A STUDY OF MEMBRANE PROTEINS AND ION TRANSPORT PROCESSES IN RED BLOOD CELLS OF THE SPONTANEOUSLY HYPERTENSIVE AND WISTAR-KYOTO RATS

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Frank Harvey Shiffman

Department of Pharmacology and Therapeutics

Faculty of Medicine

Winnipeg, Manitoba

ISBN 0-315-33991-8

A STUDY OF MEMBRANE PROTEINS AND ION TRANSPORT PROCESSES IN RED BLOOD CELLS OF THE SPONTANEOUSLY HYPERTENSIVE AND WISTAR-KYOTO RATS

BY

FRANK HARVEY SHIFFMAN

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

THIS THESIS IS DEDICATED TO

MY FAMILY:

MY PARENTS DAVID AND MARGARET

MY BROTHERS AND SISTER

MARTIN, LAWRENCE, MURRAY AND SHARON

MY NEPHEW KENNY

AND MY NIECE SIMCHA

ABSTRACT

Essential hypertension is a pathological condition manifest as a sustained elevated blood pressure, the etiology of which remains unknown. Indications are that the pathogenesis of this disease is partly dependent on hereditary factors. We studied red blood cell membranes of spontaneously hypertensive rats and their controls as a model for genetically transmitted membrane abnormalities. Our first objective was to determine whether or not a simple diagnostic test for the identification of the spontaneously hypertensive trait could be developed based on the rates of lactate release of the red blood cells. This endeavor was partially successful as we were able to measure differences in the rates of lactate release when the cells were incubated in a medium containing manganese, furosemide and ouabain. Manganese by itself reduced the rates of lactate release of the cells, however, the cells of the hypertensive rats responded with ten times the sensitivity than that observed in the control strain. These results suggested that a site of interaction between cellular calcium pools and sodium and potassium transporting systems may be the location of a defect in the membrane's ion regulatory processes in hypertension. A direct study of the role of calcium in the regulation of the sodium, potassium pump activity indicated that the presence of calcium has an inhibitory effect on the sodium, potassium pump. Removal of calcium from the medium stimulated the pump activity to a greater extent in the cells of the hypertensive rats than in the controls, suggesting that the membranes of the hypertensive rat cells had a lower affinity for the binding of calcium. Removal of calcium also increased the sensitivity of the (Na^+-K^+)-ATPase to ouabain, hence it is conceivable that in cells of

SHR, where the membrane binding of calcium is decreased, the (Na^+-K^+)-ATPase may have an increased sensitivity to circulating ouabain-like factors.

In order to determine whether or not calcium handling mechanisms were indeed a location of the primary defect(s) in hypertension, calcium influx was studied using a radioactive calcium isotope. Manganese has been shown to produce a contraction in SHR vascular smooth muscle, a defect present prior to development of hypertension. It was observed that manganese increased the net accumulation of calcium by the cells and that this increase was greater in the cells of the hypertensive rats. Calcium uptake in the absence of manganese was not different between SHR and WKY rat red blood cells. These results therefore suggested that the site of action of manganese was in the calcium handling processes of the cells. $(Ca^{2+}-Mg^{2+})$-ATPase activity was found to be slightly lower in the SHR and manganese inhibited the ATPase. The Ki for manganese was not found to be significantly different. The calcium pump was shown to be less efficient in SHR red blood cells and smooth muscle by others. Intracellular calcium has been found to be higher in SHR red blood cells. These results taken together would suggest that a defective (Na^+-K^+) cotransport leads to a higher intracellular sodium and consequently a higher intracellular calcium which can be further increased in the SHR cells by manganese inhibition of the $(Ca^{2+}-Mg^{2+})$-ATPase.

Studies of the kinase dependent phosphorylation-dephosphorylation process indicated the presence of a defective membrane protein of molecular weight of 140,000 daltons in the cells of the spontaneously hypertensive rats. Since the molecular weight of the red blood cell calcium pump has been identified to be approximately 140,000

daltons it would appear that it is the calcium pump which is defective in these cells.

In summary the data in this thesis suggest that the primary defect in hypertension resides in the membrane protein responsible for the extrusion of calcium and that this defect can secondarily alter the activity of sodium transport mechanisms.

ACKNOWLEDGEMENTS

I would like to thank the following people who have helped me, both academically and spiritually.

Dr. Ratna Bose: for taking me into her laboratory and sharing with me her knowledge, skills and experience. I would also like to thank her for her words of encouragement and endless patience;

Dr. Deepak Bose, Dr. Clive Greenway, Dr. Ivan Bihler, Dr. John McKenzie and the rest of the Faculty and Staff at the Department of Pharmacology and Therapeutics: for the opportunity to study in the Ph.D. program and their assistance in my project and course work;

The people whom I have had the pleasure of working with, Ms. Pat Konantz, Mrs. Teressa Chau, Miss. Cindy Moore and Mr. Dan Moquin: for their friendship as well as technical assistance;

Mrs. Peggy Merrick: for her friendship, aid in preparation of the figures and cheerfulness each morning;

Dr. Luisa Bigornia, Dr. Frank Burcyznski, Miss. Cara MacIntyre Dr. Ken Seaman and all of the other graduate students of the Department of Pharmacology and Therapeutics: for their friendship and for sharing with me their knowledge and good times;

My Family: for continued support and understanding in my career endeavors;

Elaine Barrett-Cramer: for her support, encouragement, assistance and companionship. Words cannot express my appreciation and gratitude for her;

The Canadian Heart Foundation: for the award of a Research Traineeship.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

SECTION I

INTRODUCTION

In this thesis red blood cell membranes are studied in order to elucidate possible defects in membrane proteins which may have integral roles in the development of hypertension. The following introduction therefore will review the liturature pertaining to the proteins which have been identified within the membranes of red blood cells as well as ion transport processes related to these proteins. Furthermore this review will include information on the activities of these transport mechanisms in hypertension.

A. RED BLOOD CELL MEMBRANE PROTEINS

1. CHARACTERIZATION

i. DETERMINATION OF MOLECULAR WEIGHT

A useful technique for separation of proteins is the sodium dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (Freifelder, 1976). At neutral pH, in a 1% SDS, 0.1% mercaptoethanol solution, most proteins are completely denatured by complexing with the SDS and mercaptoethanol. The mercaptoethanol breaks disulfide linkages while the SDS complexes with protein in a constant ratio of 1.4 grams of SDS per gram of protein. This results in randomly coiled peptides which behave as if they have a uniform shape, hence equal charge to mass ratios. An electrical current is employed to move the proteins through a polyacrylamide gel which contains the detergent SDS. Under these conditions the effective mobility of each protein through the gel is directly related to its molecular weight. When a series of proteins with known molecular weights are treated in this manner and electrophoresed, the distance of the proteins from the point of origin plotted against the log of their molecular weights produces a straight line. This technique therefore allows for the estimation of the molecular weights of unknown proteins by simply measuring the distance migrated and comparing to proteins of known molecular weights.

Fairbanks et al. (1971) employed this technique to study the proteins of human erythrocyte membranes. Electrophoresis of the ghost proteins in a 5.6% polyacrylamide gel containing 1% SDS consistantly separated six well defined coomassie blue stained bands, which Fairbanks named bands I-VI. Periodic Acid-Schiff reagent (PAS) was employed as a stain for the identification of carbohydrate bands on the gel. The electrophorograms displayed one PAS-positive band just before the tracking dye and three others which were designated PAS-1, 2, and 3. None of the PAS bands coincided with coomassie blue stained bands. Fairbanks estimated the stoichiometric ratios of bands (I+II):IV:V:VI to be 1:1:1:1. The band III proteins were 2.5 times more abundant than the VI proteins. The molecular weights were calculated to be aproximately 250,000 for bands I and II, and 89,000 D, 77,500 D, 41,300 D and 36,200 D for III, IV, V and VI respectively. The PAS-stained bands have the molecular weights of 83,500 D, 45,600 D and 25,500 D (Fairbanks et al., 1971).

ii. LOCALIZATION

Further studies of these protein bands in relation to the plasma membrane were performed by Steck et al. (1971). In addition to the methods for preparation of membrane ghosts outlined by Fairbainks, they employed techniques which enabled them to prepare and purify inside-out membrane vesicles in which the membrane's cytoplasmic surface is susceptible to selective attack. Each of these membrane surfaces were studied independently by partial digestion with impermeable proteolytic enzymes and subsequent SDS polyacrylamide electrophoresis.

Intact red blood cells which were subjected to the proteolytic enzyme trypsin, chymotrypsin, and papain, prior to isolation and electrophoresis of the membranes, did not reveal any proteolytic

digestion of the coomassie blue staining proteins. However, the major PAS-stained sialoglycoproteins were readily digested by all three enzymes. Isolated ghosts proved to be more suseptible to the enzymes since all but band VI displayed evidence of partial digestion. When band VI was isolated from the ghost membranes by 0.15M NaCl, it remained resistant to these enzymes. Only following treatment with 1% SDS did this protein band become susceptible indicating that its resistance to proteolytic digestion is not a consequence of its position within the membrane but that it is an intrinsic property of the protein.

The proteins of right-side-out vesicles were similarly affected by the three digestive enzymes. However, when inside-out vesicles were treated with these enzymes, the electrophoresis patterns revealed a more selective action. Only band III diminished, resulting in the appearance of a new band between IV and V. This band did not appear following digestion of right-side-out vesicles. Trypsin, chymotrypsin and papain all produced the same result. The three PAS staining bands were susceptible to these treatments in both the inside-out and right-side-out vesicles.

Based on these findings, Steck and coworkers formulated the following model. All red blood cell proteins are exposed at the outer surface of the membrane. Only the PAS-stained sialoglycoproteins and the band III proteins penetrate the membrane and are exposed at the inner surface. Both are asymmetrical in their orientation. The sialic moieties of the sialoglycoproteins are exposed exclusively at the outer side. The proteins of bands I, II and V are loosely associated with the membrane, compared to band IV. The localization of band VI could not be determined due to its intrinsic resistance to proteolysis.

iii. DETERMINATION OF ISOELECTRIC POINT

For greater resolution than the SDS electrophoresis, proteins can also be separated by their isoelectric points. This technique is dependent on the amphoteric properties of proteins. Proteins can be either neutral, or positively or negatively charged depending on the surrounding pH. The pH at which the protein has no net charge is the isoelectric point, which is an intrinsic property of proteins. When an electrical current is applied across a pH gradient, the charged proteins will migrate towards either the anode or cathode, depending on whether they are negatively or positively charged. However, as they move through the gradient, the surrounding pH is changed. When the protein reaches a pH which is equivalent to its own iso-electric point, it is no longer charged and ceases to move. Providing that the current is applied to the proteins for a long enough period or time, they will end up at a position in the gradient at which the pH corresponds to their iso-electric points. Isoelectric focusing and gel-electrophoresis employed together is referred to as a 2-Dimensional Electrophoresis (O'Farrell, 1975).

2. PROPERTIES

Proteins of the red blood cell plasma membrane are generally considered to be of either structural or metabolic importance and can be classified as either peripheral or integral.

Cytosolic peripheral proteins, which are metabolically active, include enzymes such as acetylcholine esterase and are involved in cellular processes. Other metabolic membrane proteins are transport proteins which are responsible for removing waste products from the cell, supplying the cell with required substances from the extracellular fluid, maintaining proper ion gradients and buffering the intracellular

pH. The structural proteins form a membrane cytoskeleton which is exclusive to the red cells. In order to traverse the capillaries, the flexible cytoskeleton allows the red blood cell to conform to the small capillary openings, yet, it is highly resistant to the stressful forces incurred while circulating through the cardiovascular system. It consists of a network of proteins, some of which are deeply embedded into the lipid bilayer (integral proteins), and others which are bound to the membranes' inner surface (peripheral proteins). Peripheral proteins can be removed from the membrane by alterations to the ionic environment. The integral proteins, which can only be isolated from the membrane by treatments which drastically disrupt the membrane, generally span the bilayer and are exposed on both sides of the membrane.

The functional importance of the cytoskeleton proteins, has been demonstrated in several different ways. The peripheral proteins, spectrin, actin, and band 4.1 can be extracted by low ionic media. This treatment will cause membranes to lose their structural integrity and undergo extensive vesiculation. Treatment of red cell membranes with the non-ionic detergent triton X-100 will solubilize the lipids and integral proteins. The remaining proteins form a skeleton which retains the original shape of the red cell (Tomselli et al., 1981).

Many inherited disorders of abnormally shaped cells are associated with defective or absent cytoskeleton proteins. For example, red cells from a strain of mice with hereditary spherocytosis have been analysed by SDS gel electrophoresis and found to be deficient in proteins of both spectrin bands (Greenquist et al, 1978). In this disease the red cells lose their normal biconcave shape and become spherical. In hereditary elliptocytosis of human red cells, the amounts of the spectrin components remain normal, however the spectrin itself is defective (Lui et al., 1982).

i. Spectrin

Spectrin, identified as bands 1 and 2 by SDS polyacrylamide-gel electrophoresis, is the largest component of the membrane cytoskeleton. It accounts for 25% of the total red cell protein and 75% of the cytoskeleton mass. It is a cytoplasmic peripheral protein consisting of two subunits. The alpha subunit has a molecular weight of 240,000 daltons and the beta has 220,000 daltons. Spectrin can exist as an alpha-beta dimer or an $alpha_2$-$beta_2$ tetramer. The tetrameric complex is a result of head to head association of two dimeric proteins. (Shotton et al., 1979) The beta chain is phosphorylated by cAMP-independent (Fairbanks et al., 1978) and possibly cAMP and calmodulin-dependent mechanisms (Boivin et al., 1981). In addition, there have been reports of auto-phosphorylation and also the involvement of Ca^{++} stimulated Mg^{++}-dependent ATPase (Baskin and Langdon, 1981).

ii. Actin

Actin is a relatively ubiquitous eucaryotic cell protein also found in red blood cells. Generally it exists in vivo as double-helical filaments, 5nm in diameter. Each monomeric subunit is a protein of molecular weight 42,000 daltons, identified as band 5 on the SDS-electrophorograms (Tilney and Detmers, 1975). Erythrocyte actin is very similar, although not identical, to muscle actin. Iso-electric focusing revealed erythrocyte actin to be primarily the beta isomer while muscle actin is exclusively an alpha isomer (Nakashima and Beutler, 1979).

iii. Band 4.1

Band 4.1 is a globular peripheral protein with a molecular weight between 78,000 daltons and 82,000 daltons. When separated in a discontinuous buffer system two distinct proteins bands are revealed,

bands 4.1a and 4.1b. Both have similar peptide sequences. They are phosphorylated to the same extent by cAMP-dependent and possibly cAMP-independent kinases. It is believed that this protein is associated with spectrin in the membrane since it complexes with purified spectrin in vitro. (Goodman et al., 1982)

iv. Band 4.9

Band 4.9, a 48,000 dalton phosphoprotein and is present in equimolar concentrations with the spectrin tetramers. Although it is believed that this protein may interact with actin filaments to reduce the rate of actin polymerization, and increase stability of the actin oligomers, its exact function is unknown. (Siegel and Branton, 1982)

v. Ankyrin

Ankyrin, was identified as band 2.1 with a molecular weight of 200,000 daltons. It is not an essential component of the cytoskeleton, since cytoskeletons prepared by triton treatment maintain their shape without the presence of ankyrin. It is a peripheral protein and contains specific high affinity, binding sites for spectrin. Ankyrin also maintains a tight association with the cytoplasmic side of the integral band 3 protein in a 1:1 stoichiometric ratio (Bennett and Stenbuck, 1979), thereby performing the structural function of linking the cytoskeleton with the plasma membrane.

vi. Glycophorins

PAS staining of gels by Fairbanks's procedure reveals 4 bands. The three major bands PAS 1, 2, and 3 have molecular weights of 83,000 daltons, 45,000 daltons, and 25,000 daltons respectively (Fairbanks et al., 1971). Band PAS 4 appears between PAS 1 and PAS 2. Glycophorin A is believed to be present in both PAS 1 and PAS 2 bands. Evidence suggests that PAS 1 is a dimeric form of the protein in the PAS 2 band. The dimer

is formed by two glycophorin A molecules bound to each other by their hydrophobic regions (Furthmayr and Marchesi. 1976).

Glycophorin A comprises 75% of RBC membrane sialoglycoproteins. It is a simple polypeptide chain of 131 amino acid residues with 16 oligosaccarides, 15 of which are tetrasaccharides attached to either serine or threonine residues (Thomas and Winzler, 1969). The 16th residue is a large asparagine bound oligasaccharide (Kornfeld and Kornfeld, 1970). These amino acid residues are all located in the amino-terminal end of the polypeptide chain. The majority of the aminoacids of the central region are non-polar. This implies that this section of the peptide chain spanning from residue 71 to 90 is embedded within the lipid structure.

Human erythrocyte glycophorin A has been shown to be phosphorylated on the C-terminal end. When intact cells are studied, only 1% of the glycophorin molecules are phosphorylated by incubation with radioactive phosphate. This appears to be due to a very selective action of the phosphorylating mechanisms (Shapiro and Marchesi, 1977).

vii. Band 3

Band 3 appears as a large diffuse band on SDS-gel electrophoresis, exhibiting a molecular weight of 90,000 daltons. This protein penetrates the plasma membrane and may exist in the membrane as a dimer. Carbohydrates compose 5 - 8% of its mass, and heterogeneity of the carbohydrate may be responsible for the separation of the purified protein as a diffuse band (Yu and Steck, 1975). The protein can be separated into three distinct regions. Chymotrypsin treatment of intact erythrocytes cleaves off a 38,000 dalton peptide. This is the portion of the protein which is exposed on the outer surface of the membrane. The remaining 55,000 dalton fragment, which is embedded in the membrane can

be further separated into two fragments by mild trypsin and S-cyanylation cleavage (Steck et al., 1976). Of the resulting two fragments, 41,000 and 23,000 dalton peptides, the latter is phosphorylated and therefore probably represents the region exposed within the cell cytosol (Drikamer, 1977).

The Band 3 protein is now known to function as the mediator of anion transport. This was demonstrated with a stilbene derivative ^{3}H-DIDS which inhibits anion permeability and forms covalent bonds with whichever polypeptide it binds to. This agent has been found to bind to the band three protein of intact erythrocytes. (Cabantchik and Rothstein, 1974)

viii. $(Na^{+}-K^{+})$-ATPase

The sodium potassium pump is an ubiquitous enzyme responsible for maintanence of the Na^{+} and K^{+} gradients. In some cells, such as nerves, it plays a major role in cell function. In the red blood cells the contribution of this pump to cell function is not as significant. Hoffman (1969) estimated that human red blood cells contains as few as 200 $(Na^{+}-K^{+})$-ATPase's per cell, each having a turnover rate of 6000 K^{+} ions per min. The functional complex contains two different glycoprotein subunits as well as a phospholipid component, which accounts for 30% of the total weight.

The molecular weights of the subunits have been determined most accurately by sedimentation analysis in the absence of detergents (Peters et al., 1981). Prior estimates were complicated by the solubilizing of disproportionate amounts of the lipids by the detergents. The larger glycoprotein, alpha subunit, has a total molecular weight of 131,000 daltons, the protein component being 120,600 daltons. The beta subunit, is a total of 61,800 daltons, 42,800 daltons

for the protein. The alpha : beta ratio has been determined to be 1:1. Considering the estimated molecular weights of the complex, which have been as high as 380,000 daltons (Hastings and Reynolds, 1979), it appears that the enzyme is an $alpha_2$: $beta_2$ tetramer. (Peters et al., 1981) This postulate is further supported by electron microscopic studies (Haase and Koepsell, 1979).

The alpha subunit is believed to be the catalytic subunit since it contains the phosphorylation site (Hokin et al., 1973) and the binding sites for ATP (Haley and Hoffman, 1974) and ouabain (Kott et al., 1975). Ouabain and ATP bind at opposite sides of the membrane hence the alpha subunit penetrates the membrane, being exposed on either side.

The beta-subunit is a sialoglycoprotein, 13-31% carbohydrate. Variations in the sugar moiety result in a heterogenous population, detected when separated by isoelectric focusing (Marshall and Hokin, 1979). Shamoo and Myers (1974) suggest that this subunit functions as a Na^+ ionophore, its activity being modulated by interactions with the alpha-subunit and K^+. Beta-subunit specific antibodies inhibit ouabain from binding to the alpha subunit but do not affect phosphorylation. This implies that the alpha and beta subunits interact on the outer side of the membrane (Jean and Albers, 1977).

The number of ATP and ouabain binding sites per enzyme is presently debated. Originally Jorgensen and Petersen (1977) reported only one site of each per enzyme. Considering that the (Na^+-K^+)-ATPase is most likely a tetramer containing two identical alpha subunits, this finding is difficult to explain. Peters et al., (1981) suggested that due to previous errors in molecular weight estimation and Lowry protein determinatons, the phosphorylation capacity has been underestimated. His calculation estimates 1.9 phosphorylation sites on each enzyme complex.

