral cisternea (LC) stain in the control and ouabain treated preparation.
Figure 7

Phosphatase Activity in Myocardium
Fixed with One Fourth Percent Glutaraldehyde, One Percent Paraformaldehyde and Ouabain Treated

This myocardium was fixed by perfusion in 0.25 percent glutaraldehyde and 1.0 percent paraformaldehyde. The incubation media included 10 mM magnesium chloride, 10 mM potassium chloride, 5 mM PNPP, 20 mM strontium chloride, 10 mM ouabain and 100 mM Tris base pH 9.0 (16,000x).
This myocardium was treated as the tissue in Figure 7; except, 10 mM cysteine was included in the incubation media and ouabain was not included in the incubation media (16,000x).
Figure 9
Phosphatase Activity in Myocardium
Fixed in One Percent Glutaraldehyde
and Treated with Ouabain

The myocardium was fixed by perfusion with 1 percent glutaraldehyde for 10 minutes. The incubation media included 10 mM magnesium chloride, 10 mM potassium chloride, 5 mM PNPP, 20 mM strontium chloride and 100 mM Tris-base pH 9.0. Note an apparent stain upon T-tubules (T-T) and plasma membrane (PM) (16,000x).
Figure 10
Phosphatase Activity in Myocardium
Fixed in One Percent Glutaraldehyde
and Treated with Cysteine

The myocardium was treated as the tissue in Figure 9 except that 10 mM cysteine was included in the incubation media and ouabain was excluded. Note the diminution of stain in T-tubules (T-T) and plasma membrane (PM) (16,000x).
(absent from cysteine preparations) not found in the 1 percent paraformaldehyde-0.25 percent glutaraldehyde preparation. Tissue fixed in 2 percent glutaraldehyde demonstrated similar staining (less intensity) and better tissue preservation than tissue incubated in 1 percent glutaraldehyde.

It is interesting to note that in similar experiments where 0.1 percent, 0.5 percent, or 0.75 percent glutaraldehyde or 1.0 percent, 2.0 percent or 3.0 percent paraformaldehyde or 0.75 percent paraformaldehyde and 0.1 percent glutaraldehyde was used as a perfusion fixative for 20 minutes at zero degrees centigrade, it was determined that either 0.75 percent glutaraldehyde, 1.0 percent paraformaldehyde or 0.75 percent paraformaldehyde plus 0.1 percent glutaraldehyde gave minimal fixation. minimal fixation for purposes of these experiments was defined as that fixation adequate to preserve some Golgi apparatus and plasma membrane.

The incubation media used in the next experiments contained one half the strontium (10 mM strontium chloride) as in the experiments represented by Figures 1 through 10. Decreasing the strontium concentration should do two things: (1) increase ouabain inhibitable PNPPase activity and (2) decrease the strontium trapping efficiency. Apparently 10 mM strontium chloride is not sufficiently high to efficiently precipitate the phosphate liberated by
cardiac contractile tissue. However, at 10 mM as well as 20 mM strontium chloride concentrations, capillary pinocytotic vesicles stained very heavily which was not inhibitable with ouabain. Figure 11 is an example of such staining. Using the Wachstein-Weisel media, Marchesi and Barrnett (1963) demonstrated a (Mg\(^{++}\) ATP) dependent stain in rat heart capillaries which is identical to that staining seen in Figure 11. Similar capillary pinocytotic (Mg\(^{++}\) ATP)-dependent staining has been reported in rat lung (Marchesi and Barrnett, 1964) and rat brain (Torach and Barrnett, 1964).

**In Vivo Ouabain Pretreatment**

When the author's minimal fixation or 0.75 percent paraformaldehyde 0.1 percent glutaraldehyde was used at room temperature for 20 minutes in tissue treated in vivo to 300 \(\mu g/kg\) ouabain, morphological and cytochemical changes were noted. Ouabain was injected one hour before sacrifice in order that the hypothesized ouabain stimulation of Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase might increase Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase activity upon specific epicytotic sites. This concentration of enzyme should facilitate cytochemical staining.

As predicted cytochemical localization of precipitate was found upon the epicytotic structures described by Lindower and Marks (Lindower and Marks, 1976) as can be seen upon "T" tubules in Figure 12 and plasma membrane in
Figure 11

Pinocytotic Vesicular Staining in Capillaries

This stain is an example of the pinocytotic vesicular stain common to cardiac capillaries. Although this micrograph is of a capillary incubated with PNPP and strontium, this type of ouabain resistant stain was common to lead ATP incubations as well (16,000x).
One hour prior to sacrifice, the guinea pig was injected with 300 μg/kg ouabain. The fixative, 0.75 percent paraformaldehyde and 0.1 percent glutaraldehyde was perfused 20 minutes, then superfused for five minutes. The incubation media included 5 mM PNPP, 5 mM potassium chloride, 5 mM magnesium chloride, 10 mM strontium chloride and 100 mM Tris·base pH 9.0. Note the heavier stain which appears to be pinocytotic or epicytotic budding vesicles (V). Lateral cisternae (LC) and T-tubules (T-T) may be more heavily stained as well (16,000x).
Figure 13. These epicytotic structures were not apparent in tissue which had not been treated in vivo to ouabain. Pinocytotic or epicytotic vesicles staining on cardiac muscles has not been reported in the literature. However, Bartoszewicz and Barrnett demonstrated similar stain in urinary bladder smooth muscle of toad (Bufo marinus) (Bartoszewicz and Barrnett, 1964). Lateral cisternae (LC) staining is also apparent in Figure 12.

There was no noticeable loss of stain when 1 mM ouabain was included in the incubation media. Apparently in vivo administration of ouabain, indeed, did cause the lead deposition, its increased concentration or localized activation of adenosine triphosphatase on the plasma membrane and "T" tubule.

**Phosphatases in Whole Heart Homogenates**

Though homogenates are considerably different from intact heart tissue, it was decided, at this point, to measure Na\(^+\), K\(^+\), Mg\(^{++}\)-ATPase activity in whole heart homogenates using the Ernst incubation media. The incubation media was to include either no inhibitor, 10\(^{-2}\) M cysteine, 10\(^{-2}\) M ouabain or 10\(^{-2}\) M cysteine plus 10\(^{-2}\) M ouabain.

Hearts were excised and the aorta was cannulated. The following solutions were back perfused through the heart at room temperature. First, a balanced bicarbonate buffered salt solution with heparin was perfused for 5 to
Figure 13

Phosphatase Activity in Plasma Membrane
Induced by One Hour *In Vivo* Ouabain Pretreatment

Tissue treated as that in Figure 12 shows the same type of vesicular stain (V) upon plasma membrane (PM). The plasma membrane also is well stained. Note that one cell demonstrates what appears to be an artifactual stain in contractile elements when the adjacent cell does not (16,000x).
10 minutes. Second, 1 percent paraformaldehyde, 0.25 percent glutaraldehyde in 100 mM cacodylate buffer pH 7.4 was perfused for 10 minutes and third, 20 ml of 300 mM sucrose was perfused for 3 minutes. Hearts were removed from the perfusion apparatus and homogenized in 100 mM Tris-base pH 9.0 with a tissumizer and teflon to glass Potter-Elvejehm tissue homogenizer. Two tenths of the 13 percent homogenate was then introduced into a 12 x 75 mm test tube containing basic media minus strontium or 1.8 ml of 10 mM potassium chloride, 5 mM magnesium chloride, 5 mM PNPP, 100 mM Tris-base pH 9.0 plus or minus ouabain, plus or minus cysteine. At 30 minutes the tubes were centrifuges for 3 minutes in a table top centrifuge at 2,000x gravity. Final clarification of the solutions was achieved by addition of 0.2 ml of 10 N sodium hydroxide followed by centrifugation for 3 minutes. Parallel experiments with basic media plus 10 mM strontium chloride were also run.

Para-nitrophenyl phosphatase activity was measured by absorption at 420 μm in a Gilford Spectrophotometer. Samples were read against unincubated complete media.

The results of the experiment are summarized in Table I. Although there is high variability in the ability of the hearts to metabolize PNPP, one can learn from these data that there is some ouabain sensitive activity in preparations incubated without strontium. This ouabain sensitive activity seems to be masked by high background
Table 1

The Effect of Ouabain and 10^{-2} M Cysteine upon P-nitrophenylphosphatase Activity of Aldehyde-Fixed Whole Heart Homogenates

<table>
<thead>
<tr>
<th>Inhibitors Used (10^{-2} M)</th>
<th>Optical Density at 240 mu*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strontium Absent</td>
</tr>
<tr>
<td>None</td>
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</tr>
<tr>
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<td>Cysteine</td>
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<td>Ouabain + Cysteine</td>
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<tr>
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<td>Ouabain</td>
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<tr>
<td>Cysteine</td>
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<tr>
<td>Cysteine</td>
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</tr>
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<tr>
<td>Cysteine</td>
<td>.228</td>
</tr>
<tr>
<td>Ouabain + Cysteine</td>
<td>.152</td>
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* Values given are the average of two optical density readings at 240 mu. The incubation conditions are described in the text.
PNPPase activity and unless $10^{-2}$ cysteine is included in the incubation media, ouabain sensitive PNPPase activity is not apparent.

It was decided to determine whether high cysteine concentrations were inhibiting ouabain sensitive PNPPase as well as background PNPPase activity. For this experiment $10^{-3}$ M cysteine was used. The results in Table 2 indicate that $10^{-3}$ M cysteine is not a sufficiently high concentration to inhibit background PNPPase activity. Decreasing the cysteine concentration apparently only raised background activity without increasing ouabain sensitive activity.

Since ouabain inhibitable activity that survived fixation was totally inhibited by strontium, other capture ions were considered. The ions considered all formed insoluble phosphate salts. These ions were aluminum, ammonium molybdate, bismuth, nickel, thorium, tin, and uranyl. Criteria for use of the ion in a cytochemical experiment included, solubility of the ion in a complete media including potassium chloride, magnesium chloride and PNPP within the pH range of 7.0 to 10.0, and insolubility in 5 mM phosphate in that same pH range. Five millimolar solutions of chloride salts of the above mentioned ions and 5 mM PNPP, 10 mM potassium chloride and 5 mM magnesium chloride were prepared and titrated to pH 1.0 with 1 N hydrochloric acid. The final volume was
Table 2

The Effect of Ouabain and $10^{-3}$ M Cysteine upon P-nitrophenylphosphatase Activity of Aldehyde-Fixed Whole Heart Homogenates

<table>
<thead>
<tr>
<th>Inhibitors Used</th>
<th>Optical Density at 240 μm*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Strontium Absent</td>
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<td>None</td>
<td>.716</td>
</tr>
<tr>
<td>$10^{-2}$ M Ouabain</td>
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<tr>
<td>$10^{-3}$ M Cysteine</td>
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</tr>
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<tr>
<td>$10^{-3}$ M Cysteine</td>
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<tr>
<td>$10^{-2}$ M Ouabain + $10^{-3}$ M Cysteine</td>
<td>.747</td>
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* Values given are the average of two optical density readings at 240 μm. The incubation conditions are described in the text.
within one half a ml of 20 ml. The solution was then slowly back-titrated with stirring to pH 10 with freshly diluted (from 10 N NaOH) 1 N sodium hydroxide. The only ion that fulfilled the criteria of solubility in the pH range of 7.0 to 10.0 was aluminum. This ion also demonstrated phosphate (5 mM aluminum and 5 mM phosphate) insolubility in the pH range of 3.0 to 10.0.