The phospholipid component has also been a subject of considerable study. The purified functional (Na^+-K^+)-ATPase complex contains about 270 moles phospholipid per mole of enzyme. A minimum of 90-100 are required to maintain maximal activity, demonstrated by the observation that depletion of selective phospholipids by treatments with specific phospholipases and other enzymes, did not significantly affect enzyme activity (De Pont et al., 1978). The types of fatty acids surrounding the enzyme appear to be of great importance to enzyme activity. There are about 50 moles of lipid, most of which are negatively charged and directly associated with the protein. Within this annulus a certain fluidity must be maintained (Brotherus et al., 1980).

iv. (Ca^{2+}-Mg^{2+})-ATPase

Prior to 1979, little information about the physical properties of the calcium-transport protein of erythrocyte membranes was known. This was due to the difficulties that existed in obtaining purified preparations.

The (Ca^{2+}-Mg^{2+})-ATPase represents only a minor component of the total membrane protein, and its molecular weight is near the range of the abundant band 3 protein. It was known to have a specific requirement for acidic phospholipids (Ronner et al., 1977), and to be directly activated by calmodulin (Gopinath and Vincenzi, 1977; Jarrett and Penniston, 1977; Niggli et al., 1979b). Niggli and coworkers were able to exploit the calcium-dependent formation of the calmodulin-ATPase complex in order to purify the calcium-ATPase using a calmodulin affinity chromatography column (Niggli et al., 1979a). The result of this isolation procedure yielded three proteins identifiable by SDS-polyacrylamide electrophoresis. A band exhibiting an apparant molecular weight of 125,000 daltons represented the majority of the

protein. Two minor bands with molecular weights of 205,000 daltons and 90,000 daltons represented 11% and 6% of the protein respectively. Labelling with [^{32}P]-ATP revealed that only the 205,000 dalton and 125,000 dalton molecular weight bands were phosphorylatable. In a subsequent paper they reported a corrected value of the molecular weight for the major band to be 140,000 daltons (Niggli et al., 1981a)

The (Ca^{2+}-Mg^{2+})-ATPase can be interconverted between two states, one of low calcium affinity (Km; 10-14 uM) and another of high calcium affinity (Km; about 1 uM). Conversion from the low affinity to the high affinity state is induced by calmodulin binding, or when reconstituted into liposomes containing a high content of acidic phosphospholipids or the unsaturated fatty acids, oleic and linoleic acid. Limited proteolysis with trypsin also mimicks calmodulim activation. (Niggli et al., 1981b)

B. RED BLOOD CELL ION TRANSPORT MECHANISMS

Intact red blood cells have been shown to possess several active and passive mechanisms by which various ions and substrates are transported across the cell membrane. A brief review of sodium, potassium and lithium transport in human red cells has recently been presented by Tosteson (1981a). Of these, the sodium-potassium pump has the most significant role the maintainance of the ion gradients. Normally it exchanges three intracellular sodium ions for two extracellular potassium ions at the expense of one ATP but can also mediate other ion exchanges and also an uncoupled Na^+ extrusion (Tosteson, 1981a). Other sodium and potassium transport mechanisms present in human red blood cells are Na^+-Na^+ exchange, Na^+-K^+ cotransport, a calcium activated potassium channel and a choline transporter. The Na^+-Na^+ exchanger and the Na^+-K^+ cotransport have been implicated in many hereditary disorders (Parker and Berkowitz, 1983). Calcium induced potassium transport was discovered in red blood cells by Gardos in 1959, yet its function in this cell remains unknown. The choline transport system primarily transports the choline ion but Na^+ or K^+ ions can be substituted. Two other transport systems of major importance are the calcium ATPase and the anion transporter. The calcium ATPase is an ATP consuming pump which maintains a low intracellular calcium concentration. The anion transport mechanism, primarily for HCO_3^-, is an important component of respiration.

1. SODIUM - POTASSIUM PUMP

The discovery of an ATP dependent, sodium and potassium pump in red blood cells was the result of cummulative efforts of several investigators over a period of time beginning around the early 1940's. Post et al. (1960), finally demonstrated in human red blood cells the

existence of an adenosine triphosphatase activity linked to obligatory Na^+ and K^+ transport. Bonting et al., (1962), reported specific activity to be 0.03 micromoles Pi X h^{-1} X mg^{-1} protein. The stoichiometry of this pump is now well accepted to be three sodium and two potassium exchanged for each ATP hydrolysed, which occurs on the cytosolic side of the membrane only (Sen and Post, 1964). The Km for ATP binding at this site varies between 1 micromolar, in the absence of potassium, and 20 micromolar, in the presence of 0.1 millimolar potassium (Post et al., 1965). A second ATP binding site, of lower affinity (Km; 0.48 mM), was later reported. This site is involved in allosteric activation of the enzyme (Post et al., 1972).

Ouabain sensitive 1-1, Na^+-Na^+ exchange, first observed by Glynn (1956, 1957) was characterized further by Garrahan and Glynn. Increasing concentrations of Na^+ in the external media linearly increased this exchange process (Garrahan and Glynn, 1967a). It was inhibited by small amounts of K^+. A minimal amount of ATP (0.3 mM) is an absolute requirement, although no significant ATP hydrolysis has been detected (Garrahan and Glynn, 1967b). Raising the ATP concentration from 0.3 to 1.5mM does not affect ouabain sensitive Na^+-Na^+ exchange, however a linear relationship to the ADP concentration does exists (Glynn and Hoffman, 1971).

Ouabain sensitive K^+-K^+ exchange in human red cells was characterized by Simons (1974). Working with resealed erythrocyte ghosts, he determined that this system required 10mM internal K^+ and 0.3mM external K^+ for maximal activation and was strongly inhibited by internal or external Na^+. Internal Pi stimulated activity, therefore indicating that pump reversal may be involved. ATP was necessary (Km; 0.1mM) although non-metabolizable ATP analogues could be

substituted suggesting an ATP requirement of the low affinity, non-phosphorylating site (Simons 1975).

Ouabain sensitive uncoupled Na^+ efflux has been demonstrated to occur in the absence of external Na^+ and K^+ (Beauge and Ortiz, 1973). Hydrolysis of ATP is involved and external Na^+ inhibits this transport in favor of Na^+-Na^+ exchange (Glynn and Karlish, 1976).

2. CALCIUM PUMP

Human red blood cell ATP and Mg^{2+} dependent Ca^{2+} extrusion was first reported by Schatzmann who studied Ca^{2+}-loaded resealed ghosts (Schatzmann, 1966). The ATP binds to the cytosolic side of the enzyme with a high affinity (Km; 50uM) for hydrolysis, in order to provide the energy for calcium translocation (Wolf, 1972). The Mg^+ must be present on the cytosolic side of the enzyme, and it is not transported (Schatzmann and Vincenzi, 1969). Intracellular calcium (0.1-1.0uM) stimulates the pump to 50% maximal activity (Wolf, 1972). The maximum rate of calcium efflux is 4-10 mmoles X $l^{-1}$of cells X h^{-1} (Sarkadi et al., 1977). Extracellular Ca^{2+} concentration does not affect activity (Schatzmann and Vincenzi, 1969).

The stoichiometry of this pump in intact cells was originally determined to be 1 Ca^{2+} per ATP hydrolyzed (Schatzmann and Vincenzi, 1969). This value however, was an underestimate due to the presence of Ca^{2+}-stimulated ATPase, not associated with calcium transport. A ratio of 2:1 was later determined by comparing lanthanum-sensitive Ca^{2+} efflux to lanthanum-sensitive ATP hydrolysis (Sarkadi et al., 1977).

Jarrett and Penniston (1977) and Gopinath and Vincenzi (1977) found that this Ca^{2+} and Mg^{2+}-dependent ATPase could be activated by the protein calmodulin. In human erythrocytes this activation is a result of a direct interaction between the calmodulin and the transport enzyme (Niggli et al., 1979b).

(Ca^{2+}-Mg^{2+})-ATPase activity is present in a few different tissues. However, besides the erythrocyte, only in dog heart sarcolemma (Caroni and Carafoli, 1980) and sarcoplasmic reticulum (SR) has it been directly linked to calcium pumping activity. In most respects these enzymes appear to be quite similar. The SR-pump transports two calcium per ATP (Makinose and Hasselback, 1971), with affinities for ATP and calcium binding which are similar to those of the red blood cell (Hasselbach et al., 1975) and sarcolemma (Caroni and Carafoli, 1981). The major differences between the enzymes are in their ability to function in reverse and the mechanism by which Ca^{2+} modulates the activity. The SR-calcium ATPase is capable of synthesizing ATP during backwards calcium transport (Makinose and Hasselbach, 1971), while the erythrocyte calcium ATPase does not. In the erythrocyte and sarcolemma, calcium modulates activity by direct interaction between calmodulin and the enzyme. In cardiac SR, a 22,000 dalton molecular weight phosphoprotein, phospholamban, is involved (Tada et al., 1982).

3. ANION EXCHANGE

The anion exchange capability of the red blood cell is an important aspect of respiration. In tissue capillaries, CO_2, a by-product of cellular metabolism, diffuses across the red cell membrane into the cells. The carbonic anhydrase enzyme catalyses hydration of the CO_2 molecule to form HCO_3^-. This intracellular anion is transported back across the membrane into the extracellular fluid in exchange for Cl^-. Hence the bulk of the CO_2 is eliminated from the tissues as HCO_3^- dissolved in the plasma. In the lung capillaries this process is reversed, thereby liberating CO_2 gas which is cleared into the alveoli. Since transit time of the red cells through capillaries is very short, a rapid, high capacity anion transporter is necessary. This was

demonstrated by Harris and Pressman (1967). They treated cells with valinomycin, which acts as a potassium ionophore. However, even with an increased permeability to potassium, the cells did not exhibit rapid potassium efflux. They reasoned that the K^+ loss was prevented by increasing negativity within the cell. This conclusion implies that although Cl^- moves rapidly across the membrane, it can only do so in exchange for another anion.

Transport of HCO_3^- and Cl^- can be competitively inhibited by $NaCO_3^-$, $LiCO_3^-$, HCO_3^-, halides, sulfate, phosphate and some organic anions, which are also transported (Tosteson, 1981a; Passow, 1969; Wieth, 1970). The anion transporter is also non-competitively inhibited by anion binding at another site, of low affinity (Dalmark, 1976).

The anion exchange mechanism is one of the few red cell transport processes for which a protein has been identified. Cabantchik and Rothstein (1972; 1974) labeled intact red blood cells with radio-active 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS), a potent irreversible inhibitor of anion transport (Funder, 1978). Greater than 90% of the label bound to a single intrinsic membrane protein of 95,000 dalton molecular weight, corresponding to Fairbanks band III protein. The remainder was found in the lipid and bound to a glycoprotein. Mild proteolytic digestion which removes the glycoprotein bound DIDS does not affect anion transport, therefore Cabantchik and Rothstein concluded that the anion transport is a property of the band III protein.

4. SODIUM-SODIUM EXCHANGE

i. HUMAN RED BLOOD CELLS

In human red blood cell membranes there exists a ouabain-insensitive 1:1 Na^+-Na^+ exchange diffusion process (Hoffman and

Kregenow, 1966). Most studies on this transport mechanism take advantage of its capability to transport lithium in place of sodium in either direction and measure ouabain-resistant lithium fluxes as an indicator of transport activity. Hence the term "sodium-lithium" countertransport is often used.

Lithium transport in RBCs was initially suggested by the observation that for patients on chronic lithium therapy the ratio of lithium in the red blood cell to lithium in the plasma is about 3:1. Assuming simple diffusion for the monovalent cation, a value of 1.2 would be expected. This seemingly 'active' transport has been attributed to a Na^+-Li^+ exchange mechanism on the basis of observations that low sodium media enhanced lithium accumulation by normal RBCs and reduced lithium extrusion from lithium loaded RBCs in vitro (Haas et al., 1975; Duhm et al., 1976; Pandey et al., 1978). In vivo this pathway is believed to be the major route of lithium extrusion (Tosteson, 1981b).

Further characterization of this pathway revealed sensitivity to phloretin (1mM) (Duhm et al., 1976; Pandey et al., 1978), furosemide (1mM), ethacrynic acid (1mM), quinine and quinidine (Pandey et al., 1978) and resistance to the agents ouabain and amiloride (Duhm and Becker, 1977a; Pandey et al. 1978). Inhibitory effects of furosemide or ethacrynic acid could not be observed by Duhm and Becker (1977). Depletion of cellular ATP did not appear to affect its activity (Duhm et al., 1976; Pandey et al., 1978).

Kinetic analysis by Sarkadi et al. (1978) found the exchange of Na^+ for Li^+ to be one for one. They reported an affinity for Li^+ almost 20-fold the affinity for Na^+ on both sides of the membrane. The ionic concentrations required for half-maximal activity are 9.0mM Na^+ and 0.5mM Li^+ on the inside and 25.0mM Na^+ and 1.5mM Li^+ on the outside.

Duhm and Becker (1977b) reported similar K_m values of 1.3-1.5mM Li^+ for efflux and 1.4-1.8mM for influx. Both authors reported considerable variation of the maximal rate, among the individuals tested, 0.14 to 0.37 mmol/(liter cells X hr) (Sarkadi et al., 1978) and 0.47 to 1.40 umol/(ml cells X hr) (Duhm and Becker, 1977b) for Li^+ efflux. It now appears that the maximum rate is dependent on several genetic and pathological factors (Parker and Berkowitz, 1983).

The physiological role of this transport mechanism is uncertain although there is evidence that it may function as a Na^+-H^+ exchanger (Dissing and Hoffman, 1982; Milanick and Hoffman, 1982).

ii. RENAL PROXIMAL TUBULE EPITHELIA

Mammalian kidneys secrete hydrogen ions through a sodium-hydrogen exchange mechanism found in the proximal tubule (Murer et al., 1976). This mechanism has been localized to the luminal membrane and may be similar, if not identical to the sodium-sodium exchange mechanism of red blood cells (Aronson, 1982). The sodium-hydrogen exchange is a component serving the overall cell function of proton secretion and/or sodium reabsorption.

Several characteristics have been defined and show considerable similarity to those of the red blood cell sodium-lithium exchanger. Lithium is easily accepted and transported in place of hydrogen (Kinsella and Aronson. 1981), and hence competitively inhibits sodium-hydrogen exchange. The affinity of the transporter for lithium is much greater (Km=1.2$\pm$0.1 mM) than its affinity for sodium (Km=14.3$\pm$0.8 mM) on the outside of the cell. This high affinity for external lithium resembles that of the red blood cell transport protein. The Vmax of lithium induced proton efflux is 2.4$\pm$0.1 acridine orange fluorescence units / (sec. X mg protein) and that of sodium induced efflux is 7.1$\pm$0.2

units / (sec. X mg protein) [Acridine orange will fluoresce upon binding acids.] Lithium also inhibited the transporter at a second site in a noncompetitive manner, for which the $K_{1/2}$ of inhibition was 50-100 uM. The second site may be the same site to which the diuretic amiloride binds and inhibits transport activity, $K_{1/2}$ of inhibition = 90 uM (Ives et al., 1983).

5. SODIUM-POTASSIUM COTRANSPORT

Sodium-potassium cotransport represents unidirectional coupled movement of sodium and potassium ions across the cell membrane. This system is relatively specific. Rubidium has been successfully employed as a substitute for potassium and only lithium has been found able to replace sodium. Chloride is required for activity and in fact may be transported along with the cations. Many have suggested that NaCl-KCL cotransport is a regulator of cell volume. Recent evidence links cotransport activation to the phosphorylation of a specific membrane protein. In some species it appears that the phosphorylation process is mediated by cAMP.

i. HUMAN RED BLOOD CELLS

Initial evidence for the existence of Na^+-K^+ cotransport in human red blood cells can be found in the early work of Hoffman and Kregenow (1966). Observing ouabain resistant sodium ion extrusion, against its concentration gradient, they postulated the existence of a second ion pump, "Pump II". Sach (1971) reported this transport system to be antagonized by increasing the extracellular K^+ concentration and also to be sensitive to the loop diuretic furosemide. Beauge and Adragna (1971) studied ouabain-resistant uptake of $^{86}Rb^+$, $^{86}Rb^+$ accumulation representing K^+ influx. They found this flux to be activated by external sodium and dependent on internal ATP. It represented about 15% of the

total $^{86}Rb^+$ influx. Wiley and Cooper (1974) have further characterized ouabain resistant, furosemide-sensitive transport of Na^+ and K^+. They reported Na^+ influx was stimulated by addition of K^+ to the medium and K^+ influx required the presence of Na^+ in the medium. Both the K^+ stimulated Na^+ influx and Na^+ dependent K^+ influx were inhibited by furosemide. Ouabain insensitive efflux of these cations was also inhibited by furosemide. They concluded that human red blood cells contained a reversible Na^+-K^+ cotransport distinct from an exchange diffusion process which was inhibited by furosemide but insensitive to ouabain. These conclusions were independently supported by the work of Beauge (1975) with his observations that; 1) raised concentrations of intracellular sodium will decrease the net sodium gain by increasing sodium efflux, 2) furosemide causes an increase net sodium gain only when cells have a high intracellular sodium content, and 3) 50mM external potassium will decrease sodium efflux only in low sodium cells while stimulating sodium influx only in high sodium cells. These data fit the concept of a reversible furosemide sensitive mechanism which passively shuttles sodium and potassium across the membranes in either direction. The effect of external potassium simply suggests that when intracellular sodium is low the influx is down the sodium ion concentration gradient and hence independent of the potassium ion gradient. The efflux is against the sodium gradient and therefore driven by the potassium ion gradient. Conversely in high sodium cells the opposite applies.

Garay et al., (1981) studied ouabain-resistant, furosemide sensitive Na^+ and K^+ efflux into Na^+ and K^+ free media, as representative of cotransport. The efflux media contained; 75mM $MgCl_2$, 85mM sucrose, 0.1mM ouabain, 10mM Tris-MOPS (pH 7.4, 37^oC), 10mM glucose

with and without 0.5mM furosemide. Fresh cells exhibited furosemide sensitivity to 30-60% and 10-30% of their net Na^+ and K^+ effluxes, respectively. The dose response curve for furosemide inhibition was sigmoidal, being maximal with 0.5mM. They varied the internal cation concentrations and found the efflux of both cations to be linear functions of their internal concentration. Simultaneous presence of both ions was required for furosemide-sensitive efflux of either Na^+ or K^+, as both appeared to be tightly coupled in a 1:1 stoichiometry.

More detailed studies of human erythrocyte Na^+-K^+ cotransport revealed an absolute requirement for extracellular chloride (Dunham et al., 1980; Chipperfield, 1981). Canessa et al. (1982) reported that lithium can be transported in place of sodium but not potassium.

The physiological role of this transporter remains an uncertainty. Some authors have suggested that it is actually a NaCl-KCl cotransport mechanism similar to chloride transport of epithelial tissue (Frizzel et al., 1979). Ellory and Stewart (1982) have confirmed that the potency of various loop diuretics coincides with the ability of these agents to inhibit the red cell Na^+-K^+ cotransport. Garay and his coworkers have proposed that cotransport is important for the maintanence of low intracellular sodium. Furthermore, they have suggested that defective cotransport is involved in the pathogenesis of hypertension through the increase of intracellular sodium (Garay et al., 1980a). Duhm and Gobel (1984) have presented observations of furosemide sensitive Rb^+ transport being correlated with Mean Cellular Hemoglobin Content (MCHC). A high MCHC indicates low cellular water content. In addition the MCHC was negatively correlated to the cellular potassium content. They could not observe a correlation of furosemide-sensitive Rb^+ transport to cellular sodium content. This evidence suggests that in

vivo the cotransporter is a K^+ and water extruding mechanism involved in the regulation of cell volume.

Activity of this transport appears not only to be dependent on ion gradients but also regulated by intracellular messengers. Garay (1982) reported an inhibition of transport by intracellular cAMP and intracellular calcium. He found only minimal inhibition by intracellular cGMP. From several eicosanoids tested, prostacyclin was observed to have a stimulatory effect (Garay et al. 1983a). In addition, this transport was found to be sensitive to Mg^{++} and other divalent cations; Sr^{++}, Ba^{++}, Mn^{++} and Co^{++} (Ellory et al., 1983).

ii. AVIAN RED BLOOD CELLS

A similar loop diuretic sensitive (Palfrey et al., 1980) transport of ions exists in avian erythrocytes and is believed to be Na-K-Cl cotransport (Palfrey and Greengard, 1981). In turkey red blood cells ion transport is activated in response to catecholamines, mediated through the second messenger cyclic AMP (Gardner et al., 1976).

A membrane protein of 240,000 daltons has been identified as a phosphoprotein possibly involved in activation of cotransport (Rudolph and Greengard, 1974). Alper et al. (1980b) reported isoproterenol to activate cotransport and ^{32}P incorporation into a 37,000-dalton fragment of the phosphoprotein. Both processes exhibit similar half-times and both were reversed with propranolol (Alper et al., 1980a). With tryptic hydrolysis five distinct phosphopeptides can be derived. Two of which can undergo cAMP-dependent phosphorylation and two in which phosphorylation can be stimulated by purified calmodulin and Ca^{2+} (Alper et al., 1980b). This evidence suggests that regulation of ion cotransport may be by two independent second messenger systems.

Studies with duck erythrocytes has delineated a physiological role in volume regulation (Kregenow, 1981). In a hypertonic medium these cells will shrink, however continued incubation in this medium results in a regulatory response by which the cell volume is returned to normal. For this increase to occur, sodium, chloride and a minimum of 2.5 mM potassium must be present in the external medium supporting the cotransport of the three ions. A similar response can be evoked in isotonic medium with catecholamines. Catecholamines activate the cotransporter through an increase in intracellular cAMP, resulting in the cell swelling beyond the original volume. The hypertonic medium induced activation of cotransport does not involve cAMP.

iii. RAT RED BLOOD CELLS

Na-K cotransport in rat RBCs is not well characterized, but there is sufficient evidence in the literature to support its existence. Duhm and co-workers reported observing ouabain-resistant Na^+-dependent $K^+(Rb^+)$ and $K^+(Rb^+)$-dependent Na^+ movements that are sensitive to the loop diuretics and replacement of Cl^- with NO_3^- (Duhm et al., 1983). Duhm and Gobel (1984) also reported its activity to be highly dependent on the cell volume hence suggesting a role in volume regulation.

iv. VASCULAR AND EPITHELIAL CELLS

Observations suggesting the presence of Na-K-Cl cotransport in cells of vascular smooth muscle and kidney epithelial origin allow for speculations as to the role in blood pressure regulation. Frizzell et al. (1979) discussed the evidence which implicated sodium-coupled chloride transport as the mechanism responsible for Cl^- secretion in various epithelial cells. Although this review does not mention coupled potassium transport, the described characteristics are similar to known sodium-potassium cotransport systems. Similarly sodium-chloride coupled

cotransport has been identified in vascular smooth muscle (Widdicombe and Brading, 1980; Kreye et al., 1981).

Saier and Boyden, (1984) have reviewed studies on transport in the Mandin-Darby Canine Kidney (MDCK) cell line. Kinetic analysis of transport in MDCK cells support a 1:1:2 Na:K:Cl stoiciometry. Transport of each ion is dependent on the other two and the overall process is electroneutral (Saier and Boyden, 1984). In epithelial tissues of rabbit gallbladder absorption of Na and Cl is electroneutral and requires both ions. In taenia of Guinea-pigs the transport of these ions is also dependent of the presence of the other ions on the same side. All of these transport systems are inhibited by furosemide and other loop diuretics.

Studies by Rindler et al. (1982) suggest an ATP dependency of the NaCl/KCl cotransport in MDCK cells. Although this cell possesses an adenylate cyclase activated by PGE_1, vasopressin and glucagon (Rindler et al., 1979) hormone stimulation of cotransport has not as yet been observed. Saier and Boyden have therefore postulated a cAMP independent protein kinase phosphorylation mechanism of activation. In many epithelial tissues chloride ion secretion is enhanced by interventions which raise intracellular cAMP (Frizzel et al., 1976) and calcium (Frizzel et al., 1977).

6. CHOLINE TRANSPORT

The choline carrier of human erythrocyte membranes represents another mechanism by which the sodium, potassium or lithium ions can cross the membrane. It is believed to be a counter-transport mechanism with a very high affinity for choline as half maximal influx can be reached with 20-30uM choline. Other monovalent cations which are transported and therefore act as competitive inhibitors to choline

transport are, in order of decreasing affinity; Cs Rb K Na (Martin 1972).

The choline transport remains functional in ATP depleted cells and is capable of concentrating choline within the cells. In a physiological environment, it is the K^+ gradient which provides the energy for choline accumulation. There is, however, an inconsistancy with a simple countertransport model. At steady-state, the distribution of choline should be dependent solely on the K^+ gradient, independent of the external choline concentration. Martin (1972) has demonstrated that the ratio of intracellular to extracellular choline decreases as the extracellular choline concentration is increased. This information is more compatible with a pump-leak model. Presently the exact mechanism of choline transport is not well defined.

Choline transport is inhibited by other ions which compete for the binding site and are transported. Hemicholinium competes for binding to the transport site but is not transported. It is the only competitive inhibitor with a greater affinity than choline. Non-competitive inhibition can be achieved with a variety of SH-reagents. These and other aspects of choline transport have been reviewed by Martin (1977). Cysteamine, p-chloromercuribenzene sulphonic acid (PCMBS) and N-ethylmaleimide all produce almost complete inhibition. Various SH-reagents were tested for their ability to inhibit choline transport and lipid solubility was found to be a major determinant of inhibitory ability. This suggests that the reactive SH group is within the lipophilic environment.

7. CALCIUM INDUCED POTASSIUM PERMEABLITY (GARDOS EFFECT)

Calcium induced K^+ flux which is now known to be present in several tissues, was originally observed in red cells by Gardos in 1957,

hence the name "Gardos Effect". Gardos reported that incubation of human red cells in a medium containing iodoacetic acid, adenosine and calcium resulted in a rapid loss of cellular potassium (Gardos, 1959). The mechanism by which this effect can be induced has been explained by Lew and Beauge (1979).

First the cells are depleted of ATP by the presence of iodoacetic acid and adenosine in the medium. This treatment reduces ATP concentration from 1mM to approximatly 1uM due to increased ATP consumption by the substrate while ATP synthesis is blocked by the inhibitor. Following ATP depletion there is a decrease in the active Ca^{2+} extrusion from the cell, which leads to a higher intracellular calcium level due to passive calcium leakage at a rate between 1 to 10 umol/(l cells)/hour. Some of the calcium is buffered by hemoglobin but one third to one half remains in the ionized form. It is the ionized calcium only that is involved in the interaction with the K^+ gating mechanisms and the subsequent movement of the K^+ through these selective channels. Once activated the channels remain as such until the calcium is removed.

Oligomycin, furosemide, ouabain (Blum and Hoffman, 1971), quinine and quinidine (Armando-Hardy et al., 1975), as well as some calmodulin inhibiting agents (Lackington and Orrega, 1981), have been reported as inhibitors of the Gardos Effect. Only quinine and quinidine act directly on the potassium channel (Armando-Hardy et al.,1975), the others act on earlier steps in the process. The action of ouabain may be a consequence of its indirect effect on intracellular ATP concentration by inhibition of the (Na^+-K^+)-ATPase (Lew, 1971).

The protein(s) which mediate the Gardos Effect are not known, however the (Na^+-K^+)-ATPase was always considered as a likely

possibility (Blum and Hoffman, 1971). This now appears not to be the case since calcium stimulated potassium transport has been observed in dog red blood cells, which do not possess a (Na^+-K^+)-ATPase (Richardt et al., 1979). Also Na^+, K^+ pump activity has been reconstituted in proteoliposomes without the appearance of a calcium-activated K^+ efflux (Karlish et al., 1981).

8. LACTATE TRANSPORT

Mature mammalian erythrocytes are well documented as not having the intracellular organelles, mitochondria. They therefore do not have the benefit of oxidative metabolism which further converts glycolytic end products to additional ATP. These products, lactate and pyruvate can easily cross the erythrocyte membrane and are equilibrated with the extracellular fluid. The transport processes involved have been recently defined (Deuticke et al., 1982). Only 5% of the lactate transport can be inhibited by stilbene disulfonates, which completely inhibits the known anion transporter. Another 5% is insensitive to all inhibitors but does exhibit pH dependency. This component is non-ionic diffusion of lactate. The majority of the lactate transport appears to be handled by a specific monocarboxylate transport system.

The monocarboxylate transport system is inhibited by SH-reagents which do not affect the band 3 anion exchange system. Its activity is affected by both intracellular and extracellular pH. Increasing extracellular pH will stimulate lactate efflux and increasing intracellular pH will attenuate the flux. This transport system is therefore most likely a lactate-proton cotransport, or alternatively a lactate-OH^- countertransport.

C. ION TRANSPORT MECHANISMS AND HYPERTENSION

1. OBSERVED ABNORMALITIES

Essential hypertension is defined as a spontaneous rise in blood pressure, which cannot be attributed to any known pathological condition. Although this term refers to any stable hypertension of unknown cause, as more secondary forms of hypertension are identified, the remaining classification of essential hypertension becomes a more homogeneous group. This is not to suggest that all cases of genuine essential hypertension are of the same etiology. It remains to be seen if the disease classified as "essential hypertension" may be subdivided into groups with different primary defects.

The literature on hypertension research contains many theories on the mechanism of how a spontaneous rise in blood pressure occurs and is maintained. A recently proposed theory that is widely gaining popularity suggests that essential hypertension is the result of improperly maintained ion gradients in specific tissues (de Wardener and MacGregor, 1980; Haddy, 1983; Blaustein, 1977). Indeed many publications report various abnormalities in ion gradient and transport in vascular smooth muscle, neuronal tissue, cardiac muscle and even the red blood cells. However, there still remains some doubt as to their primary or secondary nature. Even among those authors who believe that ion gradient abnormalities have a primary role in the development of hypertension there remains uncertainty as to which tissue the presence of this defect is critical.

Development of experimental models of hypertension allowed for the study of tissues other than the clinically accessible red blood cells. The most popular is the Japanese rat model for genetic hypertension, the Spontaneously Hypertensive rat (SHR) with its control,

the Wistar-Kyoto (WKY) rat. Another rat model which receives considerable attention are the Dahl salt-sensitive and salt resistant strains. This model relies on a combination of genetic and environmental determinants for the development of hypertension.

In the tissues studied, reports of defects in specific ion transporting mechanisms included the sodium-potassium pump, sodium-potassium cotransport and sodium-lithium exchange. Unfortunately this literature is quite inconsistent. It remains to be determined if there exists a biochemical or physical relationship between these transport processes and whether or not these observations are different manifestations of the same defect.

i. CONTENT AND NON-SPECIFIC ION FLUXES

a. RED BLOOD CELLS

Indications that spontaneous hypertension was associated with abnormalities of ion transport began appearing in the literature several years ago. These and other reports have been reviewed recently by Parker and Berkowitz (1983). Several investigators have conducted studies on ion transport in hypertension, sometimes reporting contradictory results.

The studies of human RBC ion content and fluxes are inconsistent. Some investigators have reported normal cell sodium (Weller, 1959; Schroeder, 1968; Munro-Faure et al., 1971; Canessa et al., 1980; Trevisan et al., 1981; Walter and Distler, 1982; Adragna et al., 1982; Weder, 1984). Others have observed RBCs of essential hypertensives to contain higher than normal levels of sodium (D'Amico, 1958; Fadeke Aderounmu and Salako, 1979; Urry et al., 1980; Losse et al., 1981). There even exist observations that untreated borderline hypertensives have lower RBC sodium compared to normotensives. In this

study definite hypertensives, both treated and untreated, were not significantly different from normotensives (Trevisan et al., 1983).

Similarly with rat RBCs, reports of increased sodium content (Postnov et al., 1976) and no difference in sodium content (Ben-Ishay et al., 1975) can be found in the literature on genetically hypertensive rat strains.

As for passive ion fluxes in human RBCs, Postnov et al. (1977) reported that in hypertensives the rate constant for $^{22}Na^+$ efflux is twice the rate constant observed in normotensive patients. Henningsen et al. (1979) and Fitzgibbon et al. (1980) reported a greater rate of $^{22}Na^+$ turnover in essential hypertension. A lower potassium efflux rate was observed by Fadeke Aderounmu and Salako (1979). Faster $^{22}Na^+$ accumulation was reported by Mahoney et al. (1982). Poston et al. (1981) could not find differences in passive sodium fluxes and Adragna et al. (1982) observed similar rate constants of passive efflux of Na^+ and K^+ but a significantly higher rate constant of Li^+ efflux was detected in hypertensives.

In rats with genetic hypertension, increased passive $^{22}Na^+$ efflux was observed by Ben-Ishay et al. (1975). Friedman and coworkers demonstrated an increased rate of Li^+ accumulation (Friedman et al., 1976) and a faster net movement of Na^+ and K^+ determined by glass electrode measurements (Friedman et al., 1977). Postnov et al. (1976) also found greater permeability of the erythrocyte membrane to $^{22}Na^+$ and $^{42}K^+$.

b. VASCULAR SMOOTH MUSCLE

The literature contains an abundance of publications which report findings of abnormal ion gradients in a large variety of tissues, in both man and animals. The pioneering studies by Tobian and Binion

(1952) of the water and cation content of renal artery obtained postmortem from hypertensive and normotensive patients concluded that the tissues obtained from patients diagnosed as hypertensive contained a significantly higher precentage of water and sodium. This paper also reported a similar trend of higher sodium content in frontal lobe brain tissue. Jones reported similar findings in vascular smooth muscle from hypertensive rat strains. Comparing SHR to Wistar rats (WKY, the proper controls to SHR were not available) There was a greater rate of turnover for $^{36}Cl^-$ and $^{42}K^+$, and a decreased ability to extrude Na^+, by the vascular smooth muscle of aorta (Jones, 1973). These observations were later extended to smaller blood vessels (Jones, 1974). A further observation by Jones and Hart (1975) that alterations in aortic vascular smooth muscle ion handling occurred prior to the onset of hypertension in experimentally produced Deoxycorticosterone Acetate (DOCA) hypertension, suggested to these authors that ion transport is primary to the changes in vascular reactivity and hypertrophy associated hypertension. Experiments by Friedman also showed that vascular smooth muscle tissue from hypertensive rats exhibited a significant increase in leakiness to ions. He made this observation by incubating freshly excised rat tail arteries in a physiological salt solution in which lithium replaced sodium at 2^oC. Arteries of DOCA-salt hypertensive rats contained more intracellular and extracellular Na and accumulated lithium at a faster rate than controls (Friedman, 1974). Similar observations were made with tail arteries of SHR, in which he reported accelerated passive downhill movement of sodium ions (Friedman and Friedman, 1976).

ii. SODIUM-POTASSIUM PUMP

a. HUMAN RED BLOOD CELLS

The function of the ATP-dependent, Na^+,K^+ pump in essential hypertension is presently an unresolved issue. This is mostly due to the varied and contradictory literature resulting from differences in methodology and assay conditions. A review of this literature presented by Parker and Berkowitz (1983) reveals an almost even split among authors reporting increased and decreased activity. Reports of decreased activity refer to decreases in the maximal rate (ouabain sensitive sodium efflux from sodium loaded cells) (Fadeke Aderounmu and Salako, 1979) and a decrease in the rate constant (ouabain sensitive $^{22}Na^+$ efflux) (Walter and Distler, 1982). Walter and Distler also observed that in spite of the lower rate constant, ouabain sensitive fluxes remained the same due to a higher intracellular sodium concentration. Of the papers reporting an increase of Na^+,K^+ pump activity, Garay et al. (1980b) reported an increase in ATPase activity (sodium and potassium dependent release of inorganic phosphate). More recently Cole (1983) observed a significant elevation of sodium-potassium pump activity measured as ouabain-sensitive $^{22}Na^+$ efflux. Sodium and potassium dependent ATPase activity was elevated, although not significantly. However the maximum flux rates measured from sodium-loaded cells were not changed. Accordingly the author suggested that the altered activity was due to a defect in a regulatory mechanism rather than the transporter itself. Similarly, Postnov et al. (1977) had earlier reported differences in calcium binding to the inner side of the plasma membrane correlating with differences in ouabain-sensitive ATPase activity, hence suggesting a defect in a regulatory mechanism.

Other ways in which net sodium-potassium pump activity may be altered are: changes in a number of membrane transport sites (Takayama et al., 1984) and by the presence of a circulating endogenous inhibitor (Hamlyn et al., 1982).

b. RAT RED BLOOD CELLS

The literature on the sodium, potassium pump of rat red blood cells is in more agreement. De Mendonca observed red cells of SHR to have a greater rate of ouabain-sensitive potassium influx, when the cells were first depleted of potassium (De Mendonca et al., 1982). Duhm reported an increased rate of ouabain-sensitive rubidium uptake, however, he could not find an accompanying increase in ouabain-sensitive sodium extrusion (Duhm et al.,1983).

In cells other than erythrocytes, pump activity has been reported to be increased (Brock et al., 1982) and decreased (Lee et al., 1983).

c. REGULATION BY CALCIUM

Dunham and Glynn (1961) observed an inhibiting effect of calcium on strophanthin-sensitive ATPase in human red blood cell ghosts. This observation, however, received little attention until Davis and Vincenzi looked closely at the relationship between calcium activation of calcium ATPase and calcium inhibition of (Na^+-K^+)-ATPase (Davis and Vincenzi, 1971). They observed that as the calcium concentration is increased from aproximately 10^{-6}M to 10^{-4}M, the stimulation of the calcium ATPase was greater than the inhibition of the (Na^+-K^+)-ATPase. The observations that low intracellular calcium is necessary for (Na^+-K^+)-ATPase activity and that calcium activates the calcium ATPase before it inhibits the (Na^+-K^+)-ATPase, suggest that activity of the calcium ATPase (pump) will affect the activity of the (Na^+-K^+)-ATPase.

Paradoxically, intracellular calcium will also stimulate (Na^+-K^+)-ATPase activity. Studies by Powis et al. (1983) on neuronal cells have suggested that calcium stimulation of the the (Na^+-K^+)-ATPase is mediated by a calmodulin dependent mechanism. Postnov and coworkers (1977) observed an increase in (Na^+-K^+)-ATPase activity in red blood cells, with increasing concentrations of free calcium. They also demonstrated that in the cells from hypertensives the (Na^+-K^+)-ATPase was less sensitive to activation by calcium than in the cells of normotensives, and that for any given concentration of free calcium, between 3 and 41 micromolar, the red blood cell (Na^+-K^+)-ATPase activity of the hypertensives was less than that of the normotensives.

Hypotheses by Postnov and Orlov (1978) and Sprenger (1985) have proposed that the apparent alteration in (Na^+-K^+)-ATPase activity is secondary to defects in calcium ion handling. It is not yet known whether it is the membrane bound or free calcium which is physiologically important in determining the activity of the (Na^+-K^+)-ATPase.

iii. SODIUM - LITHIUM EXCHANGE

Noting that the maximal rate of activity of red blood cell sodium-lithium exchange was subject to unknown variations, Canessa and her group of co-workers were the first to study its relationship to the occurrence of essential hypertension (Canessa et al., 1980). To assess sodium-lithium countertransport, RBCs were first incubated in a solution of 150mM lithium for three hours in order to load them with lithium. This procedure resulted in intracellular lithium contents slightly over 13mM. Since these concentrations were at least twenty times the reported intracellular concentration of lithium required for one half maximal activity, they assumed maximal transport would occur. Subsequently the

cells were incubated in the presence or absence of external sodium and the rate of lithium efflux was measured in each case. The difference, the sodium dependent lithium efflux, represents lithium transport by sodium-lithium exchange.

Canessa reported that normotensive individuals with no family history of essential hypertension exhibited a RBC sodium-lithium exchange capacity between 0 and 0.4 mmoles per liter of RBCs per hour. In red blood cells from hypertensive patients the maximal sodium-lithium countertransport activity ranged from 0.3 to 0.9 mmoles per liter per hour. The means$\pm$SEM were 0.24$\pm$0.02 and 0.55$\pm$0.02 respectively and were significantly different from each other.

The association of elevated sodium-lithium exchange maximal rates appeared to be genetically determined and not secondary to the onset of hypertension. This conclusion was drawn from the observation that normotensive immediate relatives of hypertensive patients also had maximal rates of countertransport averaging 0.54$\pm$0.05 mmoles per liter per hour. Immediate relatives of normotensive individuals averaged 0.23$\pm$0.02 mmoles per liter per hour for this measurement.

The original paper by Canessa et al. (1980) reported a clear distinction between the maximal rates of RBC sodium-lithium exchange of hypertensive and normotensive individuals. This finding suggests possible use of this assay as a marker for essential hypertension. Other investigators confirmed that this activity is enhanced, however they questioned its accuracy in predicting the occurrence of essential hypertension and use as a diagnostic tool. Extensive overlap in the values was reported by many (Cusi et al., 1982; Adragna et al., 1982; Brugnara et al., 1983; Trevisan et al., 1983; Smith et al., 1984; Weder et al., 1984).