The aluminum ion was tested for its inhibitory effect upon PNPPase activity. As can be seen in Table 3, PNPPase activity in the presence of Al\(^{++}\) ions was similar to that in Table 1 in the absence of strontium.

**Cytochemistry Using Aluminum as a Capture Agent**

Next, a heart was prepared for cytochemical examination. The aorta was cannulated and back perfused with Krebs Henseleit followed by 0.25 percent glutaraldehyde and 1 percent paraformaldehyde at room temperature for 10 minutes. The tissue was diced into 1 mM cubes in fixative and incubated in 5 mM aluminum chloride, 5 mM PNPP, 5 mM magnesium chloride, 10 mM potassium chloride and 100 mM Tris·base pH 9.0. This was followed by a pH 9.0 wash in 100 mM Tris·base and 250 sucrose. Since aluminum is not particularly electron dense and becomes soluble at low pH's, as does strontium, this wash was followed by a 2 percent lead-250 mM sucrose replacement solution for one hour at zero to four degrees centigrade. The
Table 3

The Effect of Ouabain and Cysteine upon
P-nitrophenylphosphatase Activity of
Aldehyde-Fixed Whole Heart
Homogenates Incubated with 5 mM Aluminum

<table>
<thead>
<tr>
<th>Inhibitors Used (10^-2 M)</th>
<th>Optical Density at 240 ( \text{mu}^* )</th>
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<td>Cysteine</td>
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<tr>
<td>Ouabain + Cysteine</td>
<td>.265</td>
</tr>
<tr>
<td></td>
<td>.601</td>
</tr>
<tr>
<td></td>
<td>.664</td>
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* Values given are the average of two optical density readings at 240 \( \text{mu} \). The incubation conditions are described in the text.
replacement reaction was followed by a 250 mM sucrose wash with 3 mM Tiron to chelate any lead not precipitated in the replacement reaction. Post fixation was in 5 percent glutaraldehyde in collidine pH 7.4 overnight; osmication, dehydration and embedding followed.

Although cysteine treated, the type of reaction found in all specimens regardless of inhibitor included in the incubation media is seen in Figure 14. The stain rather than being in plasma membrane, lateral cisternae or "T" tubules, is found in what appears to be soluble cytoplasm and sarcotubular reticulum.

**Lead-Adenosine Triphosphatase Methods**

**The Traditional Wachstein and Meisel Approach**

The first lead phosphate approach discussed here was variation one as described in the Methods section. The guinea pig heart was perfused as described in the Methods section, first with Krebs Henseleit solution for 10 minutes and then with 50 ml of 2 percent glutaraldehyde in 100 mM sodium cacodylate, followed by a 15 minute perfusion of the same glutaraldehyde at an 8 ml/minute flow rate. Tissue from better perfused areas of the ventral wall of the left ventricle was excised and minced into approximately 1 mM cubes. The tissue while being minced was in a 100 mM sodium cacodylate buffer pH 7.4 with calcium-free 200 mM sucrose. The tissue was then incubated 30 minutes
Figure 14
Lack of Entrapment Using Aluminum as a Capture Agent

This tissue was fixed with 0.25 percent glutaraldehyde and 1 percent paraformaldehyde for 10 minutes. The incubation media included 5 mM aluminum chloride, 5 mM PNPP, 5 mM magnesium chloride, 10 mM potassium chloride, 100 mM Tris·base pH 9.0 and 10 mM cysteine. Notice that the stain density upon the sarcotubular system (ST), possibly mitochondrial cristae (C) and in some cases lateral cisternea (LC) (16,000x).
at room temperature in a 100 mM sodium cacodylate buffered pH 7.4 incubation solution containing 100 mM sodium as sodium cacodylate and disodium ATP, 30 mM potassium as potassium chloride, 10 mM magnesium as magnesium chloride, 3 mM lead as lead acetate, 6 mM ATP. Following incubation, the tissue was treated for one hour with 6 percent glutaraldehyde buffered with 100 mM sodium cacodylate; then the tissue was processed through osmium, dehydration, and fixation as described in the Methods section. The pre-fixation procedure with 2 percent glutaraldehyde was taken from that of Schulze and Wollenberger (1967). The composition of the incubation media was influenced by the work of Skou (1960) and the Rosenthal group (see Discussion section). Sodium and potassium levels were influenced by Skou while ATP and lead levels were calculated to be nonenzymatic hydrolysis free (Rosenthal et al., 1966) and unfortunately, at a 50 percent trapping efficiency (Rosenthal et al., 1969a). Chloride salts were used to facilitate lead precipitation in concurrence with Berg et al. (1972). A parallel experiment was run in which the incubation media contained an additional $10^{-3}$ M ouabain.

Photographs were taken from heavily staining areas of the tissue block. The state of tissue preservation was disappointing as other tissue in earlier experiments treated the same way yielded much better preservation. Figure 15 demonstrates a high level of stain in the
Figure 15

Adenosine Triphosphatase Localization in Myocardium Using a Modified Wachstein-Meisel Media and Two Percent Glutaraldehyde Fixation

The left ventricle was fixed with 2 percent glutaraldehyde. Tissue blocks were incubated at pH 7.4 in 100 mM sodium cacodylate, 6 mM disodium ATP, 10 mM magnesium chloride, 3 mM lead acetate and 30 mM potassium chloride. This tissue shows heavy stains upon the nucleus (Nu), lateral cisternae (LC) and endothelium (Endo) (16,000x).
nucleus (Nu), lateral cisternae (LC), and endothelium (Endo). Random lead deposits preclude determination of whether the "T" tubules (T-T) and plasma membrane (PM) are specifically stained. Poor preservation disallows any determination of Golgi stain. Very similar results to these have been demonstrated by Eisner et al. (1965) and Rostgaard and Behnke (1965) in rat heart and Sommer and Spach (1964) in dog heart. In Essner et al. and Rostgaard and Behnke experiments, where lead exceeded ATP concentration, the scattered nonspecific stain was not apparent. In the Sommer and Spach experiment where ATP concentrations exceeded lead concentration, the nonspecific stain is apparent. Sommer and Spach interpret this as myofilament ATPase. Other stains mentioned are identified in all three articles as being either Mg++ ATPase or Na+Mg++ ATPase (except the nuclear stain which is generally considered to be artifactual, Goldfischer et al., 1964). Sommer and Spach preperfused toxic concentrations of ouabain into their dogs and were unable to inhibit these stains. Electron micrographs of myocardium incubated in incubation media including 10^{-3} M ouabain have the same staining pattern as non-ouabain incubated tissue in Figure 15. Since no difference can be seen between ouabain and ouabain-excluded treatment, it was impossible to determine if there was any Na^{+}K^{+}Mg^{++} ATPase staining. This experiment, though it did not demonstrate the desired
ouabain inhibition of Na⁺K⁺Mg⁺⁺ ATPase, may be considered to be a confirmation of technique as it reproduces the findings of three different groups of investigators working in cardiac ventricular tissue.

The Traditional Approach Substituting the Ernst Incubation Media for the Wachstein-Meisel Incubation Media

When this experiment was repeated using a PNPP incubation media including 10 mM strontium chloride, 5 mM PNPP, 5 mM potassium chloride, 5 mM magnesium chloride, 250 mM sucrose and 100 mM Tris base pH 9.0, copious stain was found upon lateral cisternae (LC) (Figure 16). Note that the stain is not seen in sarcotubules (ST). Very little, if any stain, was seen upon plasma membrane (PM) or intercalated disc (ID). No ouabain inhibition was demonstrated.

Perfusing a Lead-Adenosine Triphosphate Media into Unfixed Myocardium

The next approach to be discussed is variation three. The rationale behind this approach was to preserve Na⁺K⁺Mg⁺⁺ ATPase activity throughout the incubation by not fixing the tissue until after the incubation was completed. Several investigators have found both glutaraldehyde and formaldehyde to be highly inhibitory to Na⁺K⁺Mg⁺⁺ ATPase (see Introduction section). The heart was first perfused with Krebs Henseleit solution for 10 minutes. Next, the heart was perfused with incubation
Figure 16
Phosphatase Localization in Myocardium
Using an Ernst Media and Two Percent
Glutaraldehyde Fixation

The myocardium was perfused with 2 percent glutaraldehyde. The incubation media which was superfused upon 1 mm cubes of fixed left ventricle, included 5 mM potassium chloride, 5 mM magnesium chloride, 250 mM sucrose, 5 mM PNPP, 10 mM strontium chloride and 100 mM Tris-base pH 9.0. Note the stain upon lateral cisternae (LC) stops where lateral cisternae becomes sarcotubule (ST) (arrow) (16,000x).
solution containing 150 mM sodium chloride, 10 mM potassium chloride, 3 mM magnesium chloride, 3 mM ATP, 2 mM lead acetate, and 100 mM imidazole buffer, pH 7.4. The high sodium level was used to simulate Krebs Henseleit solution concentrations. Potassium was high to stimulate depolarization. Magnesium was to be equimolar with ATP (Sou, 1967). The ATP concentration was kept reasonably low because it would chelate the lead and it was not clear whether or not it would diffuse into a healthy membrane. At this concentration of ATP, only 2 mM lead was necessary to achieve 0.5 mM free lead (Tice, 1969) (because of ATP-lead chelation). Imidazole buffer was used because Skou had reported that it increased $\text{Na}^+\text{K}^+\text{Mg}^{++}$ ATPase activity (Skou, 1967). Other constituents of Krebs Henseleit solution were discarded because they would precipitate the lead. The incubation solution had to be centrifuged at a 10,000 $g$ for 15 minutes to remove the precipitate that resulted when lead was added to the incubation solution.

It was hoped that endogenous ATP would be sufficient to achieve good staining if exogenous ATP did not diffuse into the cells.

Perfusion of 250 ml of incubation solution for 30 minutes at room temperature at an 8 ml per minute rate was followed by the usual 2 percent glutaraldehyde and cacodylate buffered fixative.
The results are exemplified in Figure 17. The tissue is well preserved but, no identifiable stain is apparent in the contractile cells.

Later trials included 2, 4, and 6 percent DMSO in the incubation media. At 2.5 percent concentration, DMSO has been shown to increase up to 100 times the permeability of frog skin to such agents as sucrose and mannitol (Franz and VanBruggen, 1967). It was hoped that if the difficulty in achieving a cytochemical stain was due to poor diffusion of the ATP or lead that DMSO might aid the diffusion. Even low concentrations of glutaraldehyde (0.4 percent) were used to disrupt membrane integrity. The only result of these manipulations was increasingly poorer tissue preservation.

At this point in the research it seemed more advisable to pursue other more promising approaches to Na\(^+\)K\(^+\)Mg\(^++\) ATPase cytochemistry.

The Lead-Tiron Method of Schulze and Wollenberger

Schulze et al. (1972) published an article containing details about a new lead-based incubation media for Na\(^+\)K\(^+\)Mg\(^++\) ATPase. Novel to this media was the inclusion of a lead chelating agent 4,5-dihydroxy-m-benzenedisulfonic acid or Tiron. Tiron was supposed to weakly chelate lead maintaining free lead levels below 0.1 mM and also by its dissociable binding nature, it would act as a lead buffer
The myocardium was perfused prior to fixation with 150 mM sodium chloride, 10 mM potassium chloride, 3 mM magnesium chloride, 3 mM disodium ATP, 2 mM lead acetate and 100 mM imidazole buffer pH 7.4. There is no recognizable stain (16,000x).
replacing precipitated lead.