Other factors have been found to influence sodium-lithium exchange. Mean sodium-lithium countertransport was found to be elevated in all pregnant women, both normal and hypertensive (Worley et al., 1982). Woods and co-workers (1983) observed the effects of ultrafiltration and hemodialysis on the RBC countertransport rate and came to the conclusion that the transporter activity can be altered by a dialyzable plasma factor. Brugnara reported that the age of the patient correlates with decreasing activity in normotensive males. This correlation was not observed for females or hypertensives of either sex (Brugnara et al., 1983). Weder, who studied racial differences, reported elevated cotransport with hypertension in white patients only, with extensive overlap (Weder et al., 1984). No distinction could be made between normotensive and hypertensive groups of black patients. Ibsen and co-workers were only able to find a significant elevation among hypertensive male patients, again with overlap of the values. In addition they found the offspring of hypertensive parents to have sodium lithium exchange rates similar to normotensive controls. Thus they concluded that a genetic disposition of the transport function was unlikely (Ibsen et al., 1982).

Levy et al. (1983) reported that a more accurate distinction between the hypertensive and normotensives can be achieved by comparing the effect of temperature on sodium dependent lithium effluxes. Red blood cells from hypertensive individuals exhibited Arrhenius plots of sodium lithium exchange with a change in slope at around $30^{o}C$. For the red blood cells from hypertensives this corresponding temperature was $20^{o}C$ in 75% of the cases. From these findings and the results of analysis of normotensive offspring from patients with hypertension, this group suggested that sodium-lithium countertransport remains a valid genetic marker if one looks at this parameter.

A physiological connection between sodium - lithium(sodium) exchange and hypertension is difficult to envision. A one for one electroneutral exchange diffusion process will not alter intracellular sodium concentration, or membrane potential. Speculation that abnormal activity of red blood cell sodium - lithium exchange is an indication of abnormal sodium - hydrogen exchange in epithelia of kidney, may offer an explanation (Aronson, 1982). Sodium - hydrogen exchange is found in the luminal membranes of cells of the proximal tubule. It is the major mechanism for sodium transport across the luminal membrane, which is the rate limiting step in transcellular sodium movement. It is conceivable that an increased renal salt reabsorption may occur through this process, inducing hypertension through a well defined sequence of events: 1) Increased perfusion pressure to maintain a proper sodium balance (Guyton et al., 1981) and 2) Release of natriuretic factors (Blaustein and Hamylyn, 1983).

iv. SODIUM - POTASSIUM COTRANSPORT

Na^+-K^+ cotransport was suggested to be altered in RBCs from human hypertensives (Garay and Meyer, 1979). Garay and Meyer studied RBCs of patients from three groups; 1) Normotensive patients, 2) Hypertensive patients in which the hypertension could be attributed to clinically diagnosed nephropathy, 3) Hypertensive patients with no detectable cause for the hypertension. The RBCs were first loaded with sodium and subsequently incubated in Na^+-K^+ Ringers medium at 37^oC. By measuring the changes in intracellular Na^+ and K^+ during the incubation, they estimated the net Na^+ and K^+ fluxes. They observed RBCs from normotensives and secondary hypertensives to exhibit net Na^+ efflux of approximately 3.0 mmole/(l. orig. cell X h). The Na^+ efflux rate from RBCs of essentially hypertensive patients was inversely proportional to

the mean arterial pressure of the donor. The net K^+ influx in cells from the normotensives and secondary hypertensives was also similar, approximately 1.0 mmole/(l. orig. cell X h). Influx of K^+ was elevated as high as 2.0 mmole/(l. orig. cell X h) in moderate hypertension but decreased with higher mean arterial pressures of the patients. The ratio of net Na^+ loss to K^+ gain was found to be approximately 3-4 for normotensive individuals and patients with secondary hypertension. However RBCs from essential hypertensives exhibited a ratio of approximately 1.5. This difference was attributed to an increase in net K^+ influx and a decrease in net Na^+ efflux.

Garay and Meyer explained these results as being due to a furosemide sensitive Na^+,K^+ cotransport, functioning in the RBCs of normotensives but functionally absent in the RBCs from essential hypertensive patients (Garay et al., 1980a). Thus, in essential hypertensives' RBCs, Na^+-K^+-ATPase is the primary route of Na^+ extrusion resulting in a Na^+-K^+ flux ratio of 3:2. With active cotransport additional Na^+ is extruded along with K^+, thus raising the net Na^+ extrusion and lowering the net K^+ accumulation (Garay et al., 1980b).

Na^+,K^+ cotransport, was suggested by this same group to be a valid marker for the inheritance of hypertension (Garay et al., 1980c; Dagher and Garay, 1980). For this test the maximal rate of outward Na^+ and K^+ cotransport was determined by first sodium loading and then potassium depleting the cells, then measuring outward Na^+ and K^+ fluxes into a medium of 75mM Mg^{+2}, 85mM sucrose, 10mM Tris-MOPS (pH 7.2, 37^oC) 10mM glucose and 0.1mM ouabain, with or without 1mM furosemide. They reported furosemide sensitive efflux rates of 472$\pm$112 and 501$\pm$112 umoles /(1 cells x h) for Na^+ and K^+ of cells from normotensive patients. In RBCs from essential hypertensive patients these rates were significantly

lower, 206±107 and 261±122 umole / (l cells X h) respectively. Of the group of normotensive patients with at least one hypertensive parent, half had flux rates similar to the hypertensive group, while the remainder displayed normal flux rates (Dagher and Garay, 1980).

These findings could not be supported by several other investigators (Swarts et al., 1981; Walter and Distler, 1982; Duhm et al., 1982; Davidson et al., 1982; Stessman et al., 1983; Weder et al., 1984; Smith et al., 1984). Some reported the Na^+-K^+ cotransport in the RBCs of hypertensive patients to be elevated (Adragna et al., 1982; Stokes et al., 1983). There remains however, sufficient indication that activity of cotransport is genetically determined. Stokes et al. (1983) observed male-female differences. Stessman et al. (1983) observed strong correlations of both blood pressure and furosemide sensitive Na^+ efflux between identical twins, and Weder et al. (1984) reported differences of furosemide sodium efflux rates between racial groups.

Further characterization of intracellular sodium activation of outward cotransport identified three subgroups of essential hypertensives (Garay et al., 1983b). Altering the cellular sodium content to achieve intracellular concentrations between 3mM and 22mM affected the rate and directionality of the cotransport. Less than 7-8mM (physiological intracellular sodium ion concentration) resulted in net inward cotransport. With 7mM Na^+ there was no net transport observed and increasing sodium concentration stimulated outward cotransport until a maximum rate was achieved.

In all normotensive subjects an intracellular sodium of 12.4±2.0 mmoles/l. cells (mean±SD) stimulated net outward cotransport to 50% its maximum value ($K_{50\%}$). In 19 of 55 essential hypertensive patients the $K_{50\%}$ = 12.0±2.2, which was the same as the controls. The remainder had

$K_{50\%}$ values of either 20.0±2.9 (n=40), or 33.5±1.5 (n=6). The three hypertensive groups were designated by Garay as non-Co (-), type I Co (-) and type II Co (-) essential hypertensives, respectively. Of five negro hypertensives, three were of type I and two were of type II. Normotensive offspring of hypertensive patients (cotransport status not mentioned) were distributed in all three subgroups.

Maximal rate of outward cotransport was also examined and a wide interindividual variation was reported. Excluding females taking oral contraceptives, or who were pregnant or postmenopausal, the Vmax ranged from 280 to 600 umoles / (l cells X h) in normotensives. These aforementioned groups of normotensive females exhibited a very low Vmax. The values for the hypertensive patients covered a wider range. The Co (-) tended to have a lower Vmax while the non-Co (-) had a slightly higher Vmax.

Price et al. (1984) subdivided the Co (-) hypertensives according to the apparent affinity for external potassium. They found that in cells from normotensives the concentration of external potassium required to achieve half maximal inhibition of cotransport (K_{IK}) varied between 16 and 30 mM. Of the 38 patients in the hypertensive group, most were within this range. Nine of these which were also Co (-) were designated as "Asymmetric CO (-) hypertensives". Three hypertensives with K_{IK} values less than 16 mM and six with K_{IK} values greater than 30 mM were all CO (-). These were designated as "Antisymmetric Co (-) hypertensives and Co (-) hypertensives, respectively.

v. CALCIUM PUMP

Intracellular free calcium concentration directly determines tension in vascular smooth muscle. Therefore one would intuitively suspect that abnormal activities of calcium transporting mechanisms may

have a primary role in the development of hypertension. Evidence of defective membrane calcium transport was first observed by Aoki and coworkers. In crude microsome preparations of both heart and arterial smooth muscle (of various sources), the (Ca^{2+}-Mg^{2+})-ATPase activity was increased and the maximum calcium binding capacity decreased (Aoki et al., 1974). Wei reported impaired binding of calcium to a highly purified sarcolemmal fraction from aorta of SHR, compared to normotensive rats (Wei,et al., 1976). The defect in cardiac myocyte handling of calcium was localized to the sarcoplasmic reticulum. Fractions prepared from SHR cardiac tissue were found to possess decreased calcium accumulating ability as well as decreased calcium binding capacity. Calcium dependent ATPase activity was increased (Limas and Cohn, 1976).

Red blood cells have been shown to possess the same abnormalities of calcium handling (Postnov et al., 1977; Postnov et al., 1979; Devynch et al., 1981; Zidek et al., 1982). Postnov demonstrated that EDTA removes more calcium from the outer side of membranes derived from RBCs of essential hypertensives (Postnov et al., 1977). They could not detect alterations in the total calcium bound to the outside (Postnov et al., 1979), suggesting defective calcium binding abilities. Reduced binding capacity was however reported for the inner side of the membrane of RBCs from both SHR and essential hypertensives (Postnov et al., 1979). Devynck and coworkers reported a decreased calcium binding capacity on the inner side of the membrane. They further reported reduced calcium pump activity, measured as Ca^{2+} influx into inside-out vesicles and as La^{3+}-sensitive, Ca^{2+}-dependent ATP hydrolysis. Passive (ATP independent) influx of calcium was also slightly increased. The authors noted that all three observations support an increase in

intracellular free calcium. This was confirmed by Zidek et al. (1982) who directly measured intracellular calcium ion activity by use of ion-selective electrodes.

Adipocytes from SHR were studied by Postnov and Orlov, and found to possess decreased rates of calcium accumulation into the cytoplasmic reticulum fraction, and an increased rate of calcium accumulation by the mitochondria. No differences in plasma membrane accumulation of calcium were noted. ATP-independent binding by the plasma membrane fraction was decreased in SHR (Postnov and Orlov, 1980).

2. PATHOGENESIS

i. ROLE OF KIDNEY

The relevance of ion transport abnormalities must be considered in relation to the development of high blood pressure. Some authors believe that despite the appearance of these defects in other tissues, such as vascular smooth muscle, the kidney is primarily responsible for the rise in blood pressure. Two hypotheses are considered to explain the link between impaired renal sodium excretion and hypertension.

a. THE AUTOREGULATORY HYPOTHESIS

The autoregulatory hypothesis involves a sequence of events, briefly summarized as follows;

1) Defective kidneys fail to excrete the proper amount of sodium.

2) Salt and subsequent fluid retention result in an increase in the plasma and extracellular fluid volumes.

3) Inevitably an increase in cardiac output occurs.

4) Resistant vessels sense the rise in cardiac output, which initiates autoregulatory responses resulting in increased peripheral resistance.

5) As the blood pressure rises, kidney perfusion is increased, hence pressure natriuresis occurs.

6) Salt balance is returned to normal but is maintained by a raised blood pressure.

Data describing the autoregulatory process have been reviewed by Coleman et al. (1979).

b. RELEASE OF NATRIURETIC FACTOR

This hypothesis advocated by de Wardener and MacGregor (1980) proposes that the kidney defect in sodium excretion initiates hypertension through the release of a hormone. The hormone in turn acts directly on vascular smooth muscle. The vessels constrict, increasing peripheral resistance, resulting in raised blood pressure. The hormonal factor acts by inhibiting sodium, potassium ATPase. Its primary site of action is the epithelial cells within the kidney, of which the consequence is a decrease in transcellular sodium transport resulting in a greater rate of excretion. As a circulating hormone, it also inhibits active sodium and potassium transport in the arteries, which results in vasoconstriction and hypertension.

The role of a circulating hormone was first proposed by Dahl et al. (1969) as an explanation for salt-dependent hypertension. Joining a salt-sensitive hypertensive rat to a salt-resistant normotensive rat by parabiosis (chronic suturing of skin and muscle tissue together, to allow the blood of the two animals to exchange) caused the salt-resistant rat to become hypertensive. This was prevented by bilateral nephrectomy of the salt-sensitive rat. Dahl explained that a natriuretic factor with hypertensinogenic activity was released by the salt-sensitive rat and entered the circulation of the salt-resistant rat.

This factor has been considered by some to be involved in human essential hypertension (de Wardener and MacGregor, 1980; Blaustein and Hamlyn, 1983). Hamyln et al. observed that plasma from individuals with essential hypertension inhibited (Na^+-K^+)-ATPase activity. The extent of inhibition correlated well with the mean arterial pressure of the patient (Hamyln et al., 1982). Other studies, such as the one by Bloom et al. (1976) demonstrate that plasma from hypertensives increases the sensitivity of vascular smooth muscle to agonists.

The site of release is not yet identified, however good evidence indicates that the brain is a likely source (Akagawa et al., 1984), possibly the hypothalamus.

ii. CELLULAR MECHANISMS

Opposing the hypothesis that ion transport is a hemodynamic consequence of kidney defects which initiate and maintain high blood pressure, is the hypothesis that ion transport abnormalities of vascular smooth muscle or the sympathetic nervous system are of primary importance. Three mechanisms are considered.

a. PROTEIN SYNTHESIS

Although experimental evidence is weak, there is some indication that the rate of protein synthesis is increased in smooth muscle tissue from experimental hypertensive animals, as summarized by Friedman and Friedman (1976). They have suggested that excessive sodium entry into the cells activates protein synthesizing machinery, increasing the contractile protein content, thus affecting peripheral resistance (Friedman, 1979).

It has also been considered that high sodium in sympathetic neurons initiates vascular smooth muscle protein synthesis by maintaining high levels of sympathetic activity (Abboud, 1982). Hart et

al. (1980) have demonstrated the importance of sympathetic innervation in maintaining a high wall-to-lumen ratio of vessels in stroke-prone hypertensive rats.

b. MEMBRANE POTENTIAL

Hermsmeyer has proposed that the increased reactivity of vascular smooth muscle may directly result from the ion handling abnormalities leading to alterations of membrane potentials (Hermsmeyer, 1976a; Hermsmeyer, 1976b). At 16°C, vascular muscle cells from SHR are 5 to 7 mV less negative than those of normotensive rats. This was not apparent at 37°C (Hermsmeyer, 1976a). This observation is a consequence of lower intracellular potassium ion concentration (lower membrane potential at 16°C) compensated for by increased activity of the electrogenic sodium, potassium pump. However when the cells are activated by norepinephrine, a greater extent of depolarization occurs because ion gradients contribute less to the resting potential (Hermsmeyer, 1976b). Through this mechanism altered ion handling may directly contribute to the hypertensive process. This has been recently reviewed in spontaneously hypertensive rats and other forms of experimentally produced hypertension (Harder and Hermsmeyer, 1983).

c. SODIUM - CALCIUM EXCHANGE

Blaustein formulated the hypothesis that factors which lead to increased intracellular sodium will secondarily raise the intracellular calcium concentration (Blaustein, 1977). Raised intracellular calcium levels in vascular smooth muscle directly increase vascular tone. In adrenergic neurons, raised intracellular calcium directly stimulates the release of catecholamines. Excessive catecholamine release activates more receptors on the vascular smooth muscle for longer periods of time, also resulting in an increase in vascular smooth muscle tone. In

Blaustein's proposal, the sodium -calcium exchange plays an important role, allowing the sodium ion gradient to regulate the calcium ion gradient. At a 3 sodium : 1 calcium exchange ratio, a sodium of 14:1 (extracellular : intracellular) will result in an intracellular free calcium ion concentration of 10^{-7} M, assuming the extracellular calcium to be $1.5X10^{-3}$M and the membrane potential to be -60mV. The concentration of 10^{-7}M calcium is the physiological range in which actomyosin activity is regulated.

A serious argument against this hypothesis is that the stoichiometry of the exchange may be 2:1 in vascular smooth muscle (Reuter et al., 1973). Accordingly a 14:1 sodium ion gradient will only yield a 10^{-5}M intracellular calcium ion concentration. In this case the sodium - calcium exchange does not have a physiological role in the regulation of actomyosin activity.

iii. METABOLIC REGULATORY MECHANISMS

A third aspect of the pathophysiology of essential hypertension, metabolic regulatory mechanisms, has only very recently been considered and is still not fully appreciated. Two separate, although interconnected, mechanisms are emerging as important regulators of ion channels, protein phosphorylation (Greengard, 1976) and inositol phospholipid metabolism (Berridge, 1984). Many ion transport proteins are now known or suspected to be regulated in some way by such processes. It is very realistic to consider that it may soon be known that most, if not all, mechanisms by which ions cross membranes are very tightly regulated.

a. PROTEIN PHOSPHORYLATION

The well defined series of metabolic events involved in this regulatory process have been adequately reviewed (Greengard, 1978;

Greengard, 1979). Briefly, protein phosphorylation is initiated by a signal perceived at the cell membrane. The signal may act through one of the second messengers; cAMP, calcium, cGMP. It may directly activate the enzyme adenylate cyclase, or open calcium channels, or activate a guanylate cyclase enzyme. The second messenger activates a protein kinase enzyme. The cAMP does so by binding to and removing an inhibitory subunit. The calcium acts by binding to and activating calmodulin which then interacts with the kinase. The cGMP mechanism is not well defined at this time. Protein kinase, once activated can then phosphorylate several proteins, using ATP as a substrate.

Addition of a phosphate group to specific sites alters the activity of the recipient proteins, whether they be enzymes, contractile elements, or ion transporters.

With respect to hypertension a sodium-potassium cotransport of red blood cells, kidney epithelia and other tissues can be regulated by phosphorylation (Saier and Boyden, 1984). Although the physiological role of this regulatory mechanism is unknown at this time, a defect in this process could account for the observed abnormal activity in hypertension.

Cyclic AMP responses have been observed by some to be abnormal in tissues from hypertensives. However different laboratories have produced conflicting results (Hamet et al., 1980). Nonetheless these reports may be an early indication of generalized defects in cellular regulation involved in the pathogenesis of hypertension.

b. PHOSPHOINOSITIDE METABOLISM

Phosphoinositide metabolism involves a complex series of enzymatic reactions, in which many of the products have a regulatory nature. Excellent reviews on this mechanism have been published

(Nishizuka 1984; Berridge, 1984). Phosphoinositide is a phospholipid containing myo-inositol as the polar head group. The inositol can be additionally phosphorylated in the 4 position or the 4 and 5 positions. Hydrolysis of phosphatidylinositol 4,5 bisphosphate is stimulated by activation of cell surface receptors. The resulting products, diacylglycerol and inositol 1,4,5 trisphosphate are both potential second messengers. Inositol trisphosphate is a determinant of calcium mobilization. Diacylglycerol is a direct activator of protein kinase.

Koutouzov reported differences in phosphoinositide metabolism of ghost membranes from SHR and WKY (Koutouzov et al., 1983; Koutouzov et al., 1982). These data suggest that in tissues of SHR there exists a deficiency in the enzymatic conversion of phosphatidyl inositol to diphosphoinositides and trisphosphoinositides and that this defect does not occur as a consequence of the onset of hypertension.

D. RELATIONSHIP OF RATES OF LACTATE RELEASE TO CELLULAR ATP UTILIZATION IN MAMMALIAN RED BLOOD CELLS.

1. ATP SYNTHESIS PATHWAYS

Adenosine triphosphate (ATP) is a high energy, phosphate containing molecule often referred to as the "energy currency" within cells. When an exchange of energy from one molecule to another occurs, ATP is most commonly the intermediate involved. ATP is produced through a series of pathways which catabolize organic molecules. The main substrate for ATP synthesis is glucose.

Glucose is metabolized through the Embden-Meyerhof pathway of glycolysis which results in the formation of two pyruvate molecules, the reduction of two nicotinamide adenine dinucleotide (NAD^+) to NADH + H^+, and the net formation of two ATP molecules. In respiring cells, cells which possess mitochondria, the pyruvate molecules enter the tricarboxylic acid cycle (or Krebs cycle), and are sequentially oxidized resulting in further formation of NADH + H^+. The NADH enters the electron transport chain. In this pathway the electrons are passed from high energy states to low energy states to oxygen which is the final electron acceptor. The energy lost from the electrons is utilized in the formation of high energy bonds, as ATP is formed from adenosine diphosphate (ADP) and inorganic phosphate (Pi) (Lehninger 1976).

Mammalian erythrocytes do not possess mitochondria, therefore they are non-respiring cells. Pyruvate, which is the end product of glycolysis in respiring cells, is converted by the enzyme lactate dehydrogenase, to lactate. In this process, NADH + H^+ is re-oxidized to NAD^+. The overall process of glycolysis then becomes;

(1) Glucose ------→ (2) ATP + (2) LACTATE

Therefore, in this system the rate of lactate production reflects the rate of ATP synthesis.

There are, however, additional metabolic pathways which could allow for a deviation of the 1:1 ATP-lactate formation ratio which must be considered. The Rapoport-Luebering cycle (Rapoport and Luebering, 1950) is a two enzyme shunt responsible for the production and turnover of 2,3-diphosphoglycerate (2,3-DPG). DPG-mutase synthesizes 2,3-DPG from the Embden-Meyerhof pathway intermediate 1,3-DPG. DPG-phosphatase is responsible for removing a phosphate from the 2,3-DPG to form 3-phosphoglycerate, a component of the Embden-Meyerhof pathway. Through each turnover of 2,3-DPG, the glycolytic enzyme phosphoglycerate kinase is by-passed. This enzyme is one of the steps in which ATP is produced, therefore, the ATP-lactate ratio is decreased.

The physiological role of 2,3-DPG is in the regulation of hemoglobin affinity for oxygen, and will not be discussed. Generally, the level of 2,3-DPG is maintained at 5mM but can be increased under conditions of hypoxia (Brewer, 1974). Changes in lactate production can therefore occur without concomitant changes in ATP production by variations in the rate of 2,3-DPG pool turnover. An increase in the pool turnover rate will shunt more carbon into the formation of 3-PG without the accompanying ATP synthesis via the 3-phosphoglycerate kinase enzyme. Hence, the ATP-lactate production ratio decreases. A decrease in the turnover rate will increase the ATP-lactate production ratio.

Another possible mechanism which must be considered in the relationship of lactate synthesis to ATP production was proposed by Sen and Post, (1964). In reconstituted erythrocytes loaded with large amounts of exogenous ATP (2.4mM per 5mM Hb), they observed a 2:1 ratio

of hypoxanthine lactic acid synthesis. They reasoned that for every three ATP catabolized to three hypoxanthine and three ribose phosphate moieties, eight ATP and five lactates could subsequently be formed through the metabolism of the ribose-phosphate in the pentose-monophosphate and glycolytic pathways. Therefore, ATP catabolism accounted for five-sixths of the lactate produced. The ATP-lactate ratio for this pathway, 8:5, would therefore increase the apparent ratio of cellular ATP-lactate production.

This pathway of lactate formation implies a depletion of the cellular adenosine pool. Since we are incubating the cells in the presence of glucose, glucose remained the primary substrate for ATP synthesis and the pools AMP, ADP and ATP remained relatively constant. During the initial experiments of this thesis, cellular ATP levels were randomly monitored and not found to change.

2. REGULATION OF GLYCOLYSIS

Because glycolysis is a series of metabolic reactions, regulation of this process may occur at any one of the steps. The overall rate of glycolysis is less than the Vmax of any of the enzymes involved, thus indicating that the activity of the pathway is tightly regulated by factors which keep the activity of the key enzymes below their maximal capacity (Brewer et al., 1964).

Hexokinase (HK) is the first in the sequence and is responsible for phosphorylation of glucose to glucose-6-phosphate, (G-6-P). It is subject to feedback inhibition by G-6-P and is also regulated by intracellular Mg-ATP, 2,3-DPG and inorganic phosphate (Pi). ATP is required for the forward direction of the reaction. Since the Km for ATP is near the concentration of ATP in the cell (1mM), slight changes in ATP levels may affect the activity of this enzyme. ATP also activates

hexokinase by relieving the competitive inhibition of G-6-P. Regulation by Pi and 2,3-DPG will be discussed separately.

The enzyme phosphofructokinase (PFK) is one of the more important sites of glycolysis regulation. PFK is primarily regulated by the intracellular pH, the optimum of which is pH 8.0. Other factors of importance are ATP, ADP, AMP, citrate, F-6-P and Pi. Citrate and ATP are molecules of high energy and both are allosteric inhibitors of PFK activity. The ATP inhibition is independent of its role as a substrate. Inhibition of PFK by ATP and F-6-P are both attenuated by increasing the pH so that as pH increases within the physiological range, the PFK activity increases. AMP and ADP are molecules of low energy and are activators of PFK, therefore the energy balance of the cell is an important determinant of activity.

The latter part of glycolysis is believed to be primarily regulated at two sites, pyruvate kinase (PK) and glyceraldehyde-3-phosphate dehydrogenase (GA-3-PD). Pyruvate kinase converts phosphoenol pyruvate (PEP) to pyruvate. PEP and ADP are substrates for PK and are both present in less than saturating concentrations. ATP is a product and has an inhibitory effect at concentrations found in the red cell. 2,3-DPG also inhibits PK activity and this mechanism represents a small portion of the 2,3-DPG effect. GA-3-PD catalyses the synthesis of 1,3-DPG and NADH from the substrates glyceraldehyde-3-phosphate, NAD and Pi. Activity of this enzyme is linked to the NAD/NADH ratio and the level of Pi which are also indicators of cellular energy balance.

Levels of 2,3-DPG play an intricate role in overall regulation of glycolysis. The activities of the rate regulating enzymes HK and PK are directly inhibited by 2,3-DPG. Inhibition of these enzymes is complex and is dependent on the level of ATP in the cell. A 2,3-DPG

induced fall in pH is another mechanism by which 2,3-DPG exerts an inhibitory action on glycolysis. The site of this effect is the phosphofructokinase enzyme which, as previously discussed, is highly sensitive to alterations in intracellular pH.

As alluded to previously, inorganic phosphate (Pi) can potentially contribute to the regulation of glycolysis. Intuitively since Pi is a low energy indicator, high cellular levels of Pi should stimulate glycolysis. This is, in fact, the situation. Increasing levels of Pi reduce the extent of inhibition of the PFK enzyme by ATP. In addition, the inhibition of HK by G-6-P is also decreased, particularily at low pH's. Inorganic phosphate is also a substrate for the GA-3-PD reaction and therefore a decrease in Pi levels may decrease the rate of glycolysis by limiting this reaction.

By review of these mechanisms for regulation of glycolysis, the complexity of the situation is readily observed. It is also apparent that a tight coupling of the glycolytic rate to the energy state of the cell exists. For this reason we feel that with all external factors controlled, (oxygenation, glucose availability, pH of the media), changes in cellular ATP utilization should be reflected by changes in the rate of glycolysis and hence lactate production.

STATEMENT OF THE PROBLEM

During the continued investigations into the etiology of essential hypertension, many investigators began reporting altered ion transport in cells from hypertensive patients and from animal models. In observing these abnormalities in the clinically accessible red blood cells, some investigators had proposed their use as markers in tests for a genetic component of essential hypertension. These tests were complex ion flux measurements which were time consuming and necessitated the use of expensive laboratory equipment. An initial objective of this study was to develop a related test which could be quickly performed and would involve simple techniques. We reasoned that if an abnormality was present in a cell's ion handling mechanisms, then this abnormality should be associated with an altered requirement for ATP. The basis for this reasoning is due to the tight coupling of ATP utilization to the maintenance of ion gradients. The major ion transporters, the sodium-potassium pump and the calcium pump, are known consumers of ATP. Other transporters, such as the sodium-potassium cotransport, may also require ATP in a manner which is not yet well defined.

Red blood cells do not contain mitochondria, therefore all of the ATP is produced anaerobically, hence lactate is produced as a co-product. We measured the rates of lactate release from red blood cells under the stress of different physiological and pharmacological manipulations in order to find conditions which would maximize the difference in lactate release rates between red blood cells of SHR and WKY rats. This difference would therefore be a viable marker for the hypertension trait. In addition, by determining the effects of various pharmacological interventions on lactate release, we attempted to identify a defective energy utilizing membrane process.

A procedure for detection of a hypertension trait was proposed by Garay et al. (1983b). They had reported that individuals with a genetic predisposion towards essential hypertension could be detected by a measurement of the maximal sodium potassium cotransport activity in red blood cells. In the SHR cells, maximal activity of this transporter is lower than that of the WKY rat cells. In order to observe this defect the red blood cells were loaded with sodium then incubated in an isotonic medium consisting primarily of magnesium and sucrose. In our initial approach to developing a test based on lactate release, we studied the cells under the conditions outlined by Garay and his co-workers.

Although the use of artificial media is often required for the observation of abnormalities which are present but otherwise obscured, they may alter the transport processes. It therefore makes the extrapolation of these results to the physiological situation difficult and unreliable. In order to study the roles of individual cellular processes in the hypertensive state, we studied the effects of pharmacological agents on the rates of lactate release while the cells were incubated in the physiological Krebs-Henseleit medium. These studies revealed a difference between SHR and WKY rats in their sensitivities to the effect of manganese.

The mechanism of action of manganese is unknown, however, Bohr (1974) and Shibata et al. (1973) observed that manganese induced a contraction in the vascular smooth muscle of SHR but not WKY rats. These observations suggested that the site of action of manganese may be involved in the hypertensive process. Therefore the results from our studies with manganese (as well as reports in the literature) were consistent with a hypothesis that the defect encompassed the calcium

pump or intracellular calcium pools. $^{86}Rb^{+}$ uptake studies were conducted to study the regulatory role of intracellular calcium on the sodium-potassium ATP-ase activity and its importance in hypertension. $^{45}Ca^{2+}$ uptake studies were conducted in order to directly evaluate the activity of the calcium pump in hypertension.

To study membrane proteins further, we also examined kinase dependent protein phosphorylation. The kinase dependent phosphorylation is a cellular mechanism which regulates the activity of many proteins. Since ion transport was observed to be altered, it was essential to determine if this abnormality was a function of a defective phosphorylation regulatory process.

SECTION II

METHODS

A. BREEDING AND SELECTION OF RATS

Adult Spontaneously Hypertensive Rats (SHR) and Wistar-Kyoto (WKY) rats were obtained from colonies maintained in our animal house. These colonies were inbred descendents of rats purchased from the Charles River Breeding Laboratory. Both strains were raised under the same environmental conditions and fed ad libitum a standard pellet diet (Wayne Pet Foods; Chicago Il.) along with tap water. The colony was replenished with new breeding stock from Charles River every year.

The rats were screened for blood pressure prior to each experiment by direct cannulation of the carotid artery. They were accepted to be hypertensive if their mean blood pressure was greater than 160 or 150mm Hg, for male and female rats respectively. Control WKY rats had blood pressures not greater than 125 mm Hg. Experiments were performed simultaneously on rats from both strains, paired according to sex and age. All rats were adults, three months of age or older.

B. COLLECTION AND PREPARATION OF RED BLOOD CELLS

Rats were anesthetized with ether and then a cannula of polyethylene tubing (PE 60) was inserted into the left carotid artery. The cannula was first connected to a Statham pressure transducer (Model P23Db) in order to obtain a direct measurement of arterial pressure, then used for blood removal. As the blood was drained into a syringe, saline (with 10U/ml heparin) was periodically infused into the animal to prevent volume depletion. This procedure kept the rats alive for a longer period of time and allowed us to remove a greater percentage of the red blood cells. The collected blood was immediately centrifuged (10^4g·min), and the supernatant, along with the buffy coat, was carefully removed by aspiration. The cells were washed three times prior

to use in the experiment. The composition of the washing solution varied depending on the type of experiment to follow and will be given in the individual experimental procedures outlined below.

C. LACTATE RELEASE EXPERIMENTS

1. INCUBATION IN MAGNESIUM SUCROSE MEDIUM

i. SODIUM LOADING PROCEDURE

Following the previously described procedure for obtaining red blood cells from the rats, the cells were washed three times with the following solution:

$MgCl_2$	110mM
Tris-MOPS	20mM
(pH 7.2 at 25°C)	

During the washing procedure, the washing solution and the cells were kept on ice (4°C) at all times.

Except where specified, in these experiments the cells were pretreated so as to raise the intracelluar concentration of Na^+. For this procedure the cells were suspended into a sodium loading solution to a final hematocrit of approximately 4%. Hematocrits were estimated by microhematocrit centrifugation. The sodium loading solution consisted of:

NaCl	150mM
$MgCl_2$	1.0mM
Sodium Phosphate Buffer	2.5mM
(pH 7.2 at 4°C)	

The cells were incubated in this solution overnight in a cold room (4°C). After the first six hours the solution was renewed. The total incubation period was 20 hours, following which the cells were removed,

washed three times with the 110mM $MgCl_2$ washing solution, then assayed to determine the rate of lactic acid release.

ii. ASSAY FOR LACTIC ACID RELEASE

The cells were diluted into the magnesium-sucrose incubation medium to a final hematocrit of 4%. The composition of the magnesium-sucrose medium is as as follows:

$MgCl_2$	75mM
SUCROSE	85mM
GLUCOSE	10mM
Tris -MOPS	10mM
(pH 7.4 at 37°C)	

An aliquot of the suspension was removed for protein determination and the remainder was distributed as 1ml aliquots into Eppendorf microcentrifuge tubes. Test substances were added to the Eppendorf tubes in volumes never exceeding 1% of the total. Water was used where no drug was added. The tubes were tightly sealed and incubated for 1 hour at 37°C and periodically mixed in order to avoid sedimentation. The incubation period was ended by transferring the tubes to an ice bath (4°C) for 3 minutes. Following centrifugation the supernatants were removed, deproteinized with 0.8N perchloric acid, then frozen and stored until assayed for lactic acid content.

In order to perform the lactic acid determination assay, the samples were removed from the freezer and thawed at room temperature. The lactic acid content was estimated according to the procedure originally described by Hohorst et al. (1963). This method measures changes in absorption at 340nm as NAD is reduced to NADH along with the enzymatic conversion of lactate to pyruvate.

Variations in dilution were corrected for by measuring the total protein content of each suspension. The Lowry method of protein estimation (Lowry et al., 1951) was preferred over hemoglobin estimation for normalization of values, simply because of the availability of an auto-analyzer which increases the uniformity and ease with which the samples can be analyzed. Bovine serum albumin was used as the standard.

The pH of each suspension was checked before and after the incubation and no significant changes were observed. Shape or volume changes of the cells which may have resulted from the use of some of these agents were not a major concern since only direct comparisons between SHR and WKY rats under the same conditions, were made.

iii. STATISTICAL ANALYSIS

Ion handling abnormalities are thought to be age and sex dependent. As a result, experiments were performed on age and sex matched pairs of SHR and WKY rats. Thus for statistical comparisons, unless otherwise stated, a paired Student's t-test was used for direct comparison of WKY to SHR. Level of statistical significance is indicated as; *=$p<.05$, **=$p<.01$, ***=$p<.001$.

2. INCUBATION IN KREBS-HENSELEIT MEDIUM

i. ASSAY FOR LACTIC ACID RELEASE

The procedure for the experiments measuring lactic acid release rates from red blood cells incubating in Krebs-Henseleit, is the same as that previously described for the experiments performed in the magnesium-sucrose medium, with some modification. Once isolated, the cells were washed three times with the washing solution of Fairbanks (Fairbanks et al., 1971):

NaCl	150mM
Sodium-Phosphate	5mM
(pH 8.0)	

They were then suspended (4% hematocrit) in the Krebs-Henseleit medium for the lactic acid release experiments. The composition of the Krebs-Henseleit medium was:

NaCl	118mM
KCl	4.7mM
GLUCOSE	11mM
KH_2PO_4	1.4mM
$MgSO_4$	1.2mM
$NaHCO_3$	25mM
$CaCl_2$	2.5mM

(pH 7.4 at 37°C.)

$CaCl_2$ was added after the solution was gassed with a 95% O_2: 5% CO_2 gas mixture for 15 minutes. For low calcium Krebs-Henseleit medium, calcium was simply omitted from the solution. For the experiments in which the calcium concentration was varied from 0.5mM - 6.0mM, a 1M stock of $CaCl_2$ was diluted to concentrations which were 100 times the desired final concentration. These $CaCl_2$ solutions were then added to the appropriate cell suspensions in volumes equivalent to 1% of the total volume. No adjustment was made to compensate for the changes in osmolarity resulting from the altered $CaCl_2$ concentrations in the cell suspensions.

The concentration of lactic acid in the samples were determined before and after a 120 minute incubation period, at 37°C, with a Model 27A Stat Lactate Analyser manufactured by Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A. This instrument contains the enzyme L-lactate oxidase which is immobilized on a membrane and metabolizes L-lactate according to the following equation:

$$\text{L-Lactate} + O_2 \text{ ------} \rightarrow H_2O_2 + \text{Pyruvate}$$

The H_2O_2 is oxidized by a platinum probe anode as follows:

$$H_2O_2 \text{ ------} \rightarrow 2H^+ + O_2 + 2e^-$$

to produce a probe signal current (Mason, 1982). Aliquots of 25ul were used for each determination.

D. $^{86}Rb^+$ UPTAKE EXPERIMENTS

The radioactive tracer $^{86}Rb^+$ can be used as a substitue for K^+ to be taken up by the (Na^+-K^+)-ATPase pump. Measurement of $^{86}Rb^+$ accumulation which is sensitive to the presense of ouabain is an indication of the activity of the (Na^+-K^+) pump. Prior to these experiments the cells were washed three times in the solution of Fairbanks which has been described previously. The media for incubation were similar to the Krebs-Henseleit medium with the exception that the bicarbonate buffer was omitted and replaced with HEPES, with or without 2.5mM calcium. These media are referred to as HEPES Krebs-Henseleit and Low calcium HEPES Krebs-Henseleit. The compositions of the HEPES Krebs-Henseleit was:

NaCl	120mM
KCL	4.7mM
$MgCl_2$	1.2mM
GLUCOSE	11mM
KH_2PO_4	1.4mM
HEPES	25mM
$CaCl_2$	2.5mM
(pH 7.4)	

For Low calcium HEPES Krebs-Henseleit medium the 2.5mM Ca^{2+} was omitted. No correction was made to compensate for the resulting change in osmolarity.

Suspensions of 10% Hct. were prepared, one with calcium and one without calcium. Aliquots of 125ul of each suspension were distributed to a series of eppendorf tubes.

To obtain final concentrations of ouabain ranging from 10^{-9}M to 2.5mM, a 10mM stock solution was serially diluted to create additional stocks of 1.0mM, 0.1mM, 0.01mM, 0.001mM, 0.0001mM, and 0.00001mM. Two sets of stocks were prepared, one using HEPES Krebs-Henseleit and one using low calcium HEPES Krebs-Henseleit as the diluent. Appropriate amounts of ouabain were added from these stocks to the eppendorf tubes in order to obtain the desired final concentration. The ouabain was added in volumes not less than 12.5ul and never exceeding 75ul. The appropriate buffers were added in the required amounts to make a final volume of 200ul in each tube.

To start the assay 50ul of buffer containing 0.6uCi $^{86}Rb^{+}$, was added to each tube. The tubes were incubated at 37^{o}C for the desired time period, usually 20 minutes unless otherwise stated. The incubation period was terminated by rapidly adding 1ml of cold HEPES Krebs-Henseleit solution. The cells were washed twice with the same solution. A third wash was found not to affect the counts. Following the final wash, the cells were digested by adding 100ul of a 1:1 mixture of nitric acid and perchloric acid. Digestion was completed either overnight at room temperature or within 2-3 hours at 60^{o}C. Aliquots of the digest were transferred to 5ml of scintillation cocktail for scintillation counting.

The scintillation cocktail was prepared in volumes of 2.5 liters. It is a mixture containing; toluene, 2 liters; 2,5-diphenyloxaxole (PPO) 11.3 grams; 1,4-bis(2-(5-phenyloxazolyl)) benzene (POPOP), 0.2 grams; ethylene glycol monomethyl ether (EGME),500 ml.

For statistical comparison of WKY to SHR non-paired Student's t-test was employed.

E. $^{45}Ca^{2+}$ UPTAKE EXPERIMENTS

The radioactive isotope $^{45}Ca^{2+}$ was used in the experiments to determine the effect of manganese on net calcium uptake by the red blood cells. For the preparation of the cells for this experiment, following isolation, the cells were washed three times in the previously described washing solution of Fairbanks and then given a final wash in the same solution which was to be used as the incubation medium. For the experiments in which $^{45}Ca^{2+}$ uptake was studied in a low sodium medium, a low calcium, sodium free washing solution was used.