A major disadvantage to Schulze's 1972 paper was its brevity. While more details on this study were being sought through inter-library loan, it was decided to try some ideas of our own. The first idea was to use this incubation media in variation 3 experiments (as described in the Methods).

Perfusion of Unfixed Heart with a Lead-Tiron Incubation Media, the Sequential Addition of Incubation Media Constituents

The heart was first perfused with Krebs Henseleit balanced salt solution at room temperature. After most of the blood was washed out (when the spent perfusate became a pale pink color after approximately 10 minutes of perfusion), the heart was then perfused with a buffered solution containing 135 mM sodium chloride, 5.6 mM potassium chloride, 2 mM ATP, 2 mM magnesium chloride, and 100 mM s-collidine buffer pH 7.4. After 5 minutes of perfusion at room temperature with this solution, a second solution identical to the first was perfused except it contained 1 mM lead acetate and 3 mM Tiron. This second solution was also perfused for 5 minutes.

The first of these two collidine buffered solutions was used to wash out calcium, phosphate, carbonate, and other constituents of Krebs Henseleit solution which might precipitate lead or phosphate. Upon perfusion of this
solution the heart immediately stopped beating. This was expected as a result of the lack of calcium, requisite for muscle contraction. It is known however (Marshall, 1968) that electrical activity continues after mechanical activity stops as a result of low calcium.

When the second collidine solution was perfused with the lead entrapping agent and Tiron chelate, the Na\(^+\)K\(^+\)Mg\(^++\) ATPase should have been functioning at or near normal tissue levels since the tissue had neither been fixed nor had the lead-Tiron combination detectably inhibited Na\(^+\)K\(^+\)Mg\(^++\) ATPase from guinea pig heart microsomes (Schulze et al., 1972).

A collidine buffered 1 percent paraformaldehyde solution (pH 7.4) followed. This was perfused by syringe at 25 ml per minute flow rate for 2 minutes followed by 5 minutes of perfusion at a rate of 7 ml per minute. Six percent glutaraldehyde was then perfused for 10 minutes at 0-4 degrees centigrade. The tissue taken for microscopy was then excised and diced in 6 percent glutaraldehyde and superfused for 1 hour in fresh 6 percent glutaraldehyde. Normal osmication, dehydration, and embedding followed.

The resultant fixation was poor. There was no evidence of glycogen particles or ribosomes remaining in the cells. The mitochondria were in many cases ruptured or partially devoid of cristae. Golgi apparatus was missing from all cells examined. Plasma membrane, nuclear
membrane (both layers), "T" tubules and myofibrils were the only structures relatively well preserved. Most disappointing of all was the lack of anything resembling lead phosphate entrapment.

As stated in the Introduction, the three major goals of this project were (1) preserving the tissue adequately, such that Golgi apparatus, plasma membranes and "T" tubules would be intact; (2) staining a specific phosphatase, and (3) being able to inhibit that phosphatase stain with ouabain (Glynn, 1957; Skou, 1957) and being able to see this inhibition clearly. None of these goals had been achieved in this experiment and consequently a new approach was tried.

It was determined at this point to work primarily upon the first goal: adequate tissue preservation under cytochemical conditions.

**Perfusing Fixed Heart with a Lead-Tiron Incubation Media**

Schulze and Wollenberger (1967) reported success in staining Na⁺K⁺Mg++ ATPase using 2 percent glutaraldehyde prefixation followed by superfusion in a Tiron-lead incubation media.

The next two experiments were, therefore, of variation 1 (see Methods). Two percent glutaraldehyde buffered in collidine was perfused following the ever present Krebs Henseleit solution wash of the heart. A
fifteen minute perfusion with the incubation media fol-
lowed. In the first experiment lead and Tiron levels
were 2.0 mM and 10 mM; in the second experiment, they
were 3.0 mM and 30 mM respectively. The rest of the incu-
bation media remained the same. Six percent glutaral-
dehyde perfusion and superfusion followed as in prior
experiments. All steps were carried out at 0-4 degrees
centigrade.

There was no recognizable stain. In some cases
tissue was not osmicated in order not to add any electron
dense material other than that from the incubation lead.
The complete lack of electron density in these non-osmi-
cated sections made photography extremely difficult; as
there was so little contrast, focusing was by trial and
mostly error. Surprisingly, the structure was still poor.
Mitochondria (M) were improved, but still poorly preserved.
The nuclear pole (NP) was still washed out.

The buffer was changed in both the incubation and
fixation media to cacodylic acid and 2 percent formal-
dehyde was added to the final 6 percent glutaraldehyde
fixation solution. This resulted in significant improve-
ment (Figure 18). Mitochondria (M), Golgi apparatus (GA),
plasma membrane (PM), "T" tubules (T-T), nuclei (Nu), and
nuclear pole (NuP) were all in relatively good shape.
There was a smudgy appearance about the myofibrils. It was
suggested that formaldehyde was responsible for this.
Cacodylate buffered 2 percent glutaraldehyde was perfused through the myocardium prior to the perfusion of the incubation media. Two percent paraformaldehyde was included in the 6 percent glutaraldehyde post fix. The incubation media included 135 mM sodium chloride, 5.6 mM potassium chloride, 2 mM ATP, 2 mM magnesium chloride, 100 mM s-collidine, 3 mM lead and 30 mM Tiron. The mitochondria (M), Golgi apparatus (GA), plasma membrane (PM), "T" tubule (T-T), and nuclei (N) are all well preserved (16,000x).
When formaldehyde was removed from the 6 percent glutaraldehyde solution during the next experiment, the smudge also disappeared.

Now that the first goal had been achieved, it was decided we could expend structural integrity in order to achieve a stain.

The first change along this line of thought was to increase the incubation temperature to room temperature. Prefixation temperature was also increased to room temperature. Tiron and lead concentration were returned to 10 mM and 2 mM respectively. As the result of not taking into account the sodium hydroxide used to titrate caco-dylic acid to pH 7.4, sodium levels were extremely high (approximately 250 mM). The resultant lack of stain was somewhat offset by the remarkable increase in structural quality of the tissue (Fig. 19). The conclusion that increasing the prefixation temperature to room temperature favors better fixation had earlier been reported in the literature (Palay et al., 1962). According to Palay, room temperature perfusion increases vasodilation and fixative diffusion rates.

**Perfusion of 2 Percent Glutaraldehyde Fixed Heart with a Lead-Tiron Incubation Media, the Sequential Addition of Incubation Media Constituents**

Skou (1965) described the interaction of Na\(^+\)-Mg\(^{++}\)-K\(^+\) and ATP upon the Na\(^+\)K\(^+\)Mg\(^{++}\) ATPase this way. Sodium and
Figure 19

The Lack of Adenosine Triphosphatase Activity
When Lead-Adenosine Triphosphatase Media Was
Perfused Following Cacodylate Buffered Two Percent
Glutaraldehyde Prefix, Two Percent Paraformaldehyde
Was Deleted from the Six Percent Glutaraldehyde Post Fix,
Resulting in the Loss of Myofibrillar Smudge

The myocardium was treated as the tissue in Figure
18; except, the 2 percent paraformaldehyde was deleted
from the post fixation media and preperfusion of 2 per­
cent glutaraldehyde, as well as the perfusion of incuba­
tion media, was with room temperature perfusates. The
mitochondria (M), Golgi apparatus (GA), plasma membrane
(PM), "T" tubule (T-T), and nuclei (N) are all well pre­
served (16,000x).
magnesium are required for ATP hydrolysis and upon hydrolysis, the inorganic phosphate binds the enzyme and potassium is required to liberate the phosphate from the enzyme. Incidentally, potassium and cardiac glycosides apparently compete at the potassium site. The glycosides do not release phosphate.

It was decided to try to take advantage of this arrangement to improve upon the cytochemical aspects of these experiments. The rationale was as follows. If lead and Tiron are the inhibitory factors upon Na⁺K⁺Mg⁺⁺ ATPase, then ATP hydrolysis should be facilitated in their absence. And since Mg⁺⁺ Na⁺ are the only other constituents necessary for ATP hydrolysis, it would be advantageous to incubate the tissue in ATP, Mg⁺⁺, and, Na⁺ prior to the addition of Pb⁺⁺ and Tiron. The lead and Tiron could be followed by K⁺ which would release all of the phosphate from the enzyme simultaneously and facilitate lead phosphate nucleation.

After perfusion of the heart with Krebs Henseleit solution, a 2 percent glutaraldehyde cacodylate buffered solution was perfused at room temperature for 15 minutes. A solution containing 100 mM sodium, 3 mM ATP, and 10 mM magnesium chloride was perfused next for 10 minutes at 0-4 degrees centigrade. Lead and Tiron were added to the incubation solution (with stirring) such that final concentrations would be 3.0 mM and 10 mM respectively. This
solution was perfused for 6 minutes before potassium was added (with stirring) to a final concentration of 30 mM. The high potassium concentration was to facilitate rapid diffusion. The remainder of the procedure was the routine 6 percent glutaraldehyde post fix, osmium tetroxide, etc. The resultant lack of membrane staining but excellent tissue preservation was nearly identical to the results of the previous experiment (Fig. 19).

The Lack of Membrane Adenosine Triphosphatase Activity When DOC Was Used to Increase Permeability in Fixed Heart

Auditore (1962), Duel and McIlwain (1961), Jarnefelt (1964), and Jorgensen and Skou (1969) using, respectively, rabbit ventricle, guinea pig cerebrum, rat cerebrum, and rabbit kidney microsomes, demonstrated that pretreatment with desoxycholate (DOC) in concentrations ranging from 0.1 percent to 1 percent caused a change in the specific activity of membrane ATPases. The DOC caused a 2 to 5 fold increase in Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase activity. Rostgaard and Moller (1969) reported that these concentrations of DOC caused the microsomes to open and concluded that the increase in Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase activity was due to increases in availability of substrate to enzyme ("uncovering latent sites").

It was decided in light of these results that it might be advantageous to include 0.1 percent DOC into our incubation media. If, in fact, DOC did work to stimulate
Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase in microsomes, it might also increase the activity in whole cells by the same mechanism or by possibly increasing capillary permeability. Any agent that would decrease the activity of nonspecific ATPase was also welcome. The concentration of 0.1 percent DOC was selected because it was the lowest effective concentration in the before mentioned studies; as, there was concern that DOC might solubilize cell components.

Marchesi and Palade (1967) reported that decreasing the duration of fixation time increase Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase activity. Since the ultra structure of the last experiments was more than adequate for the stated goals, it was decided to decrease the fixation time to five minutes.

After Krebs Henseleit solution and a 5 minute room temperature perfusion of 2 percent glutaraldehyde in cacodylate, the heart was perfused with the incubation media for 20 minutes at 0-4 degrees centigrade. The media was the same as the final media in the last experiment except that 0.1 percent DOC was included. The result was poorer structure and absolutely no stain.

The Use of Distilled Glutaraldehyde as a Prefixative

Somogyi (Somogyi et al., 1971) reported successful Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase localization using the Schulze and Wollenberger technique and fixing with 4 percent vacuum distilled glutaraldehyde. Somogyi's preparation was
sensitive to $10^{-5}$ M ouabain, 2 mM calcium, or potassium withdrawal.

Anderson (1967) reported that distilled glutaraldehyde left more enzymatic activity in fixed tissue than charcoal washed glutaraldehyde ($\text{Na}^+\text{K}^+\text{Mg}^{++}$ ATPase was not one of the enzymes tested in Anderson's articles). It seems likely that distilled glutaraldehyde would denature fewer enzymes since Richards and Knowles (1968) reported that the most reactive protein cross-linking forms of glutaraldehyde are the glutaraldehyde polymers. Since Robertson and Schultz (1970) reported that the impurity removed by distillation (the ultraviolet 2350 Å peak) is a dimer of glutaraldehyde, it would be more specific for certain protein configurations. It would also seem probable that fixation might be poorer since some protein configuration cross-linked by dimers may not be cross-linked by a monomer.

Glutaraldehyde was distilled (see Appendix section) and diluted to 2 percent in cacodylate buffer and perfused for 10 minutes at room temperature. Prior to perfusion of the heart with incubation media plus 0.01 percent DOC, the right ventricle was removed, diced, and, incubated according to the method of Somogyi in incubation media plus or minus DOC. The basic incubation medium was the same as described earlier and was not altered until cryostatic sections were used. This medium consists of 100 mM
cacodylic acid titrated to pH 7.4 with 10 M sodium hydroxide, 10 mM MgCl₂, 10 mM Tiron, 3 mM lead acetate, 30 mM potassium chloride, and 6 mM ATP. Sodium concentrations were calculated to be 100-110 mM. The source of the sodium was disodium ATP, disodium Tiron, and sodium cacodylate.

Figures 20 and 21 are examples of the kind of membrane stain found in both superfused or perfused tissue. This type of stain was apparent whether or not 10⁻³ M ouabain was included in the incubation medium. This stain was not seen when Tiron and lead were deleted from the incubation media. It was also abolished when ATP was deleted or 0.01 percent DOC was included in the incubation medium. It was concluded that this did not represent Na⁺K⁺Mg++ ATPase but, most likely, was the Mg++ ATPase which Auditore (1962) found to be inactivated by DOC in rabbit ventricle (Inhibited must be loosely interpreted; the enzyme might be solubilized by DOC.).

Fixed Frozen Sections

Dunham and Glynn (1961) found in human erythrocyte ghost preparations that freezing and thawing increased Na⁺K⁺Mg++ ATPase by up to 5 times. Rostgaard and Moller (1971) reported that freezing and thawing opened microsomes and increased Na⁺K⁺Mg++ ATPase activity by the same mechanism as DOC. Schulze and Wollenberger (1971a) used
Figure 20

Plasma Membrane Adenosine Triphosphatase Stain
Seen When Two Percent Distilled Glutaraldehyde
Was Used as a Prefixative and a Lead-Tiron
Incubation Media Was Used

The myocardium was perfused for 10 minutes in 2 percent distilled glutaraldehyde. One millimeter cubes from the right ventricle were immersed in incubation media including 100 mM sodium cacodylate, 30 mM potassium chloride, 10 mM magnesium chloride, 10 mM Tiron, 3 mM lead and 6 mM disodium ATP. Note the large crystals indicating slow crystal growth (16,000x).
Figure 21

Transverse Tubular Adenosine Triphosphatase Stain
Seen When Two Percent Distilled Glutaraldehyde
Was Used as a Prefixative and a Lead-Tiron
Incubation Media Was Used

This tissue was perfused with the same medium as
the tissue in Figure 20 (16,000×).
cryostatic section when localizing Na⁺K⁺Mg++ ATPase in cardiac muscle. It was decided to copy their medium for use in cryostatic sectioning. The medium included sodium (100-110 mM), potassium (10 mM), ATP (3 mM), lead (2 mM), Tiron (30 mM), and cacodylate buffer (100 mM), pH 7.2-7.4. Incubation time was 40 minutes at room temperature. Basically, the procedure was variation 4 (see Methods). Distilled glutaraldehyde was used in two experiments, but it yielded extremely poor fixation. Apparently distilled glutaraldehyde was unable to maintain fixation throughout freezing, thawing, and cytochemical incubation. Later, charcoal treated 2 percent glutaraldehyde with 100 mM cacodylate buffer (pH 7.4) was perfused through guinea pig heart for 10 minutes at room temperature. The fixative was washed out of the heart for 20 minutes at room temperature with 100 mM cacodylate buffer (pH 7.4) containing calcium-free sucrose (200 mM) for osmolarity purposes. Excised left ventricle specimens were frozen in liquid nitrogen. Tissue was stored frozen from 1 to 3 days.

Skou (1962), Schwartz (1962, 1963), and Hokin and Reasa (1964) had demonstrated increases in specific activity of Na⁺K⁺Mg++ ATPase by aging frozen microsomes in rabbit brain, rat heart, rat liver, and human erythrocyte ghosts, respectively. Skou and Schwartz attributed the increase in specific activity to loss of nonspecific phosphatases with minimal loss of Na⁺K⁺Mg++ ATPase
activity. Hokin and Reasa found increase in Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase activity concurrent with decreases in nonspecific ATPases activity. Based upon the above studies, aging the specimens for cryostatic sectioning could not be expected to decrease and possibly increase Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase activity.

Frozen specimens were cut as 60 \( \mu \) thick frozen sections. These sections were floated upon the incubation medium just described, with or without lead or ouabain \((10^{-3} \text{ M})\) added; similarly, the Ernst incubation medium was also used. Figure 22 is representative of the results obtained. In no case was there difference seen between any of the sections, regardless of aging or incubation media, or incubation media inclusions, or exclusions.

**Increased Membrane Stains with Decreased Tiron Concentrations**

At this point, it was decided to test what would happen if Tiron were decreased in concentration. The heart was washed, fixed, and washed as usual by perfusion. A section of left ventricle was excised and minced into 1 m\textsuperscript{m} square blocks. These blocks were incubated in an incubation media containing 100 mM sodium cacodylate, 10 mM potassium chloride, 3 mM magnesium chloride, 3 mM ATP, 30 mM Tiron and 2 mM lead acetate, with the exception that some blocks were incubated in that medium with only 20 mM, 10 mM or no Tiron included in the medium.
Figure 22

The Lack of Adenosine Triphosphatase Staining in Fixed Frozen Myocardium

After perfusion with 2 percent glutaraldehyde, pieces of left ventricle were excised and dropped into liquid nitrogen. These frozen pieces were sectioned still frozen to 60 μ thickness. These sections were incubated in 100 mM sodium cacodylate, 10 mM potassium chloride, 3 mM magnesium chloride, 3 mM disodium ATP, 30 mM Tiron and 2 mM lead acetate. This section looks the same as those which had 10^{-3} M ouabain included into the incubation media (16,000x).
Figure 23 is a photograph of a thin section from a block incubated in 10 mM Tiron. It is representative also of those blocks incubated in 20 and 30 mM Tiron. At this point, it was decided that the author would no longer attempt to reproduce the results of Schulze and Wollenberger, and Somogyi et al. Figure 24 is representative of the type of stain seen when no Tiron was included in the incubation media.
Increased Adenosine Triphosphatase Staining When Incubation Media Tiron Concentrations Are Reduced

The myocardium was fixed by perfusion with 2 percent glutaraldehyde, then tissue blocks were incubated in 100 mM sodium cacodylate, 10 mM potassium chloride, 3 mM magnesium chloride, 3 mM ATP, 10 mM Tiron and 2 mM lead acetate. Tissue incubated in media which contained 30 mM Tiron and 20 mM Tiron looked much as this section looks (16,000x).
Figure 24

Further Increased Adenosine Triphosphatase Staining When Incubation Media Tiron is Deleted

This tissue was treated as that in Figure 23; except, Tiron was excluded from the incubation medium (16,000x).
DISCUSSION

The Problem

The original question asked in this dissertation (as stated in the Introduction: Are cardiac plasma membrane-bound enzymes placed there by epicytotic means?) posed considerable technical problems which had to be resolved. Demonstration of whether newly synthesized phosphatases specifically Na⁺K⁺Mg++ ATPase is transported from Golgi apparatus (Mucopolysaccharide synthetic areas) by Golgi vesicles to plasma and "T" tubular membranes by Golgi vesicles (which become a part of these vesicles), it is necessary to answer the following question. Is Na⁺K⁺Mg++ ATPase found in Golgi apparatus and Golgi vesicles? Approaches which were considered to answer this question were (1) differential centrifugation and enzyme identification, (2) cytochemical localization and (3) electron microscopic immunological localization of the enzyme in Golgi apparatus and vesicles. Differential centrifugation was discarded on the grounds that homogenization required for muscle tissue was so severe that 20 to 40 percent of the microsomal fraction is vesiculated.
mitochondria (Inesi et al., 1964). This meant that methods as those of Schachter (Schachter et al., 1970) for isolating whole Golgi apparatus from liver would most likely fail because of the violent homogenization necessary in the tougher heart tissue which would vesiculate Golgi apparatus. Other Golgi apparatus isolation techniques rely upon special functional characteristics of liver Golgi apparatus as markers, such as very low density lipoproteins in alcohol treated rats (Ehrenreich et al., 1973). Golgi enriched fractions would not be definitive in this study since Golgi fractions are usually contaminated with plasma membrane (de Duve, 1971), a membrane strongly suspected of high Na\(^+\)K\(^+\)Mg\(^++\) ATPase concentration. In view of the difficulty in separating Golgi vesicles from vesiculated Golgi the likelihood of success seemed highly problematic. For these reasons this approach was discarded.

Immunological localization is an attractive alternative but was discarded since at the time this study was initiated, there was no Na\(^+\)K\(^+\)Mg\(^++\) ATPase antibody and Na\(^+\)K\(^+\)Mg\(^++\) ATPase isolation studies though imminent apparently had not been perfected to immunological purity standards. Consequently, cytochemical studies were chosen for these reasons. In addition, earlier studies of Lindower and Marks (1976) had been done with the electron microscope and there was therefore the experience and facilities necessary for this approach.
Lead-Adenosine Triphosphatase Methods, A Critique

(1) Lead Inhibition of ATPases:

Several problems must be considered when lead and ATP are used as the capture ion and substrate in cytochemical localization of enzymes. Among these are that lead is an ATPase inhibitor in the concentrations most often used and lead causes the non-enzymatic hydrolysis of ATP. These two problems combine to cause a situation where artifactual stain and dislocation of precipitate from the site of phosphate genesis are possible. Whatever enzymatic activity survives the tissue preparation for cytochemistry must now cleave ATP to ADP and $P_i$ for this procedure to work. Lead, a part of the incubation media, then must in some way render the phosphate immediately insoluble.

Lead, also, is an inhibitor of ATPases. Novikoff (Novikoff et al., 1958) demonstrated a 75 percent decrease in ATPase activity in rat liver when lead concentrations were increased from 0.36 mM to 3.6 mM. Marchesi and Palade (1967) reported the ATPase most inhibited by lead in guinea pig erythrocyte ghosts was the ouabain sensitive $Na^+K^+Mg^{++}$ ATPase, although other $Mg^{++}$ dependent ATPases were also inhibited. In Marchesi and Palade's work, significant inhibition of $Na^+K^+Mg^{++}$ ATPase did not occur until lead concentrations reached 0.4 mM. At 0.3 mM the inhibition was practically nonexistant, at 0.4 mM there was 50 percent inhibition and at 0.8 mM lead, the inhibition had
reached 90 percent. Schulze (Schulze et al., 1972) reported 1 mM lead concentration inhibited Na\(^+\)K\(^+\)Mg\(^{++}\) ATPase activity in guinea pig cardiac muscle by 90 percent. Other authors including Bonting (1962), Tormey (1966), Moses and Rosenthal (1968), and Charnock et al. (1972) have reported significant lead inhibition of various Mg\(^{++}\) dependent ATPases.