The solutions employed for the washing and incubation procedures were similar to the Krebs-Henseleit medium, however, because a sodium free solution was required the sodium carbonate was omitted and replaced with tris buffer. The composition of the Tris Krebs-Henseleit solution was:

NaCl	120mM
KCl	4.7mM
$MgCl_2$	1.2mM
GLUCOSE	11mM
$CaCl_2$	2.5mM
Tris-HCl	25mM
(pH to 7.3 with HCl)	

To prepare sodium free Tris Krebs, the NaCl was replaced with 240mM sucrose. For the low calcium solution the $CaCl_2$ was omitted.

The final suspensions which were incubated varied in volume, depending on the experiment, but all contained a final hematocrit of 10%

red blood cells. Manganese stocks of 100 times the desired final concentration were prepared in water. These stocks were added to the incubation media in volumes equivalent to 1% of the total volume. Where no manganese was required, water was added (1% of final volume) in order to control for any changes in osmolarity.

At the start of the incubation period $^{45}Ca^{2+}$ was added (0.2uCi per 250ul). The incubation period was for 45 minutes, except where indicated and was terminated by diluting an aliquot of the suspension with large volumes of 50mM Lanthanum solution. For blanks, $^{45}Ca^{2+}$ was added after dilution with the lanthanum solution. The lanthanum ion has the property of displacing extracellular calcium and blocking calcium fluxes across the membrane. This technique has been adapted from the method of Godfraind (1976) for the study of calcium movements in vascular smooth muscle. The composition of of this solution was:

NaCl	122mM
KCl	5.9mM
$MgCl_2$	1.25mM
GLUCOSE	11mM
$LaCl_3$	50mM
Tris-Maleate	15mM

(pH to 6.8 with maleic acid)

The cells were washed twice with the lanthanum solution and then digested with 100ul of a 1:1 nitric acid, perchloric acid mixture. The digestion was completed overnight at room temperature or within 2-3 hours at $60^{o}C$. Aliquots of the digest were transferred to 5ml of scintillation cocktail for scintilaton counting.

For statistical comparison of WKY to SHR non-paired Student's t-test was employed.

F. ELECTROPHORESIS AND PHOSPHORYLATION EXPERIMENTS

Electrophoresis was performed on the proteins of isolated red blood cell membranes (ghosts) by the procedure of employed by Fairbanks et al. (1971) for the study of human erythrocyte membrane proteins. In order to prepare the hemoglobin-free preparations, the cells first were washed three times in cold washing solution of 5mM sodium phosphate and 0.15M NaCl (ph 8.0). The cells were lysed by rapidly mixing 1 to 1.2 ml. aliquots of cells into 40 mls. of a cold 5mM sodium phosphate solution. Following centrifugation the hemoglobin remained in the supernatant fraction, which was removed by aspiration. The pellet consisted of a top layer, the "ghosts", and an opaque, cream-colored button, which was also aspirated. This was an important step in order to minimize the contamination of the ghosts by proteases. This wash was repeated until the remaining ghost pellet was homogenous, white and packed to a concentration of 3-4 mg/ml. The creamy white ghosts were stored in 25mM imidazole buffer at pH 8.0.

The incubation of the ghosts with radioactive ATP gamma-^{32}P (1mM ATP with 4uCi/100u/) was undertaken for 20 minutes at 37^{o} C according to the procedure of O'Farrell (1975). All assay mixtures contained 24mM imidazole buffer at pH 7.0, 5mM $MgCl_2$ and either 0.1mM EGTA or 0.1mM $CaCl_2$. The reaction was stopped by the addition of a mixture of sodium dodecylsulphate (SDS) (0.5%) and mercaptoethanol (1mM). Following electrophoresis, the gels were fixed in an isopropyl alcohol solution and stained with coomassie blue protein stain. Destaining of the background was accomplished with a 10% acetic acid solution.

The gels were dried and then subjected to autoradiography for visualization of the radioactivity incorporation. X-ray films (X Omat AR) were exposed to the gels for different durations by keeping

them in contact with the dried gels in an intensifier, to obtain optimum impressions of the phosphorylated bands. The radioactivity of specific proteins, such as spectrin, 100,000 dalton and the 140,000 dalton proteins were determined from the autoradiograms with a Helena Quick Scan densitometer. The 100,000 dalton protein was equally phosphorylated in SHR and WKY (confirmed by CPM/mg protein and its intensity on autoradiograms, therefore its level of phosphorylation served as an internal marker. Hence the quantitation of the phosphorylation of the 140,000 dalton protein was expressed as the ratio to the phosphorylation of 100,000 dalton protein. The 100,000 dalton protein was not thio-phosphorylated to detectable levels hence spectrin phosphorylation was used as the internal standard in the experiments with gamma ^{35}S-ATP.

For statistical comparison of WKY to SHR non-paired Student's t-test was employed.

SECTION III

RESULTS AND DISCUSSION

STUDIES OF LACTATE RELEASE BY RED BLOOD CELLS INCUBATING IN MAGNESIUM-SUCROSE MEDIUM

1. RESULTS

i. BASAL RATES

Since other investigators were only able to demonstrate abnormal ion fluxes in red blood cells under non-physiological conditions, we attempted to duplicate these conditions and determine their effect on rates of lactate release. Garay and his group observed altered ion fluxes in cells which were loaded with sodium, using the PCMBS procedure of Garrahan and Rega (1967), prior to being incubated in a medium of magnesium and sucrose (Garay et al., 1983b). We avoided the use of PCMBS and raised the intracellular sodium concentration by storing the cells in a 150mM NaCl, phosphate buffer at $4^{o}C$ for 20 hours. The resulting intracellular sodium and potassium ion concentrations are presented in Table I. Washing the cells three times in 110mM $MgCl_2$, buffered with tris-HCl, ensured that extracellular sodium and potassium have been removed. Lactate release rates were studied in an incubation medium consisting of 75mM magnesium chloride, 85mM sucrose, 10mM glucose and 10mM tris-MOPS (pH 7.2 at $37^{o}C$). This condition promotes the outward movement of sodium and potassium through the furosemide-sensitive co-transport mechanism. Although this situation is highly non-physiological, it has been successfully employed by other investigators who have reported observing different rates of ion fluxes between cells obtained from hypertensive and normotensive patients (Dager and Garay, 1980).

Our studies indicated that the rate of lactate release remained constant for up to two hours. Figure 1 illustrates typical time studies from 0 to 60 minutes for cells from WKY rat and SHR strains. The coefficient of determination (R^2) $\pm$ S.E. values were 0.988 ± 0.039 and 0.996 ± 0.027 for WKY and SHR respectively. Lactate release rates were

Figure 1. Time study of lactate release from sodium loaded red blood cells incubating in the magnesium-sucrose medium. The SHR is represented by the open symbols and the broken line. WKY is represented by the solid circle and solid line.

LACTATE RELEASE

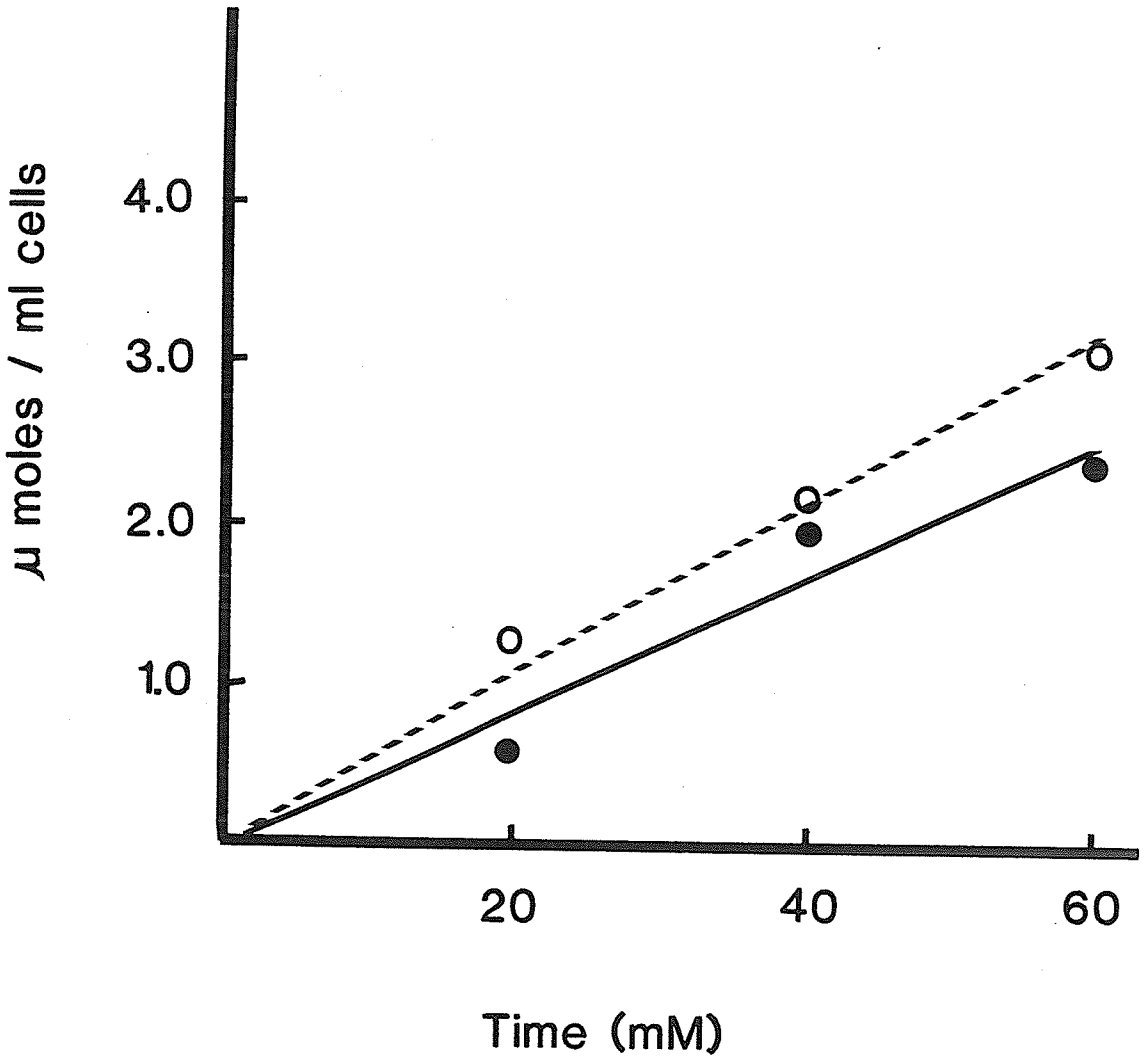


Table I. Intracellular concentrations of Sodium and Potassium in fresh red blood cells and cells which were subjected to the sodium loading procedure.

COMPARISON OF INTRACELLULAR ION CONCENTRATIONS BETWEEN FRESH AND SODIUM-LOADED CELLS

	FRESH (mM)	SODIUM-LOADED (mM)
SODIUM		
WKY	6.17 ± 0.37	16.70 ± 3.03
SHR	5.76 ± 0.14	19.53 ± 3.87
POTASSIUM		
WKY	118.7 ± 3.4	100.9 ± 6.8
SHR	116.9 ± 1.2	99.4 ± 4.4

calculated from the slopes, as determined by linear regression. In this typical experiment the rate of lactate release from the WKY rat red blood cells was measured to be 2.51 micromoles per ml cells per hour and the measured rate from SHR red blood cells was 3.22 micromoles per ml cells per hour.

The rates of lactate release for cells incubated in this medium have been estimated from the means of 18 experiments. These values are illustrated in Figure 2. The rate of lactate release by SHR cells, 1.23 $\pm$ 0.13 micromoles per ml cells per hour, is 16.6% greater than the rate of release from WKY rat cells, 1.06 $\pm$ 0.11 micromoles per ml cells per hour. The two means were compared by Students' paired t-test and the difference was not found to be significant.

A measurement of the concentration of lactate was also performed after hemolysis of the red blood cells. It was observed that hemolysis of the red blood cells had no effect on the concentration of lactate measured. This ensured that the equilibration of lactate across the membrane was rapid enough so that changes in the extracellular concentrations were an accurate reflection of changes in the intracellular concentration.

ii. THE EFFECT OF RECEPTOR AGONISTS AND ANTAGONISTS

A group of agents, representative of well known membrane receptor agonists have been tested for their effect on the rate of red blood cell lactate release. These agents are listed in Table II. Table II also lists the rates of lactate release from both WKY and SHR cells, in the presence of these agents.

Although cholinergic receptors have not as yet been identified on the rat red blood cells, the cholinergic agonist carbachol was tested. We had observed earlier that smooth muscle tissue of dog trachea

Figure 2. Rates of lactate release of sodium loaded red blood cells of WKY (filled bars) and SHR (open bars) incubating in the magnesium-sucrose medium. Bars represent the mean and SEM of 8 experiments.

LACTATE RELEASE

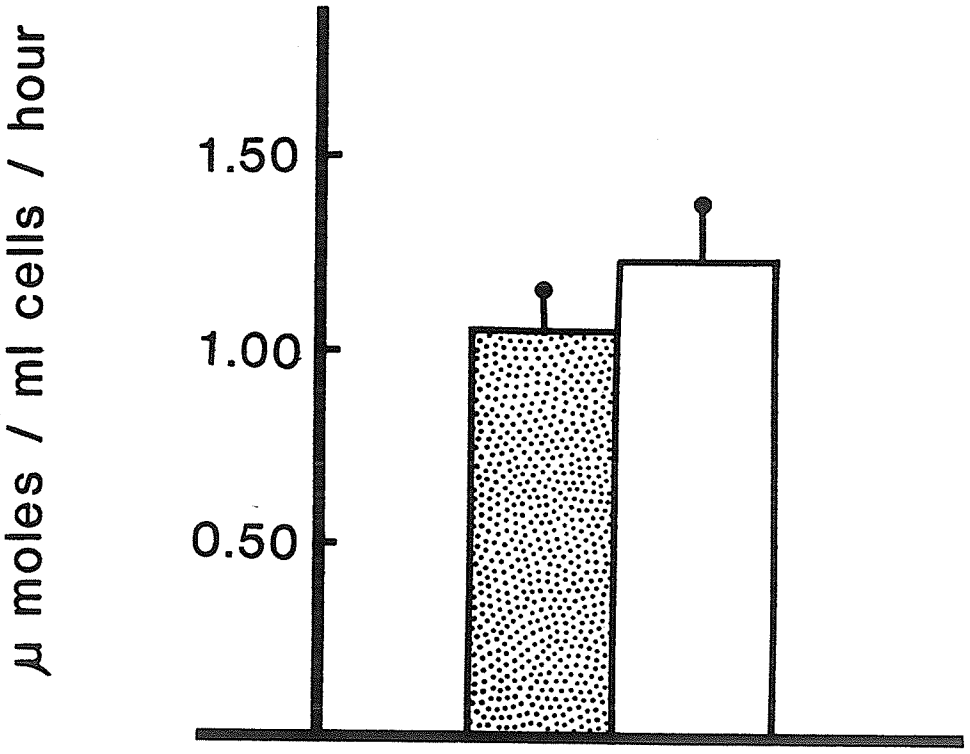


Table II. Rates of lactate release of red blood cells incubated in the magnesium-sucrose medium with the addition of various receptor agonsist and antagonists. The number of experiments for each agent is indicated on the right of the table.

TEST SUBSTANCE	LACTATE RELEASE RATES (μ moles / ml cells / hour)		
	WKY	SHR	(n)
CARBACHOL (10^{-5}M)	0.76 ± 0.17	0.78 ± 0.14	(4)
ISOPRENALINE (10^{-5}M)	0.85 ± 0.12	0.97 ± 0.10	(4)
PHENOXYBENZAMINE (10^{-5}M)	0.85 ± 0.20	1.32 ± 0.07	(5)
PROPRANOLOL (10^{-5}M)	1.14 ± 0.07	0.94 ± 0.18	(6)
SEROTONIN (10^{-5}M)	0.87 ± 0.17	1.25 ± 0.16	(6)
PHENOXYBENZAMINE AND NORADRENALINE	1.15 ± 0.09	0.91 ± 0.18	(6)
PROPRANOLOL AND NORADRENALINE	1.08 ± 0.09	0.97 ± 0.67	(6)

exhibited an increased rate of lactate release when carbachol was added to the incubation medium (unpublished observation). Work by Paul et al. (1979) demonstrated that in porcine coronary arteries, the rate of lactate release is directly associated with ion fluxes. Noradrenaline, isoprenaline, phenoxybenzamine, propranolol, and serotonin are all capable of affecting adenylate cyclase activity in the rat red cell membranes (Sheppard and Burghardt, 1970). Cyclic AMP is a second messenger which modulates many energy dependent processes. Although some of the agents appeared to have an effect on lactate release, only in the presence of phenoxybenzamine was the difference between WKY and SHR statistically significant.

iii. THE EFFECT OF TRANSPORT INHIBITORS

Lactate release rates were measured following addition of furosemide (1mM), ouabain (0.1mM) or both to the incubation media. These results are summarized in Figure 3. Basal rates are also included for comparison. Statistical analysis by Duncan's multiple comparisons test did not reveal any effect of these agents on lactate release. Student's t-test was employed for WKY, SHR comparisons, and this also did not reveal any differences. The values were the means of 8 experiments.

iv. THE EFFECT OF MANGANESE CHLORIDE

Addition of 1mM manganese chloride to cells incubating in the magnesium-sucrose medium stimulated the rates of lactate release in cells of both WKY and SHR strains. The basal rates illustrated in Figure 4 represent lactate release rates measured under this condition. The effect of manganese can be seen by comparing Figure 4 to Figure 3 (rates in the absence of manganese). These data indicate increases of 20.5% in WKY rat cells, and 12.8% in SHR cells due to the manganese.

Figure 3. Rates of lactate release of sodium loaded red blood cells incubating in magnesium-sucrose medium with the additions of furosemide (1mM), ouabain (0.1mM) or both. Bars represent the means and SEM of 8 experiments. (WKY- filled; SHR- open)