(2) Nonenzymatic Hydrolysis of ATP by Lead Ions:

Another difficulty with Lead at the 3.6 mM concentration used in the Wachstein-Meisel technique (Wachstein and Meisel, 1957) was noted in 1966 by Rosenthal (Rosenthal et al., 1966). Lead at 3.6 mM causes the nonenzymatic hydrolysis of ATP when incubated with 3.7 \(\mu\)M ATP, 4.0 \(\mu\)M MgCl\(_2\), and 30 \(\mu\)M of Tris maleate or imidazole glycylglycine buffer. Nonenzymatic hydrolysis increased with increasing the time, temperature or the pH of the incubation medium. Interestingly, at ATP concentrations of over 1 mM, the rate of enzymatic hydrolysis dropped off (when other reagents were at Wachstein-Meisel concentrations and the temperature, incubation time and pH were respectively 37 degrees centigrade, 90 minutes, and pH 7.4) to hydrolysis rates similar to the spontaneous hydrolysis of ATP in solution. This stability was achieved at 6 mM ATP. When tissue (rat kidney and liver) was incubated in Wachstein-Meisel medium, less phosphate was found in the tissue by
assay using the Fiske-SubbaRow method than was liberated by nonenzymatic hydrolysis under similar non-tissue conditions (Moses et al., 1966).

(3) The Possible Diffusion of Enzymatically Liberated Phosphate Prior to Lead Precipitation:

In a later article (Rosenthal et al., 1969a) the Rosenthal group demonstrated similar findings in rat liver and kidney tissue blocks. Phosphate concentrations trapped in tissue equaled $P_i$ concentrations of enzymatically liberated $P_i$ from ATP and also equaled nonenzymatically liberated $P_i$ concentrations. Therefore, entrapped $P_i$ could be equally well explained by either enzymatic or nonenzymatic hydrolysis (concentrations of 4.0 mM ATP and 3.6 mM lead nitrate). When 8.0 mM ATP was used, nonenzymatic hydrolysis was zero (in this study there was no 6 mM ATP point on their graph) and trapped $P_i$ was equal to one fourth of the phosphate enzymatically liberated.

These studies raise two questions of major interest:

(1) If 75 percent of the $P_i$ liberated enzymatically diffuses out of the tissue without being precipitated, does the 25 percent $P_i$ trapped diffuse before it is precipitated? What is the nature of lead phosphate precipitation if, indeed, the precipitate is lead phosphate?

(2) What is the nature of the lead ATP relationship that causes the nonenzymatic hydrolysis of ATP?
In answer to the first question, we first must discuss the guinea pig peritoneal macrophages and exudate monocytes in a media containing 1 mM lead nitrate, 1 mM ATP, 20 mM magnesium sulfate, and 12.5 mM Tris HCl buffer (Poelmann and Daems, 1973). Included in the media were latex particle ranging in size from 1090 to 76,000 Å in diameter to demonstrate phagocytosis. Both plasma membrane and extracellular latex particles contained heavy lead precipitates which were not prevented by 5 mM N-ethylmaleimide or 10 mM sodium flouride. The conclusion that immediately comes to mind is that the latex particles were acting as heterogeneous nuclei. Rosenthal et al. (1969a) demonstrated in rat liver and kidney that under similar conditions (0.72 mM ATP and 1 mM lead nitrate) there was 60 percent trapping efficiency. If one assumes all of the tissue-bound phosphate to be precipitated at the site of phosphate genesis, then 40 percent of the phosphate generated is free to react with free lead and precipitate any place, such as on latex particles.

All of this seems improbable when one considers the solubility of lead phosphate 0.5 µM/liter (Seidell, 1941). In the Rosenthal experiment, just mentioned, in which phosphate was precipitated at 60 percent efficiency, he used 1 mM lead incubation solution which is 2000 times the 0.5 µM concentration needed for lead phosphate saturation. The tissue generated 1 µM phosphate per mg protein.
per hour. There was 0.1 mg protein in 5 ml volume and the incubation period was 15 minutes long. This indicates final phosphate concentration in 5 ml solution was 0.025 μM.Converted to molar concentration based upon one liter, the concentration is 5 μM phosphate. This is 10 times the amount of phosphate required. According to Rosenthal, 50 percent of this is enzymatically generated. At the sites of phosphate genesis the concentration probably would be much higher. It is possible that 50 percent of the phosphate is immediately precipitated at its site of genesis by a 2000 times excess of lead and the other 50 percent of the phosphate generated is the nonenzymatic portion spread across 5.0 ml of solution and only reaching a final 2.5 μM phosphate concentration which is only 5 times saturation. Only 20 percent of this is precipitated at heterogenic nuclei, for example latex particles or perhaps plasma membrane, especially those precipitates upon the greater membrane. Ganote (Ganote et al., 1969) expressed similar views after calculating the total phosphate liberated by nonenzymatic hydrolysis in the small volume of tissue water as opposed to total incubation volume. Ganote concludes that nonenzymatic hydrolysis in tissue is insignificant; but cautions that outer membrane lead phosphate precipitates may well be the result of nonenzymatic hydrolysis from the greater volume of the incubation media. To prove the point, Ganote pre-incubated
rat liver tissue in 4.0 mM lead nitrate and followed by immersion of the tissue in a 10 mM phosphate solution. The resultant stain was localized on plasma membrane, inside bile canaliculi and in nuclear heterochromatin. In a previous article, Novikoff (1967) was unable to demonstrate such a precipitate using similar technique; however, Ganote points out that these precipitates are only visible at the electron microscope level of resolution.

This sort of approach of reproducing and determining artifactual stain has equivocal merit.

(4) The Nature of the Lead Induced Phosphate Precipitate:

The assumption that the stain developed in Wachstein-Meisel media or other lead ATP media is simply lead phosphate is probably incorrect. Rosenthal (Rosenthal et al., 1969) demonstrated, by both chemical analysis of tissue precipitates and autoradiographs electron microscopy using titrated ATP and ADP, that the nucleotide is located within the precipitate. Chemical analysis indicates that nucleotide was in equimolar concentration with phosphate when the Wachstein-Meisel media was used. When lead concentrations were as low as 1 mM (0.76 mM ATP) in the incubation media, no nucleotide was found in precipitates but at 2 mM lead concentrations the nucleotide component was maximal.
In 1972, Berg et al. (1972) reported that the lead precipitate associated with ATPase cytochemically was a mixture of lead phosphate, adenosine nucleotide, chloride and hydroxide. Furthermore, he reported increases in precipitate when chloride concentrations in the media were raised above 100 mM. Berg determined that this chloride effect was an increase in enzymatic precipitation efficiency, rather than either an activation of the enzyme or stimulation of nonenzymatic hydrolysis. These results question the reality of Na⁺ activation of ATPase enzyme systems (when cytochemical precipitation is used as the assay system) unless other Na⁺ salts are able to activate the system.

The above mentioned affinity of ATP for lead provide a partial answer to the second question posed: what is the nature of the lead-ATP relationship that causes nonenzymatic hydrolysis of ATP?

In 1964, Berg (1964) reported that lead ion in the presence of ATP is chelated by ATP. The divalent cation lead is bound to hydroxyl ions of both the alpha and beta phosphates. In equilibrium at saturation, at the ratio of 6:1 (favoring the alpha-beta form), the lead also binds to the alpha and gamma phosphates. "Chelated lead is bound in a nonionic form and cannot be taken out of solution by such precipitating anions as sulfate and phosphate" (Berg, 1964). The alpha-gamma chelate is, however,
unstable. In addition to reducing the number of phosphate resonance forms, the lead causes a strain in bound angles. According to Berg, both chelates are subject to enzymatic hydrolysis yielding two precipitable phosphate groups, an ortho-and a pyro-phosphate.

(5) ATP as a Lead Chelate:

A different interpretation of the result of lead ATP binding is expressed by Tice (1969). This investigator finds that ATPase activity (in rat liver and kidney microsomes) is inversely proportional to Pb:ATP ratio in the incubation media. Tice found ATP chelation of lead to be inhibitory because of substrate removal. He determined this substrate inactivation to be a major form of lead inhibition of ATPases mimicking competitive inhibition. Tice also found that the reverse effect of ATP, taking lead out of solution, is significant to the point that lead phosphate precipitation is decreased. Lead phosphate precipitation was measured by adding 10 mM sodium phosphate with trace amounts of \( P^{32} \) into solutions containing 1.0 or 4.0 ml lead nitrate and 100 mM Tris maleate buffer pH 7.0. Tice demonstrated that 1.0 mM lead nitrate precipitated 1.0 mM or 10 percent of the added sodium phosphate. When the experiment was repeated with the inclusion of 4.0 mM ATP, only 0.1 mM or 1 percent of the added sodium phosphate was precipitated. When
4.0 mM rather than 1.0 mM lead nitrate was used in the media, the trapping efficiency was reduced to 18 percent. It appears that the assumption of 2000 times excess of precipitable lead is questionable.

Although no reference could be found regarding lead binding ATP as a source of lead catalyzed nonenzymatic hydrolysis of ATP, that seems to be an appealing explanation. As mentioned earlier, lead binding of ATP reduces resonance structures and strains bond angles, but Mg$$^{++}$$, Mn$$^{++}$$, Ca$$^{++}$$, and most other divalent cations bind ATP in a similar way. Unless an explanation for the difference in nonenzymatic hydrolysis can be demonstrated by chemical data the relationship between lead binding and nonenzymatic hydrolysis must remain speculative.

Finally, Ganote et al. (1969) criticized the use of p-chloromercuribenzoic acid (PCMB) and potassium fluoride (KF) as ATPase inhibitors since he considered part of their inhibitory action to be due to precipitation of lead salts from the incubation media. However, to the author's knowledge, no one has criticized the use of ouabain on similar grounds.

The conclusions concerning the validity of lead phosphate precipitation methods, especially the Wachstein-Meisel method for localizing ATPases, made by the Rosenthal, Moses, Tice and Ganote group have been disputed by Novikoff in a series of letters to the editor of the Journal of

(6) Another Source of Diffusion Artifact:

Another source of artifactual stain pointed out by van Duijn (van Duijn et al., 1967) and Holt and O'Sullivan (1958) results from poor lead ion or ATP diffusion into cell structures where enzymatic activity may be found. If the substrate does not diffuse to enzymatic sites and become hydrolyzed rapidly enough to quickly reach phosphate saturation levels, considerable phosphate will diffuse from enzyme sites to be precipitated elsewhere. Similarly, lead must diffuse at such a rate as to be able to maintain lead phosphate saturation at the area of the enzyme where lead, hopefully, is being removed at a rapid rate. If both of these criteria are not met, difficulties will likely result.

Working in the salt secreting system of young Herring gulls, Abel (1969) noted just such an effect. He
found that different types of stain and different structures staining in areas of secretory lobes appeared to be the direct result of accessibility of the substrate and the trapping agent due to diffusion barriers.