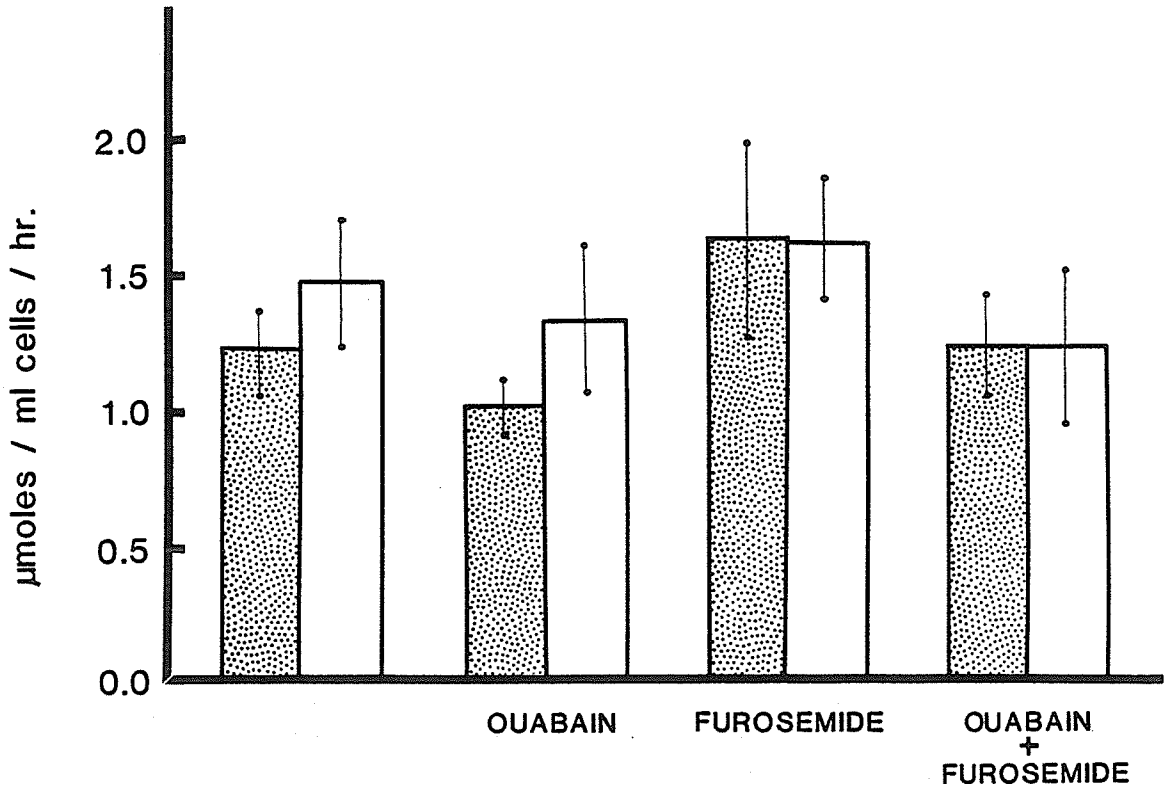
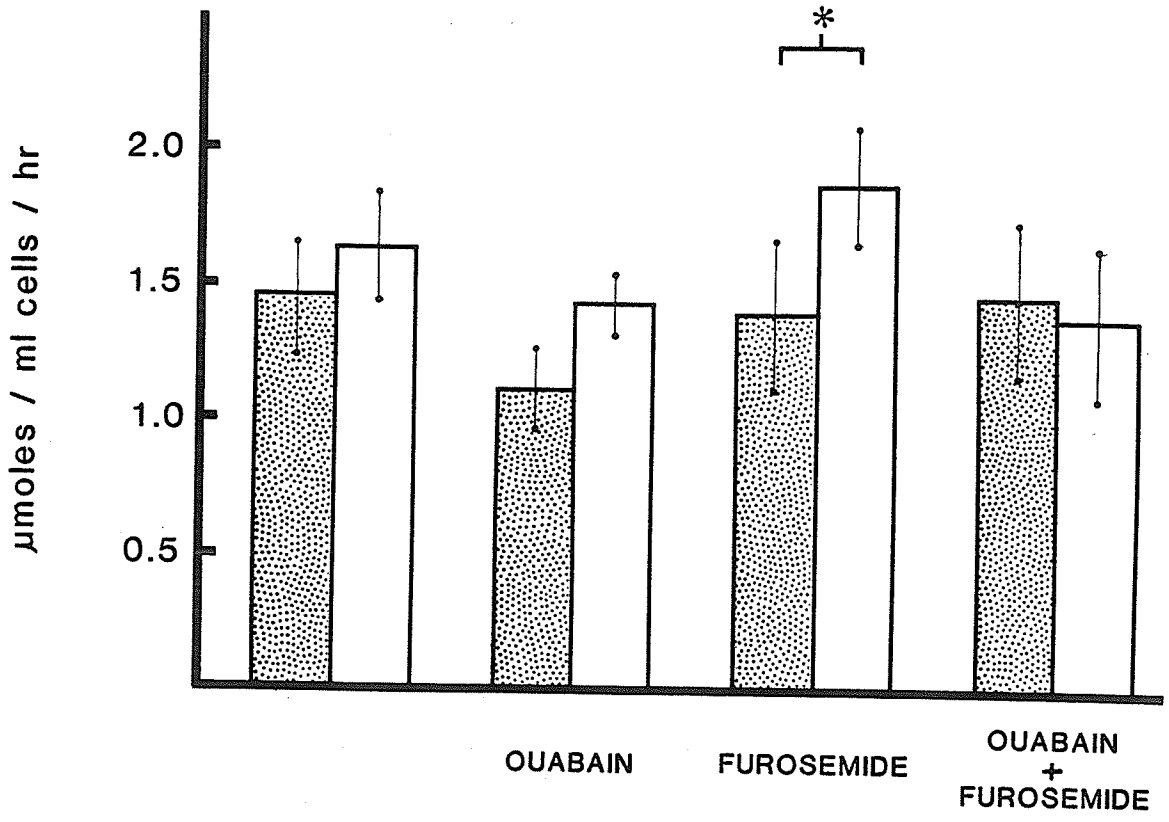

µmoles / ml cells / hr.
2.0
1.5
1.0
0.5
0.0
OUABAIN
FUROSEMIDE
OUABAIN
+
FUROSEMIDE

Figure 4. Rates of lactate release of sodium loaded red blood cells incubating in magnesium-sucrose medium containing manganese chloride with the additions of furosemide (1mM), ouabain (0.1mM) or both. Bars represent the means and SEM of 8 experiments. (WKY- filled; SHR- open)


µmoles / ml cells / hr
2.0
1.5
1.0
0.5
*
OUABAIN
FUROSEMIDE
OUABAIN
+
FUROSEMIDE

The disparity in the effects of manganese in the two strains of rats was more evident when combined with other transport inhibiting agents. With the addition of ouabain, lactate release rates were decreased in the cells from both strains. Furosemide, however, further stimulated the rate by the cells from SHRs but not WKYs. For SHR cells the increase in lactate release over the rate of release in the absence of manganese and furosemide was 28.7%, but only 15.7% for WKY rat cells. This difference was statistically significant. A combination of manganese, furosemide and ouabain eliminated the difference between the WKY and SHR red blood cells. The data of Figure 4 represent the means of eight experiments.

v. THE EFFECT OF AN INCREASED INTRACELLULAR SODIUM ION CONCENTRATION

Since all lactate release studies in the magnesium-sucrose medium were conducted on cells which were sodium-loaded to achieve a reversal of the ion gradients, we investigated the role of altered intracellular ion contents on the red blood cell lactate release. This study involved direct comparisons of sodium-loaded cells to fresh cells, both incubated in the magnesium-sucrose media.

Following the isolation and washing procedure, the cells were divided into two batches. One portion was suspended into a physiological saline solution containing ouabain. These cells were stored in this solution overnight at $4^{o}C$ for the purpose of sodium loading. The remainder of the cells were suspended in the appropriate media for determination of lactate release rates. Determinations of the lactate release rates for the sodium loaded cells were performed the following day.

Sodium and potassium ion concentrations were estimated by atomic absorption spectrophotometry in some of the experiments. Table I contains the mean values of four such determinations, both prior to and after the sodium loading procedure. Statistical significance was tested for in the comparison of WKY to SHR values, and none was observed.

The incubation media employed in this experiment was the previously described magnesium-sucrose solution, containing 1.0mM $MnCl_2$. Ouabain and furosemide were added where indicated.

Figure 5 illustrates the comparison of lactate release rates from fresh and sodium-loaded red blood cells of WKY rats. Figure 6 illustrates the same comparison for cells from SHR. Significance is indicated where rates from sodium-loaded cells were found to differ from the fresh cells under the incubation conditions indicated on the figures. For both figures the values represent the means of 7 experiments.

The rates obtained for fresh cells were subtracted from the rates obtained for the sodium-loaded cells and the differences were plotted in Figure 7. Figure 7 thus represents the changes in lactate release as a direct result of the sodium-loading and therefore indicates how intracellular sodium affects lactate release. These results clearly indicated a difference in the responses of the WKY and SHR cells to increased intracellular sodium. As seen in Figure 7, sodium loading caused an increase in the rates of lactate release in both WKY and SHR cells when these cells were incubating in the magnesium-sucrose medium containing manganese only. With furosemide present during the incubation period, sodium loading resulted in a stimulation of the rates of lactate release by the WKY cells, as is seen in the absence of furosemide. However, no change in the rate of lactate release by the SHR cells was observed.

Figure 5. Rates of lactate release from fresh (open) and sodium loaded (hatched) red blood cells of the WKY rat. Bars represent the mean and SEM of 7 experiments.

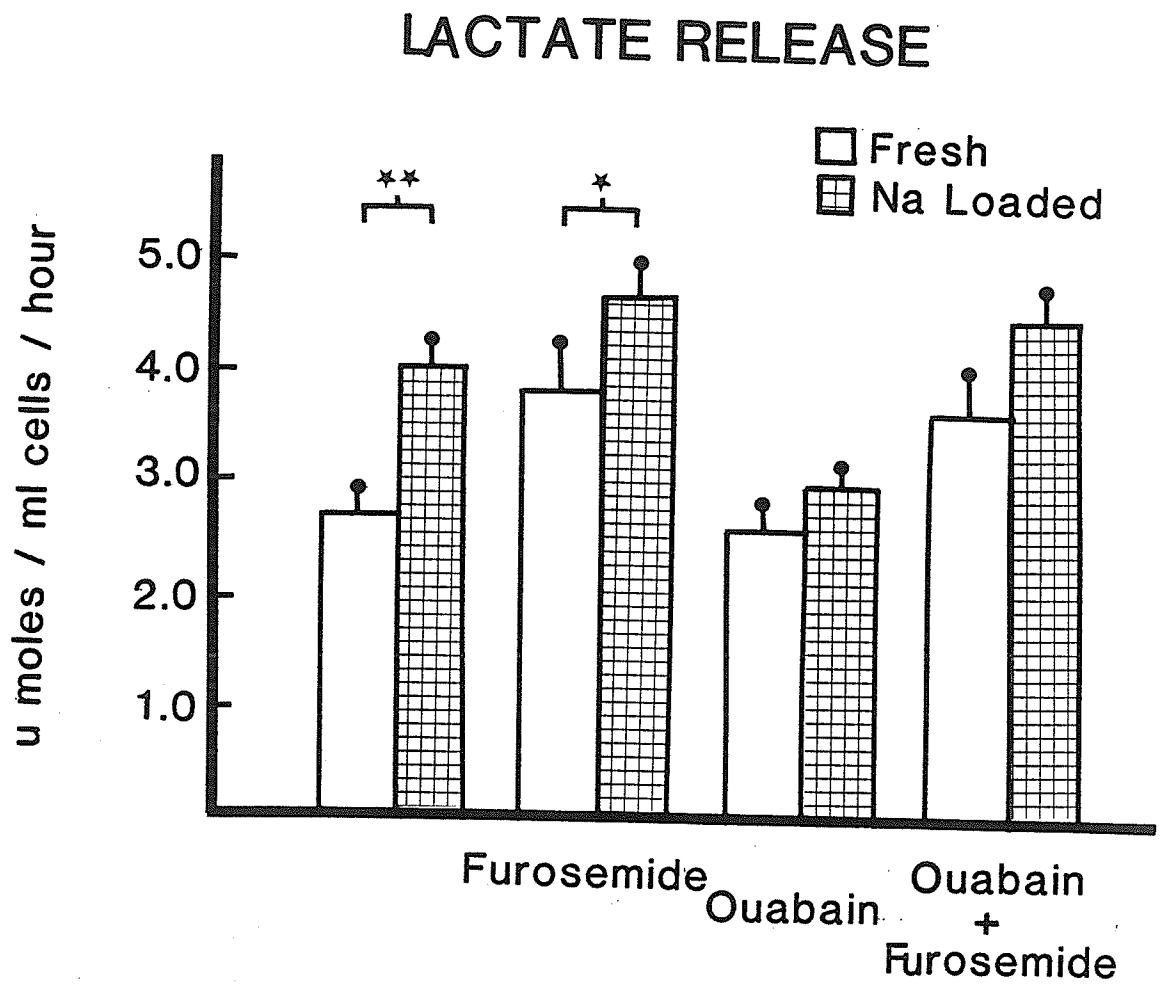

LACTATE RELEASE
Fresh
Na Loaded
u moles / ml cells / hour
5.0
4.0
3.0
2.0
1.0
Furosemide
Ouabain
Ouabain + Furosemide

Figure 6. Rates of lactate release from fresh (open) and sodium loaded (hatched) red blood cells of the SHR. Bars represent the mean and SEM of 7 experiments.

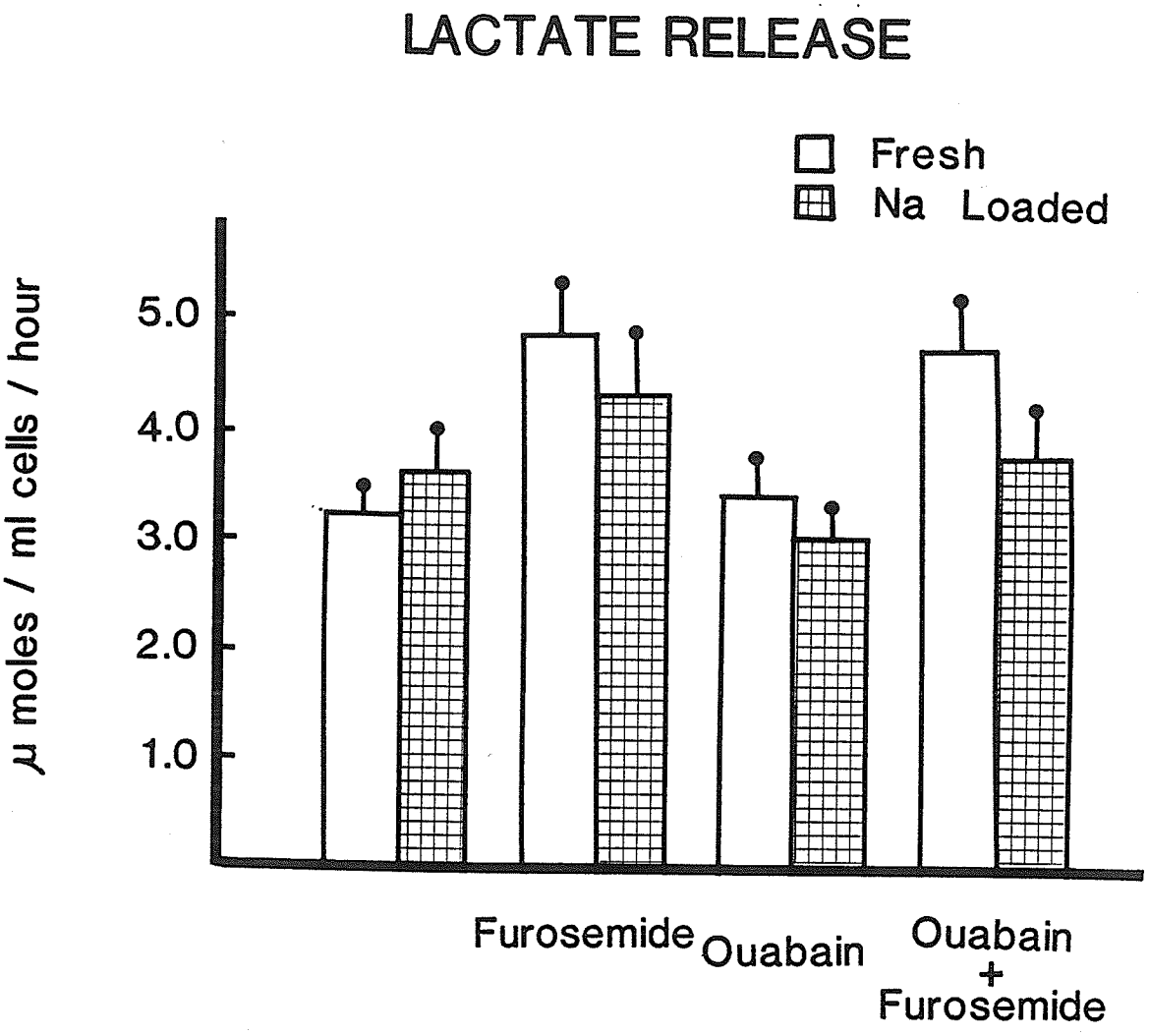
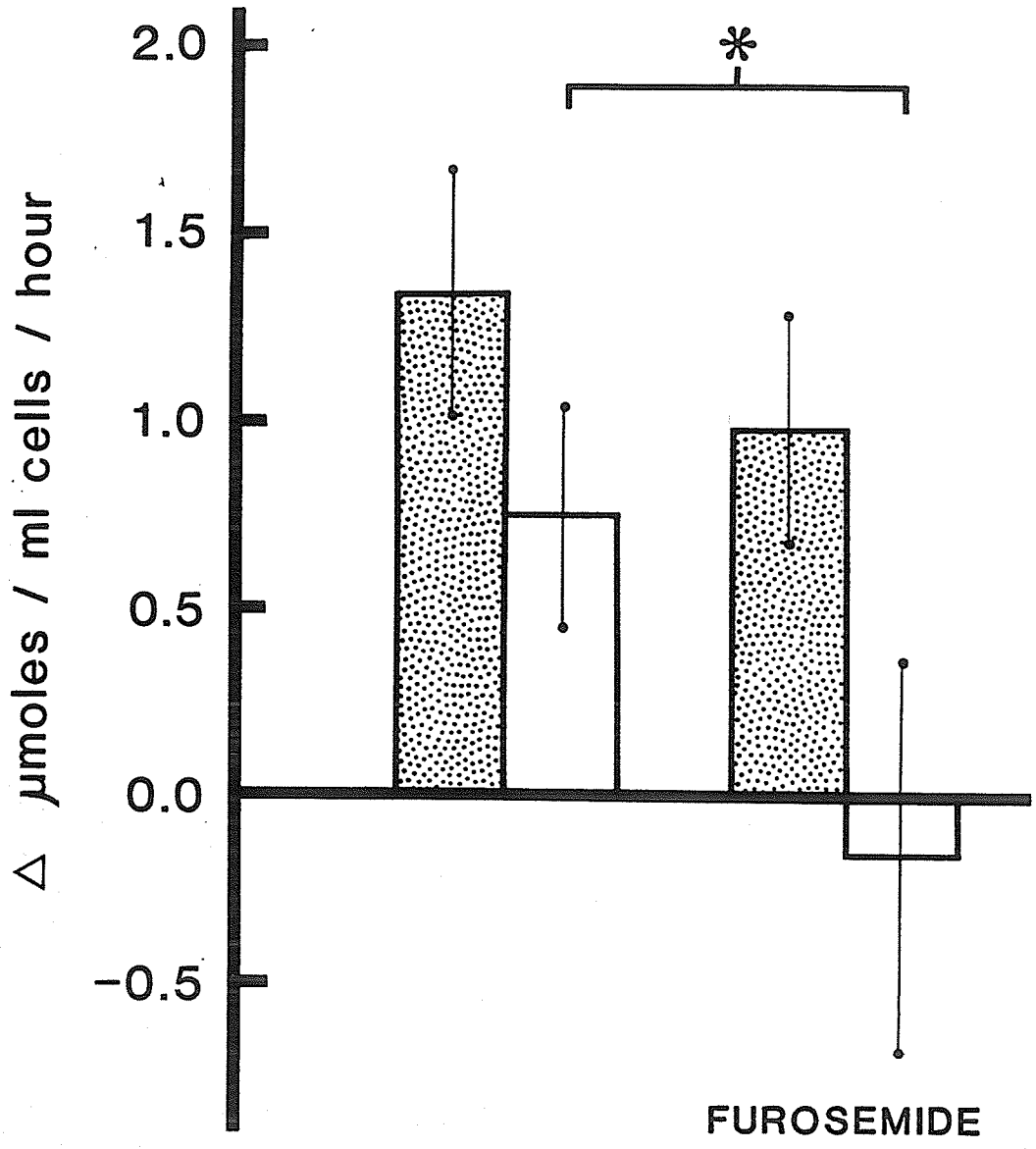

LACTATE RELEASE
Fresh
Na Loaded
μ moles / ml cells / hour
5.0
4.0
3.0
2.0
1.0
Furosemide
Ouabain
Ouabain
+
Furosemide

Figure 7. Effect of sodium loading on the lactate release rates from WKY rat and SHR red blood cells. Values were calculated as the difference between the values of figure 6 and figure 5. (WKY- filled; SHR- open)


2.0
1.5
1.0
0.5
0.0
−0.5
Δ µmoles / ml cells / hour
*
FUROSEMIDE

2. DISCUSSION

Garay et al. (1983b) had proposed that level of the sodium potassium cotransport maximal activity was associated with hypertension and could therefore be used as a marker for essential hypertension. Later, as it became apparent that this was not consistent in all essentially hypertensives observed, he subclassified the hypertensive population to explain the variability. Garay's results support the growing general belief that what is now referred to as essential hypertension is a loosely applied term for a group of patients with hypertension of unknown but different etiology. His observation, however, was shown to be a valid marker in the SHR and WKY rat models (De Mendonca et al., 1982).

The Na^+, K^+ cotransport defect was only observed when ion gradients were adjusted so as to maximize the transport activity. The procedure involved prior incubation of the cells with PCMBS, in order to load the cells with sodium. The sodium loaded cells were then incubated in an isotonic medium consisting primarily of magnesium and sucrose. Under this incubation condition the Na^+, K^+ cotransport activity was maximized.

For an initial approach to developing a test based on lactate release, we used the conditions outlined by Garay, in which observable differences in sodium-transport activity were noted. We, however, avoided the use of PCMBS because it was not known what permanent alteration this sulfhydryl inhibitor could have on cellular metabolism.

In Figures 1 and 2 it is shown that there is a slight difference in the rates of lactate release between the two strains of rats. This difference, however, was not sufficient to be used as an assay for the hypertension trait. Pharmacological agents were added to the incubation

media in an attempt to improve upon the difference in the rates of lactate release.

The use of transport inhibitors provided information which suggested a possible location for a defect in hypertension. Ouabain produced a slight, but similar inhibition of lactate release in both WKY and SHR under these conditions. Although the medium was Na^+ and K^+ free, the ouabain inhibition of lactate release may still be attributed to an effect on the ouabain sensitive sodium potassium pump. It has been shown by Glynn and Karlish (1976) that this transporter can remain operational as a Na^+-ATPase, hydrolysing ATP for uncoupled movement of sodium. The ouabain sensitive transport activity appears to be normal since the ouabain sensitive components of lactate release were similar in both strains of rats (Figure 3).