As pointed out by Novikoff (1970a), these criticisms do not preclude the use of lead phosphate ATPase cytochemistry; but are areas of which one should be well aware. One must design experiments to minimize artifactual pitfalls.

While lead phosphate precipitation methods of ATPase cytochemistry have been extensively investigated, they remain a source of considerable controversy. Consequently, the author avoided this method except in applications that would either conform maximally to the critics advice or add a new dimension to the technique.

When the Wachstein-Meisel technique was employed, it yielded no ouabain inhibitible stain. In one case when the medium was perfused through unfixed heart it produced no stain in muscle at all (perfusion of unfixed tissue is discussed under Lead-Tiron method in this section). At best Wachstein-Meisel method resulted in reproduction of the Mg$^{++}$ ATPase stains (Sommer and Spach, 1965) (see pages 74-81).
The Lead-Tiron ATP Method. A Critique

A significant modification of the Wachstein-Meisel method was introduced by Schulze and Wollenberger (1967). They used Tiron, a lead chelate, to reduce free lead levels in their cytochemical method. The advantages were clear. (1) In the incubation media there was too little free lead to inhibit Na⁺K⁺Mg⁺⁺ ATPase significantly (Schulze et al., 1972); (2) lead concentrations were low enough so that very high percentages of the added ATP would not chelate lead, therefore making more ATP available for enzymatic hydrolysis; (3) there was no evidence of lead catalyzed nonenzymatic hydrolysis of ATP (Schulze et al., 1972a); (4) Tiron prevented lead precipitation during the preparation of the incubation media; (5) without lead-ATP-complexes, the solubilizing effect of these complexes upon lead phosphate would not be a factor (Tice, 1969). A possible disadvantage was that lead trapping efficiency of phosphate would be near zero if one were to extrapolate from Rosenthal's 1969 paper to this situation (Rosenthal, 1969a); however, this system was different from Rosenthal's in that 0.1 mM free lead is not the same as 0.1 mM buffered free lead. There was nothing to indicate in his experiments that trapping efficiency was not high in the first minute of Rosenthal's incubation but lead supplies were exhausted rapidly so that final average efficiency was very low. They used 2 percent
glutaraldehyde in their successful experiments. Although this fixative seemed like an excessive concentration of fixative for Na\(^+\)K\(^+\)Mg\(^++\) ATPase enzymatic activity, Marchesi and Palade (1967) reported 2 percent glutaraldehyde diminished Na\(^+\)K\(^+\)Mg\(^++\) ATPase activity of microsomes by 98 percent; McClurkin (1964) used 4 percent formaldehyde and Palkama and Uusitalo (1968) and Palva and Palkama (1974) used 2.5 percent glutaraldehyde in their reports of successful Na\(^+\)K\(^+\)Mg\(^++\) ATPase cytochemical localization. Although Novikoff (Novikoff et al., 1966), Bosch (Bosch et al., 1967) and Tormey (1966) had not been able to reproduce the results of McClurkin (1964), no one had tested the methods of Schulze and Wollenberger, except the successful report of Somogyi (Somogyi et al., 1971). Gillis and Page in 1967 reported artifactual lead stain when testing for calcium, magnesium ATPase in rabbit skeletal muscle. However, they used 6 mM lead and 4.5 mM Tiron. These high concentrations of lead and low concentrations of Tiron alone would make Na\(^+\)K\(^+\)Mg\(^++\) ATPase localization impossible according Schulze and Wollenberger (1967). The fact that neither Na\(^+\) or K\(^+\) was included in the incubation media and that 4 mM Ca\(^++\) was included, a Na\(^+\)K\(^+\)Mg\(^++\) ATPase inhibitor, makes the report of Gillis and Page a completely different study from those of Schulze and Wollenberger.

Prior to the publication by Schulze and Wollenberger, the Rosenthal, Moses, Tice and Ganote groups had...
published several papers describing difficulties with the lead-ATP method of ATPase cytochemistry. It was the present author's feeling that many investigators had discarded the lead-ATPase method as unworkable and ignored or had not seen a successful and confirmed technique for Na⁺K⁺Mg⁴⁺ ATPase cytochemical localization (the Schulze-Wollenberger papers were all published German language or relatively obscure English language journals). This technique resolved all of the Rosenthal group's complaints except one, that being lead phosphate trapping efficiency which did not seem an insurmountable problem. If trapping efficiency was minimal, but enough phosphate was trapped to localize a ouabain inhibitible enzyme, it would be a successful technique. Any artifactual stain could be ignored or differentiated as nonspecific Mg⁴⁺ ATPase stain once resistance to ouabain inhibition was demonstrated.

Throughout most of the earlier experiments in which the Schulze-Wollenberger (lead-Tiron) technique was used, the incubation solution was perfused through the heart after fixation. This technique of perfusing the heart with incubation medium rather than superfusing it with medium is possibly one of the reasons these experiments failed to yield any cytochemical stain. However, when distilled glutaraldehyde was used, a very small amount of stain was seen. When a cytochemical incubation medium was perfused through heart prior to fixation, no
significant stain in the ventricle myocardium was detected even though the tissue had not been fixed. This medium had already demonstrated heavy stain capability in fixed superfused cardiac tissue (Figures 15 and 16). One might expect that unfixed tissue would stain more heavily than fixed tissue if the cytochemical medium was able to contact the myocardial cells. If this were not the case, one could assume that the capillaries had retained all or part of the medium. Critics might say that fixed capillaries and cells could be more permeable than the unfixed tissue; however, in the effort to increase capillary permeability, 0.1 percent glutaraldehyde was used. This fixative also failed to yield a cytochemical stain. It is the author's conclusion that these early experiments were all doomed to failure simply because the cytochemical medium was not uniformly distributed to each cell by perfusion, rather, the medium was being packaged in vasculature and isolated from the contractile cells.

The later experiments in which tissue sections were superfused, especially the experiment in which Tiron concentrations were varied, were much fairer tests of Schulze-Wollenberger technique. If one believes that the only function of Tiron is to chelate lead, and that lead is a phosphatase inhibitor, it would follow that as Tiron levels are decreased at constant levels of lead, phosphatase activity should also decrease. Marchesi and
Palade (1967) using EDTA as a lead chelator, demonstrated a significant increase in phosphatase activity as EDTA levels were increased. However, as the experiments with various Tiron levels, the staining increases as phosphatase activity should be decreasing. It was assumed that the Rosenthal results of low trapping efficiency at very low lead levels can be extrapolated to conditions in which low lead levels are maintained by a chelate.

Employing the technique of Schulze and Wollenberger in which distilled glutaraldehyde was used, the tissue was superfused. The stain found was not inhibitable by ouabain but by desoxycholate (DOC), an inhibitor of nonspecific ATPases and a stimulator of Na⁺K⁺Mg⁴⁺ ATPase. Therefore, the results of either the Schulze and Wollenberger or of Somogyi could not be reproduced.

Throughout this study the object has been to demonstrate a ouabain-inhibitable enzyme in reasonably well preserved myocardium. This object has not been realized by either modified Wachstein-Meisel or Schulze-Wollenberger techniques. All of the studies claiming success in staining Na⁺K⁺Mg⁴⁺ ATPase using the Wachstein-Meisel or variant thereof, have been (except the Palkama-Uusitalo studies) questioned in the literature by investigators who could not reproduce their results. This investigator must add his name to the list of those who were unable to produce ouabain-inhibitable ATPase stains using those
techniques. The Palkama and Uusitalo variation of the Wachstein-Meisel technique was to preincubate specimens in ouabain prior to fixation. These authors were then able to demonstrate different staining patterns in ouabain-pretreated tissue compared with tissue not pretreated with ouabain. Possibly fixation could have altered the Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase enzyme in such a way that ouabain would no longer bind to it but that already bound ouabain would not dissociate. However, Sommer and Spach (1964) conducted a similar experiment in dog heart and obtained the following results. They sacrificed a dog by ouabain overdose and subjected fixed tissue to a Wachstain-Meisel incubation medium. They were unable to elicit any difference in staining patterns between ouabain-sacrificed and anesthetized dogs sacrificed by removal of the heart. Though methods were somewhat different, they were judged to be similar enough not to warrant a trial of the Palkama-Uusitalo modification.

**Fixation Versus Staining**

It was apparent from the onset that good cytochemical staining would be inhibited by heavy fixation (Sabatini and Bensch, 1963; Essner et al., 1965; Marchesi and Palade, 1967; Ernst and Philpott, 1970) and that incomplete fixation would not preserve Golgi apparatus and could yield result either in denaturation and/or
solubilization of the desired enzyme (Novikoff, 1961a).
Therefore, the first goal in this study was to determine conditions for minimal fixation that would allow preservation of Golgi apparatus and leave enough enzymatic activity to stain \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase. Minimal fixation levels were established with either paraformaldehyde, 0.75 percent glutaraldehyde, or the mixture of 0.75 percent paraformaldehyde and 0.1 percent glutaraldehyde perfused 20 minutes at zero to four degrees centigrade. Ernst found his minimal fixative in kidney to be 1 percent paraformaldehyde and 0.25 percent glutaraldehyde perfused for 10 minutes (Ernst, 1973). At these fixative concentrations one could find, after exhaustive searching, partially intact Golgi apparatus. However, \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase activity, defined as ouabain-inhibitable enzyme activity, was not apparent on electron micrographs of myocardium at these low fixative levels.

**Para-nitrophenyl Phosphatase Method, A Critique**

When Ernst (1972) introduced a new method for cytochemically staining \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase by using para-nitrophenyl phosphate (PNPP) as substrate for \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase, the question that immediately arose: is PNPP really a substrate for \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase or is PNPPase a different enzyme? The reaction sequence for \( \text{Na}^+\text{K}^+\text{Mg}^{++} \) ATPase as described by Dahl and Hokin (1974) is as follows.
Different conformations of the phosphorylated (E₁-P and E₂-P) and non phosphorylated (E₁ and E₂) forms of the enzyme appear to be involved, as well as multiple allosteric interactions of cations and ATP with the enzyme. There is some evidence that E₂ has high K⁺ affinity and E₁ has high Na⁺ affinity. More complete sequences showing specific involvement of Na⁺ and K⁺ have been presented (Dahl and Hokin, 1974).

It is postulated that PNPP is hydrolysed during the later stages of the reaction, possibly at step four, prior to E₂ conversion to E₁.

However, Yoshida (Yoshida et al., 1969) reported that low concentrations of ATP and sodium (one mM ATP and five to twenty mM NaCl) stimulated PNPP hydrolysis, where higher concentration of either or both sodium or ATP inhibited PNPP hydrolysis. While it is not clear where PNPP would enter hydrolysis, it is generally acknowledged
that, if PNPP is hydrolyzed by ATPase it is in the second half of the reaction as a functional intermediate (Yoshida et al., 1969). This is largely based upon the absolute requirement of PNPPase for potassium and magnesium (Yoshida et al., 1966) and ouabain inhibition of both PNPPase and Na⁺K⁺Mg⁺⁺ ATP, as well as Na⁺ and ATP inhibition of PNPPase.

Fujita et al. (1966), Brooker and Thomas (1970), and Isral and Titus (1967) report that Na⁺K⁺Mg⁺⁺ ATPase and PNPPase under several different conditions are inhibited differently by different concentrations of ouabain and thereby conclude that these two may be different enzymes. It is beyond the scope of this paper to discuss the very complex relationship between ouabain, substrate, cations, and enzyme and the reader is referred to Schwartz et al. (1975) for a detailed discussion. However, it is possible that an enzyme may show a different $K_i$ in response to ouabain when subjected to different substrates, especially if the substrates enter hydrolysis at different conformational states of the enzyme. For example, the $K_i$ of ouabain for PNPPase and Na⁺K⁺ ATPase are closer when Na⁺ and ATP are added to PNPPase media (Yoshida et al., 1969). When ATP concentrations (no Na⁺ in media) in PNPPase incubation media are equal to ATP concentrations in Na⁺K⁺Mg⁺⁺ ATPase media, $K_i$'s were nearly the same (Fujita et al., 1966).
Similarities between the two systems include the observation that Rb\(^+\), Cs\(^+\), NH\(_4\)^+ or Li\(^+\) may substitute for K\(^+\) in either Na\(^+\)K\(^+\)Mg\(^++\) ATPase (Skou, 1960) or PNPPase (Bader and Sen, 1966) (Yoshida et al., 1969); both systems survive potassium iodide treatment (Fujita et al., 1966) (Yoshida et al., 1969), and both systems appear with high specific activity in purified Na\(^+\)K\(^+\)Mg\(^++\) ATPase preparations (Fujita et al., 1966) (Yoshida et al., 1969).

Askari and Rao (1972) injected a purified Na\(^+\)K\(^+\)Mg\(^++\) ATPase preparation with PNPPase activity into rabbits, in order to prepare an antibody to Na\(^+\)K\(^+\)Mg\(^++\) ATPase. The resultant antibody inactivated Na\(^+\)K\(^+\)Mg\(^++\) ATPase but, originally could not be shown to affect PNPPase activity. Later (Askari and Rao, 1973), these same workers reported that the antibody slightly lowered PNPPase's \(V_{\text{max}}\) and abolished Na\(^+\) ATP stimulation of PNPPase. Their conclusions were that the antibody bound areas involved in the first half of the half reaction and therefore, PNPPase which is involved in the second half reaction, was only subtly influenced. If Askari and Rao can prove there is only one antibody involved, their conclusion concerning PNPPase being a part of Na\(^+\)K\(^+\)Mg\(^++\) ATPase would be strong, indeed.

Whether PNPPase is a part of the Na\(^+\)K\(^+\)Mg\(^++\) ATPase or not (it seems that it is) a PNPPase cytochemical assay may still be useful to the central purpose of this study as put forth in the Introduction of the dissertation.
Since Na\(^+\)K\(^+\) ATPase and PNPPase are practically inseparable by biochemical purification, it may be concluded that the two are found upon the same membrane systems.

The best evidence of the usefulness of this technique comes from the results of the technique itself. Firth (1974) and Leuenberger and Novikoff (1974) using the Ernst technique were able to localize ouabain inhibitable stain in areas of membrane of kidney and cornea which had been indicated as Na\(^+\)K\(^+\)Mg\(^{++}\) ATPase areas by other non-cytochemical studies.

Besides using PNPP as substrate, there are other unique aspects to the Ernst technique. The most encouraging of these is that strontium, not lead, is used as a capture metal. The advantages to using strontium and PNPPase are that, as yet, none of the disadvantages of the lead-ATP interactions discussed earlier in this section have been reported for strontium. Strontium, however, does inhibit Na\(^+\)K\(^+\)Mg\(^{++}\) ATPase (Ernst, 1972a), but not as much as lead has been reported to inhibit the enzyme. A second major difficulty with the system is that at pH 7.4, strontium phosphate is too soluble in water to be an effective trapping pair. Consequently, strontium incubation media are usually adjusted to pH 8.5 to 9.0. At these pH's 90 to 95 percent of the liberated phosphate is precipitated; at pH 7.5 only 20 percent is precipitated.
Since strontium is not nearly as electron dense as lead, a lead for strontium exchange is performed after the incubation is completed. The exchange simply consists of incubating tissue in a low pH (2.0 to 3.0), 2 percent lead nitrate solution. "This procedure (Ernst, 1972) appears to be a promising one for use in such tissue as the heart" (Schwartz et al., 1975).

Earlier in this discussion the reasons for the failure to localize a stain using Schulze-Wollenberger technique were discussed. The Ernst technique has been used successfully to localize what is apparently Na\(^+\)K\(^+\)Mg\(^{++}\) ATPase or, at least, a ouabain inhibitable enzyme which the author believes to be Na\(^+\)K\(^+\)Mg\(^{++}\) ATPase in several tissues, by several investigators, including the avian salt gland (Ernst, 1972a), kidney (Ernst, 1973; Ernst, 1975; Firth, 1974; Beewwkes and Rosen, 1975), cornea (Leuenberger and Novikoff, 1974) and brain (Stahl and Broderson, 1976; Guth and Albers, 1974). The studies by Firth, Ernst and Leuenberger and Novikoff were done at the electron microscopic level of solution. Probably the most definitive experiments performed in this thesis which explain why this highly successful technique failed to work in guinea pig heart were the parallel heart and kidney experiments, as well as the whole heart homogenate experiments. The first of these two parallel experiments was run with guinea pig heart and kidney. Kidney tissue
contained ATPase activity which was ouabain-inhibitable but the heart did not. In the whole heart homogenate experiments, the whole heart was treated with a fixative demonstrated by Ernst (1973) to spare Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase in guinea pig kidney. The heart was then homogenized and assayed for Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase activity. The results show that although some activity survives the fixation, the surviving activity is inhibited almost totally by strontium employed in the Ernst method.

**Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase Specific Activity in Different Organs**

What then is the difference between heart and kidney or cornea enzyme? Bonting (Bonting et al., 1961) reported that 35 percent of kidney Mg\(^{++}\) ATPase activity is Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase; 36 percent of the cornea Mg\(^{++}\) ATPase activity is Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase, but, only 6 percent of the cat's cardiac Mg\(^{++}\) ATPase is Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase.

When the Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase activity was compared on the basis of specific activity (activity per gram protein), kidney cortex liberated 0.22 mM phosphate/gram protein/hour whereas cardiac Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase had only 1/7th the specific activity (0.03 mM/gram/hour). Cornea contains the lowest specific activity of all (0.00157 mM/gram/hour); however, corneal Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase had been shown to be concentrated on the endothelium, the surface in which Leuenberger and Novikoff located Na\(^{+}\)K\(^{+}\)Mg\(^{++}\) ATPase (Rogers, 1968).
Therefore, a relatively high Na$^+$K$^+$Mg$^{++}$ ATPase activity in a relatively localized space appears to be necessary for localization of Na$^+$K$^+$Mg$^{++}$ ATPase by a ouabain substraction type of cytochemical stain. An example of how important high specific activity phosphatases are to phosphatase cytochemistry is demonstrated in kidney. Using the Firth method (Firth, 1974), Firth and Ernst, using the Ernst method (Ernst, 1973), this author, Firth and Ernst were able to demonstrate ouabain inhibitable phosphatase activity in distal tubule, but not in kidney proximal tubule. The difficulty in localizing proximal tubule phosphatase is probably best explained by the one fifth Na$^+$K$^+$Mg$^{++}$ ATPase specific activity in proximal tubule compared with distal tubule (Schmidt and Dubach, 1971). Guinea pig heart does not fulfill either of the requirements of high specific or total Na$^+$K$^+$Mg$^{++}$ ATPase activity. Based upon a figure of 9 percent Na$^+$K$^+$Mg$^{++}$ ATPase in guinea pig heart, this is very close to that of cat heart (6.0 percent), dog heart contains 14.7 percent and frog heart 15.0 percent (Bonting, 1962).

Na$^+$K$^+$Mg$^{++}$ ATPase Organ Specific Isozymes

Further explanation of the failure to demonstrate a ouabain-inhibitable stain in guinea pig heart is suggested by the parallel kidney and heart experiments. Under
identical conditions guinea pig ventricle gave negative results however, distal tubule of guinea pig did demonstrate a ouabain inhibitable stain. There remains the possibility that different isozymes of Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase are found in the different tissues. The cardiac isozyme being more sensitive to fixation than the kidney isozyme. This idea is supported by the fact that different ouabain inhibition curves have been demonstrated for cat choroid plexus and ciliary body (Bonting et al, 1962). Further support for organ specific isozyme of Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase is demonstrated by McCans et al. (1975). The McCans group reacted a sheep antidog kidney Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase with a purified (two polypeptides) dog kidney Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase. The antibody reduced ATPase activity by 88 percent when the enzyme was saturated with antibody. When the same antibody was used on purified dog heart Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase (two major polypeptides plus several contaminant proteins), maximal inhibition was only 27 percent. The antibody inhibited purified dog brain Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase (two polypeptides plus minor protein contaminants) by only 26 percent at saturation. When the antikidney antibody was reacted with saturating levels of brain enzyme preparations, the remaining unprecipitated antibody maintained full inhibitory activity against kidney enzyme but no inhibitory activity to fresh brain enzyme. These immunological study strongly suggest isozymic differences
between dog heart and kidney Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPases. Similar results were demonstrated in rat using sheep and rabbit antidog kidney Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase antibody. Thus, it is conceivable that Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase from different organs may exhibit different sensitivity to aldehyde fixation.

**Ouabain as a Cytochemical Tool in Aldehyde-fixed Tissue**

Under normal physiological or biochemical conditions there is little doubt that ouabain in millimolar concentrations is a potent inhibitor of Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase. It is possible that aldehyde fixatives may alter the enzyme in such a way as to render it unable to bind ouabain, or unable to be inhibited by ouabain. Kyte (1976) found that glutaraldehyde destroyed antigenic sites such that his rabbit antidog kidney Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase antibody no longer reacted with the antigen. If aldehyde fixatives do alter the ouabain binding site on Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase so it no longer binds ouabain, several stains demonstrated in this dissertation may in fact be Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase. This would explain why the stain resulting from Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase activity in the epicytotic vesicle (resulting from ouabain pretreatment in vivo) was not ouabain-inhibitable. This would also explain why plasma membrane and "T" tubular ATPase using both the lead and strontium techniques was also not ouabain-inhibitable. Certainly, these stains were located where one would
expect to find Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase. However, in every case where a second inhibitor was tried, one which would not inhibit Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase, but would inhibit nonspecific phosphatases, the inhibitor prevented the non-ouabain inhibitable stain. Cysteine is not a Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase inhibitor (Padykula and Herman, 1955; Skou, 1963); but, in these experiments it abolished both "T" tubular, plasma membrane, and lateral cisternae stains. Desoxycholate is not a Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase inhibitor but, it does inhibit nonspecific ATPases (Auditore, 1962; Jarnefelt, 1964; Rostgaard and Moller, 1969). In other experiments DOC prevented formation of "T" tubular, plasma membrane and intercolated disc stains that were not inhibited by ouabain. Therefore, aldehyde fixation may very radically alter Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase. Alternatively one must assume that the stains that are not ouabain-inhibitable are not Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase ouabain receptors in distal tubules of guinea pig kidney. The above data also offers little support for the argument that Na\textsuperscript{+}K\textsuperscript{+}Mg\textsuperscript{++} ATPase activity is present, but the stain is masked by much heavior nonspecific enzyme stain.

The Significance of the Stains Achieved

Using the lead ATP method as described in Pages 74-81 of the Results section, lateral cisternae were stained in a similar way to that demonstrated by Sommer and Spach (1964), Essner (Essner et al., 1965), and
Rostgaard and Behnke (1964). This same lateral cisternae stain was seen when PNPP was used as substrate (Figure 16). When PNPP was used a substrate and fixation was less severe, this stain expanded to "T" tubules and plasma membrane. These stains are completely inhibitable with $10^{-2}$ M cysteine.

Possibly the most significant finding of this study is the observation that when hearts had been treated 1 hour in vivo with ouabain, a new stain appeared upon what looked like epicytotic structures on "T" tubules and plasma membrane (Figures 14 and 15). No other study has demonstrated such a stain. This stain is apparently caused by ouabain pretreatment.

The original goal of this study was to see if enzymes were inserted into plasma membrane and "T" tubule by means of epicytosis. Ouabain and Na$^+$K$^+$Mg$^{++}$ ATPase were used as tools because of their relevance in the study of Vaughan and Cook (Vaughan and Cook, 1972). Although success in demonstrating Na$^+$K$^+$Mg$^{++}$ ATPase upon cardiac membrane may have eluded this author, a stain was concentrated in areas that appeared to be an epicytotic membrane. One would expect the highest specific activity of enzyme at the site of new membrane. Consequently, the appearance of the half circular membrane and stain is relevant.

If, in fact, new membrane complete with Na$^+$K$^+$Mg$^{++}$ ATPase is being deposited by epicytotic means as suggested
in the Introduction, it should also contain a complete complement of enzymes including nonspecific ATPase.

How to Resolve the Problem of Cardiac Na⁺K⁺Mg⁺⁺ ATPase Localization in Cardiac Tissue

Kyte (1974) reported sheep antibody specifically for Na⁺K⁺Mg⁺⁺ ATPase. Using a microsomal preparation and a ferritin labeled second antibody, Kyte was able to localize Na⁺K⁺Mg⁺⁺ ATPase upon his microsomal preparation. Later Kyte (1976) reported the successful localization of Na⁺K⁺Mg⁺⁺ ATPase in dog kidney distal tubule and proximal tubule (Kyte, 1976a) using his sheep antidog kidney enzyme upon formaldehyde fixed ultra thin frozen kidney sections. This technique most likely could be modified to react the antibody with thin sections of embedded guinea pig heart muscle, or possibly an antiguinea pig heart antibody might be prepared for this purpose.
SUMMARY

Electron microscopic cytochemistry studies were conducted on guinea pig myocardium and renal cortex. Specific attention was given to ouabain inhibitable ATPase activity.

(1) Phosphatase activity could be shown to be present upon plasma membrane, lateral cisternae and transverse tubules of myocardium when the Ernst or modified Wachstein-Meisel incubation media were used. Phosphatase activity was resistant to 2 percent glutaraldehyde pre-fixation. These stains were present with 10^-2 M ouabain. Enzyme activity was not evident when the Schulze-Wollenberger incubation media was used.

(2) The above mentioned stains were abolished by 10^-2 M cysteine when the Ernst media was used and by 0.01 percent desoxycholate when the modified Wachstein-Meisel media was used.

(3) The use of cryostatic sections or the perfusion of incubation media prior to fixation did not increase phosphate entrapment. In most cases these maneuvers decreased the intensity of myocardial stain.
(4) An intercalated disc stain was demonstrated when 2 percent distilled glutaraldehyde and the Schulze and Wollenberger incubation medium was used. This is the only stain visualized using the Schulze and Wollenberger incubation media and was not ouabain-inhibitable.

(5) When crude homogenates of guinea pig myocardium fixed in 1 percent paraformaldehyde and 0.25 percent glutaraldehyde were assayed for para-nitrophenyl phosphatase activity, very little ouabain inhibitable activity was demonstrated. This small activity was abolished by 10 mM strontium chloride. Five mM aluminum chloride did not abolish this small activity, but a cytochemical stain failed to appear after lead replacement reactions.

(6) Parallel experiments in myocardial and renal tissue yielded ouabain-inhibitable phosphatase in distal tubules of kidney. No ouabain-inhibitable stain was demonstrable in proximal tubule of kidney or left ventricle of heart.

(7) When guinea pigs were treated with ouabain (300 μg/Kg) one hour prior to sacrifice, epicytotic vesicles were demonstrated on or near left ventricular plasma membrane and transverse tubules. Phosphatase activity of the plasma membrane and transverse tubules was concentrated in the epicytotic vesicles. This epicytotic phosphatase appearance is unique in that it is totally dependent upon in vivo ouabain pretreatment. The myocardium did
not contain phosphatase activity in pinocytotic vesicles.

It is concluded that with intact guinea pig heart tissue the author could not demonstrate ouabain-inhibitable phosphatase activity using conventional cytochemical means. Since guinea pig renal distal tubule did contain ouabain-inhibitable phosphatase activity, distal tubular phosphatase may either be a different enzyme and/or more concentrated on the renal membrane. Furthermore, it is concluded that ouabain-induced epicytotic phosphatase activity is fully consistent with the hypothesis that phosphatases are delivered to plasma membrane and transverse tubules by means of epicytotic vesicles whose origin appears to be the Golgi apparatus.
APPENDIX

Solutions

Preparation of Fixatives

Glutaraldehyde at 50 percent concentration was used and stored under refrigeration. On the day before its use, glutaraldehyde was mixed with activated charcoal and vacuum filtered using Watman No. 1 filter paper and Buchner funnel (Anderson, 1967).

Glutaraldehyde was purified by distillation as follows. Approximately 250 ml of charcoal filtered 50 percent glutaraldehyde solution was placed in a 500 ml round bottom flask; with a Vigreaux distilling column coupled to a 24 inch Liebig water cooled condenser was attached to the open end mounted in place. Distillates were collected in boiling flasks attached to the end of the Liebig condenser by means of a 130 degree angle fitting. Approximately three fourths of an inch of a six inch pasteur pipette was heated to the melting point and pulled to approximately five feet in length. This, when broken to the desired length, served the dual function of
capillary tube for nitrogen delivery into the boiling flask and agitator to prevent super heating and boiling over of the glutaraldehyde during distillation. A vacuum was drawn from a side armed angle fitting at the junction of the Liebig condenser and collecting boiling flask. Under this arrangement pure dry nitrogen was introduced into the system at one end and withdrawn from the other. Temperature readings were taken at the head of the Vigreaux column. Heat was delivered by a rheostat controlled heating mantle. A vacuum of 110 mm of Hg was maintained throughout the distillation procedure (Anderson, 1967) (Fahimi and Drochmans, 1965). Sample collection was begun when the temperature at distillation head reached 60 degrees centigrade. Upon collection, the product was immediately diluted with equal volumes of boiling distilled water (Fahimi and Drochmans, 1965a). The purity of the sample was determined by the optical density at 280 μm (Anderson, 1967) (Fahimi and Drochmans, 1966).

Fixatives were buffered in 100 mM cacodylic acid. When glutaraldehyde was being prepared, distilled water equal to eighty percent of the final volume was used to dissolve the cacodylic acid (and sucrose for osmolarity purposes when used). The acid was titrated to approximately pH 7.5 with 10 N sodium hydroxide before glutaraldehyde was added. After the addition of glutaraldehyde the pH was readjusted to pH 7.4 and the solution was
quantitatively transferred to a volumetric flask and
diluted to its final volume with distilled water.

Formaldehyde fixatives were prepared on the day of
use from paraformaldehyde powder. Forty percent of the
desired final volume of distilled water was heated to 60-
70 degrees centigrade and measured paraformaldehyde pow­
der was added to this solution with stirring. 10 N sodium
hydroxide was added slowly and dropwise until the solution
was clarified. This solution was quantitatively trans­
ferred to a beaker containing two times concentrated cacodylic acid in fifty percent of the final desired volume.
This solution was then adjusted to pH 7.4 with 10 N sodium
hydroxide and quantitatively transferred to a volumetric flask where it was diluted to the final volume.

Osmium Tetroxide Preparation

Phosphate buffered osmium tetroxide when used was
prepared by the method of Millonig (Millonig, 1961) by the
Ohio State University reagents laboratory to a final
concentration of 1 percent osmium tetroxide and 100 mM
phosphate. Cacodylate buffered osmium tetroxide was pre­
pared in two parts. Sealed ampules containing 1/4 gram of
osmium crystals were scored with a metal file and placed
in a glass bottle containing 12.5 ml of distilled water.
The remaining procedure was carried out in a fume hood,
rubber gloves and eye protection were worn. The glass
bottle was allowed to stand at room temperature until all the osmium was dissolved (usually two days). This 2 percent osmium tetroxide solution was then refrigerated. The second solution made up was 200 mM cacodylic acid solution titrated to pH 7.4 with 10 N sodium hydroxide and also refrigerated. Minutes prior to use, these two solutions were mixed in the ratio of 1:1 yielding a 1 percent osmium tetroxide 100 mM sodium cacodylate solution pH 7.4.

Preparation of Cytochemical Solutions

The lead containing medium was modified after that of Wachstein and Meisel (1957). The basic medium contained 145 mM sodium chloride, 5.8 mM potassium chloride, 2.0 mM magnesium chloride, 100 mM buffer (various), 2.0 mM lead acetate, 2.0 mM adenosine triphosphate, and 250 mM sucrose. Occasionally from 10-50 mM Tiron (4,5-dihydroxy-m-benzene-disulfonic acid disodium salt), a lead chelate, was added according to the modification of Wollenberger (Schulze et al., 1972). The solution was prepared in one of two ways. Either the entire solution was made up on the day of use, or a solution of sodium chloride, potassium chloride, magnesium chloride, buffer, and inhibitor (10⁻³ or 10⁻⁴ ouabain) and Tiron (when used) was made up days prior to use, titrated to pH 7.4 and frozen. In both cases, however, the inhibitor, ouabain was added only after the buffer had been titrated to the desired pH. Lead and
ATP were added only on the day of use and only after the solutions were titrated to pH 7.4. They were checked after ouabain addition for the proper pH.

Immediately after preparation these solutions were put into an ice bath for storage until use 30 to 120 minutes later. If the solution was not used for an hour after final preparation the pH was again checked. A decrease in the pH was interpreted as ATP degradation and the solution was discarded.

The strontium containing medium was made up according to the method of Ernst (Ernst and Philpott, 1970). The cytochemical medium contained 10 mM potassium chloride, 10 mM magnesium chloride, 20 mM strontium chloride, 100 mM Trizma HCl buffer, 5 mM para-nitrophenyl phosphate and 250 mM sucrose.

Preparation of the strontium containing solution is similar to that of the lead containing solution. Differences in the preparation include titration of the Trizma buffer (in some experiments) with 1 N potassium hydroxide rather than sodium hydroxide. Inclusion of a second inhibitor, 10⁻² M cysteine, as well as a solution containing 10⁻² M cysteine plus ouabain. The addition of para-nitrophenyl phosphate (PNPP) was treated as the addition of ATP; except, in some cases, PNPP solutions were kept frozen without formation of detectable color. The strontium containing cytochemical solution required a
complement 2 percent lead nitrate solution in order to provide successful visualization of phosphate entrapment because strontium is not a particularly electron dense metal. Following incubation in the cytochemical media and washing of specimens with Trizma buffered sucrose, this lead solution was superfused onto the specimens for one hour at room temperature.


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