Furosemide tended to increase the rates of lactate release. This observation opposed the expected result since, under these conditions the furosemide sensitive Na^+, K^+ cotransport should be maximally active in sodium extrusion and the Na^+, K^+cotransport, although not considered as an active transport mechanism, may have an ATP requirement of its own (Adragna et al., 1985). An explanation for this observation would be that the observed increase in lactate release is a reflection of an increased demand for ATP by the Na^+, K^+ pump. As the Na^+, K^+ cotransport mechanism is inhibited, sodium extrusion becomes more dependent on the (Na^+-K^+)-ATPase. The observation that the effect of furosemide is blocked by ouabain (Figure 3) supports this explanation.

The location of the primary defect in SHR is difficult to discern. These data, however, are consistent with the postulate that a membrane bound pool of calcium which inhibits the activity of the (Na^+-K^+)-ATPase (Beauge and Campos, 1983) is involved. Manganese is an

inhibitor of (Ca^{2+}-Mg^{2+})-ATPase activity, however, we observed manganese to increase the lactate release rates. This increase was primarily in the ouabain sensitive component of lactate release, hence the effect of manganese was probably a result of stimulation of the Na^+-ATPase through an interaction with the membrane bound calcium pool. In the presence of manganese, furosemide increased the rate of lactate release by SHR cells but had no effect on WKY cells. Since this effect of furosemide was not apparent in the absence of manganese, this result may involve an interference of manganese with the calcium regulation of the ouabain sensitive transporter or possibly the Na^+, K^+ cotransporter (Garay, 1982).

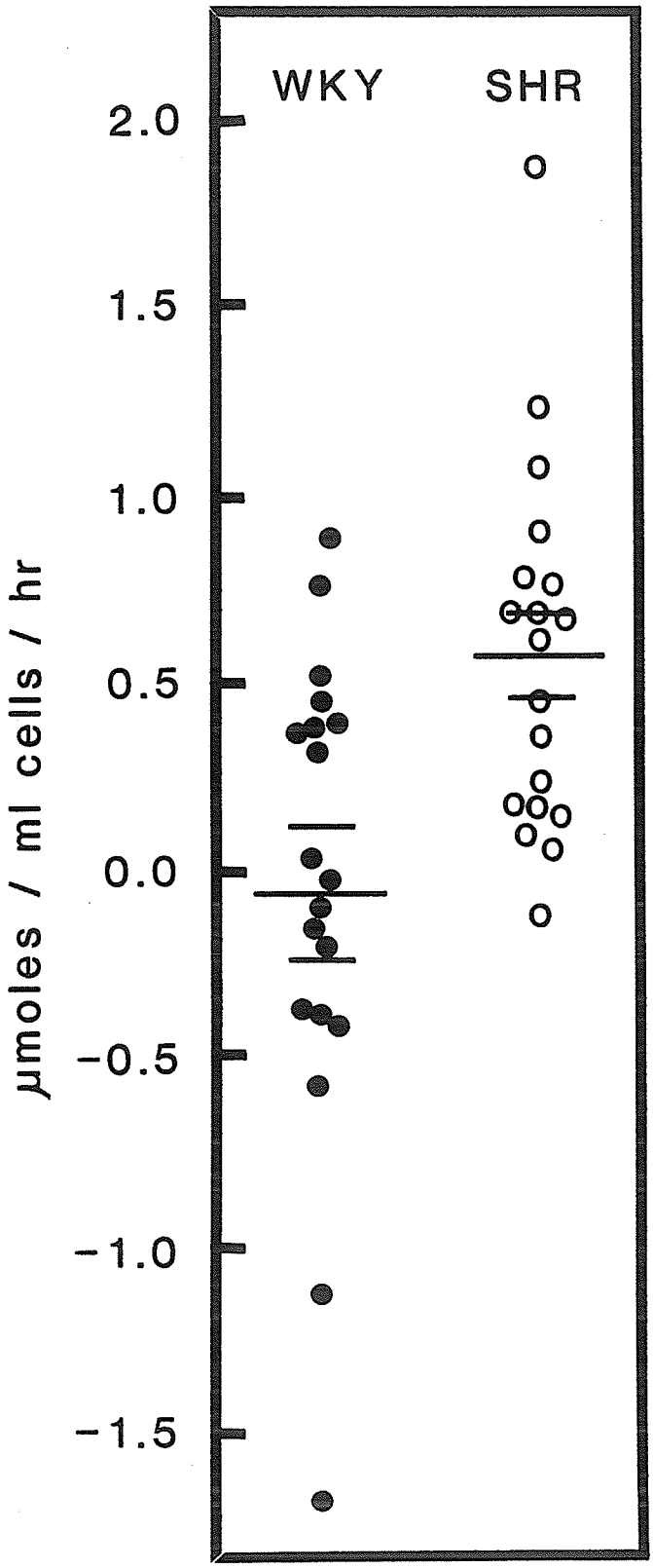
The sodium-loading procedure (Figure 7) should theoretically stimulate the Na^+-ATPase and the Na^+, K^+ cotransport activities. The increase in lactate release thus represented the greater demand for ATP resulting from activation of these two mechanisms. When the cells were incubated in a medium containing furosemide, the change in lactate release would primarily be a result of Na^+-ATPase activation. We observed that while there is a definite component of Na^+-ATPase activated by sodium-loading in WKY cells, there was none in SHR. Therefore the Na^+-ATPase in the SHR cells was not further activated by an increase in the intracellular sodium. This could either be a result of the Na^+-ATPase being in a state of full activation under normal intracellular sodium or indicate a shift in the Km for intracellular activation. Either way these data support the hypothesis that a defect is present in the regulation of transmembrane ion transport in the SHR cells. The first of these possible explanations is consistent with the postulate of a significant role of intracellular calcium in the regulation of ion transport. Postnov et al. (1977) reported that the

membranes of red blood cells from hypertensive subjects bind calcium with an attenuated affinity. Manganese, which was also present in the medium, may be able to completely displace the membrane bound calcium thus fully activating the pump.

From the data of Figure 4, it appeared that the determination of the amount of lactate release, which was both stimulated by the combination of furosemide and manganese and was sensitive to ouabain, may be a useful marker for the identification of a cellular abnormality. The difference in lactate release of cells incubating in manganese and furosemide, with and without ouabain was calculated and plotted in the scatter-diagram of Figure 8. The means and standard errors are represented by the horizontal bars. Although considerable overlap of the values makes this measurement of doubtful significance as a diagnostic test for a difference between the SHR and WKY rat, the points can be separated into two distinct populations.

In summary, this study has demonstrated that red blood cells of genetically hypertensive rats release lactic acid at a rate different from the red cells of their normotensive counterparts suggesting differing energy requirements for the two cell types. We hypothesize that in the SHR red blood cells, different rates of active ion transport resulted from alterations in the intracellular calcium pools. In addition we were interested to determine if measurement of lactate release would be a more efficient and reliable test for a predisposition to essential hypertension. From these data it appeared that determination of the amount of lactate release, which was both stimulated by the combination of furosemide and manganese and was sensitive to ouabain, may be useful for the identification of a cellular abnormality. With the development of proper conditions to reduce the

Figure 8. Scatter-diagram of ouabain-sensitive lactate release from red blood cells incubating in magnesium-sucrose medium containing manganese (1.0mM) and furosemside (1.0mM). (WKY - solid symbols; SHR - open symbols. The horizontal bars represent mean and SEM.


WKY
SHR
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
μmoles / ml cells / hr

overlap, this technique has the potential to become an easy diagnostic test.

STUDIES OF LACTATE RELEASE BY RED BLOOD CELLS INCUBATING IN KREBS-HENSELEIT MEDIUM

1. RESULTS

i. BASAL RATES OF LACTATE RELEASE

Following the washing procedure outlined by Fairbanks, the red blood cells were packed to a hematocrit of 85% - 90%. Suspending one volume of packed cells into 19 volumes of Krebs-Henseleit solution produced suspensions of 4.0% - 4.5% hematocrits. While incubating in a water bath at 37^{o}C it was found that these suspensions required periodic mixing at 15-20 minute intervals in order to prevent cell sedimentation. Under these conditions lactate was released by the cells at a constant rate for at least two hours. A typical time study is illustrated by Figure 9, in which the measured increase in the concentration of lactate in the media at the specified time points are plotted. The slopes of the resulting lines represent the rates at which lactate is released by the cells into the surrounding media. The coefficient of determination (R^2) value was 0.998$\pm$0.012 for WKY rats and 0.992$\pm$0.027 for SHR. The slopes were calculated by linear regression analysis and used as estimates of the rates at which the cells released lactate. For WKY cells the rate in this experiment was 4.220 micromoles lactate per ml cells per hour. For SHR the rate was 4.643 micromoles per ml cells per hour. For experiments in which the Lactate Dehydrogenase method was used for determining the concentration of lactate in the medium, the incubation time was usually one hour. A two hour incubation period was used when the Lactate Oxidase method was employed.

Figure 10 illustrates the basal rates of lactate release by cells from SHR and WKY rats, as estimated by the mean of twenty experiments. The bars represent the standard error of the mean. The rate of release by SHR cells, 4.38$\pm$0.23 micromoles per ml

Figure 9. Time study of lactate release from red blood cells incubating in the Krebs-Henseleit medium. The SHR is represented by the open symbols and the broken line. WKY is represented by the solid circles and solid line.

LACTATE RELEASE

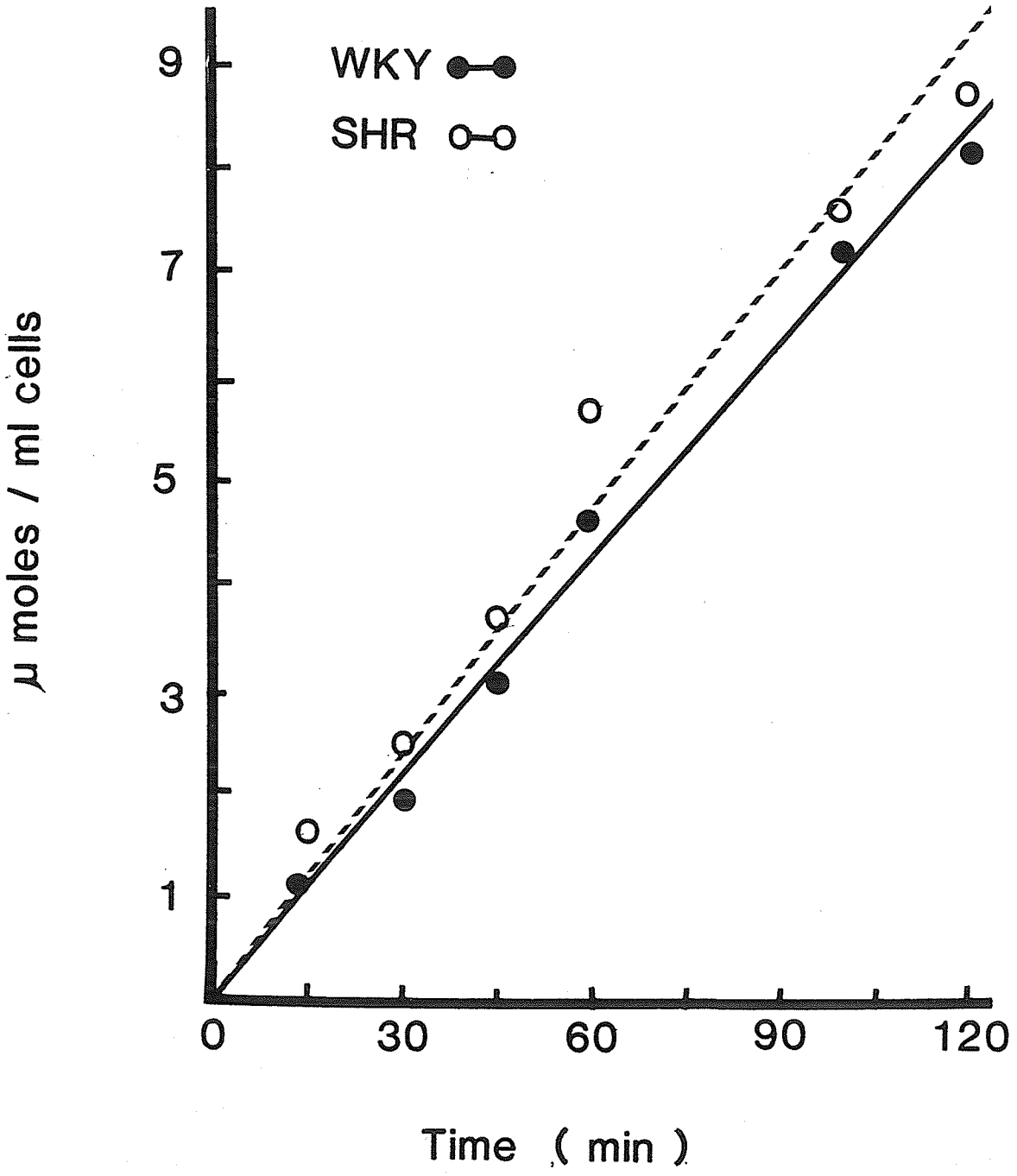
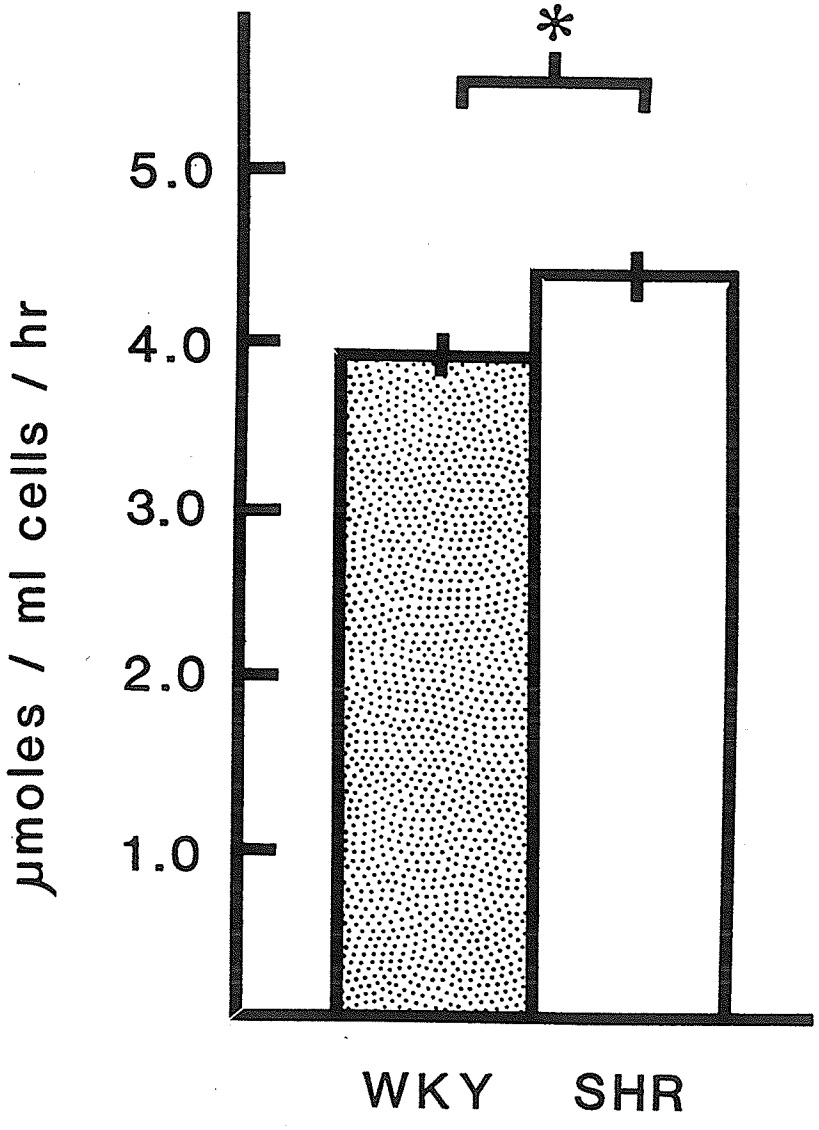


Figure 10. Rates of lactate release of red blood cells from WKY and SHR incubating in the Krebs-Henseleit medium. Bars represent the mean and SEM of 20 experiments. (WKY- filled; SHR- open)


*
5.0
4.0
3.0
2.0
1.0
μmoles / ml cells / hr
WKY
SHR

cells per hour, is 112.0% of the release rate by the WKY cells, 3.92±0.18 micromoles per ml cells per hour. This difference is statistically significant by Student's paired t-test. The standard error of the mean differences, the appropriate expression of variability for paired analysis, was calculated to be 0.56.

ii. THE EFFECT OF RECEPTOR AGONISTS AND ANTAGONISTS

The same receptor agonists and antagonists which were tested in the magnesium-sucrose medium were also tested for their effect on the rate of lactate release of cells incubating in Krebs-Henseleit medium. These results are listed in Table III.

For all of the agents tested, with the exception of isoprenaline, the lactate release rate by the red blood cells of SHR remained 10% - 15% greater than the rate of the WKY rat cells (Table III). Isoprenaline resulted in an apparent reversal of this trend although the variance was higher and no statistical significance could be observed. No change in lactate release rate occurred due to carbachol, in both WKY and SHR cells. This finding may be a reflection of a lack of cholinergic receptors on the red blood cells. Stimulation of cholinergic receptors on human red blood cells has been observed to produce a very rapid and transient increase in calcium uptake by the cells (Tang et al. 1984). It is possible that cholinergic receptors are present in rat red blood cells, however, the response would be too transient to observe an effect on lactate release over a sixty minute incubation period. Forskolin, which is an activator of adenylate cyclase, (Seamon and Daley, 1981) was also tested and found to have no effect on the rates of lactate release by either SHR or WKY rat red blood cells.

Table III. Rates of lactate release of red blood cells incubated in the Krebs-Henseleit medium with the addition of various receptor agonsist and antagonists. The number of experiments for each agent is indicated on the right of the table.

TEST SUBSTANCE	LACTATE RELEASE RATES (μ moles / ml cells / hour) WKY	SHR	(n)
CARBACHOL (10^{-5}M)	4.02 ± 0.20	4.33 ± 0.27	(14)
NORADRENALINE (10^{-5}M)	4.38 ± 0.30	4.71 ± 0.48	(7)
ISOPRENALINE (10^{-5}M)	3.92 ± 0.32	3.60 ± 0.29	(7)
PHENOXYBENZAMINE (10^{-5}M)	3.96 ± 0.37	4.69 ± 0.34	(6)
PROPRANOLOL (10^{-5}M)	4.17 ± 0.22	4.84 ± 0.25	(13)
SEROTONIN (10^{-5}M)	4.00 ± 0.29	4.66 ± 0.35	(6)
PHENOXYBENZAMINE AND NORADRENALINE	3.99 ± 0.29	4.65 ± 0.34	(6)
PROPRANOLOL AND NORADRENALINE	4.31 ± 0.21	4.64 ± 0.24	(13)

iii. THE EFFECT OF TRANSPORT INHIBITORS

The pharmacological agents ouabain and furosemide are well known inhibitors of specific ion transport systems. Ouabain inhibits the ubiquitous sodium-potassium pump (Akera et al., 1969) while furosemide is representative of loop diuretics which are considered to be specific inhibitors of the sodium-potassium co-transport system (Wiley and Cooper, 1974). Both these transporters have been implicated as components in the development of hypertension (Parker and Berkowitz, 1983).

A concentration of 0.1mM ouabain added to the cell suspension did not affect the rate of lactate release, however, inhibition of lactate release was observed with increasing concentrations of ouabain. The effect of ouabain on red blood cell lactate release is illustrated in Figure 11. Significance is indicated where values at a given concentration of ouabain are determined to be statistically different from basal values, by Duncan's multiple comparisons test. All values are estimated as the means of five experiments and expressed as MEAN$\pm$SEM.

For both WKY and SHR, the maximal effect of ouabain inhibition is achieved with 2.5mM ouabain. For WKY cells the basal rate of lactate release 0.58 micromoles per ml cells per hour (11.1%) is inhibited. In SHR cells the effect is a decrease of 0.78 micromoles per ml cells per hour (14.1%). The data was converted to an Eadie-Hofstee plot (not shown), from which the concentration of ouabain required to achieve 50% of the maximum ouabain inhibitory effect was estimated. This value was determined to be 0.49 mM ouabain for WKY and 0.52 mM ouabain for SHR.

For each concentration of ouabain tested, the resulting ouabain-sensitive components of lactate release values were calculated and plotted in Figure 12. Statistical comparisons were made between the

Figure 11. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium with the addition of ouabain (0.0 - 5.0mM) Bars represent the means and SEM of 5 experiments. (WKY- filled; SHR- open)

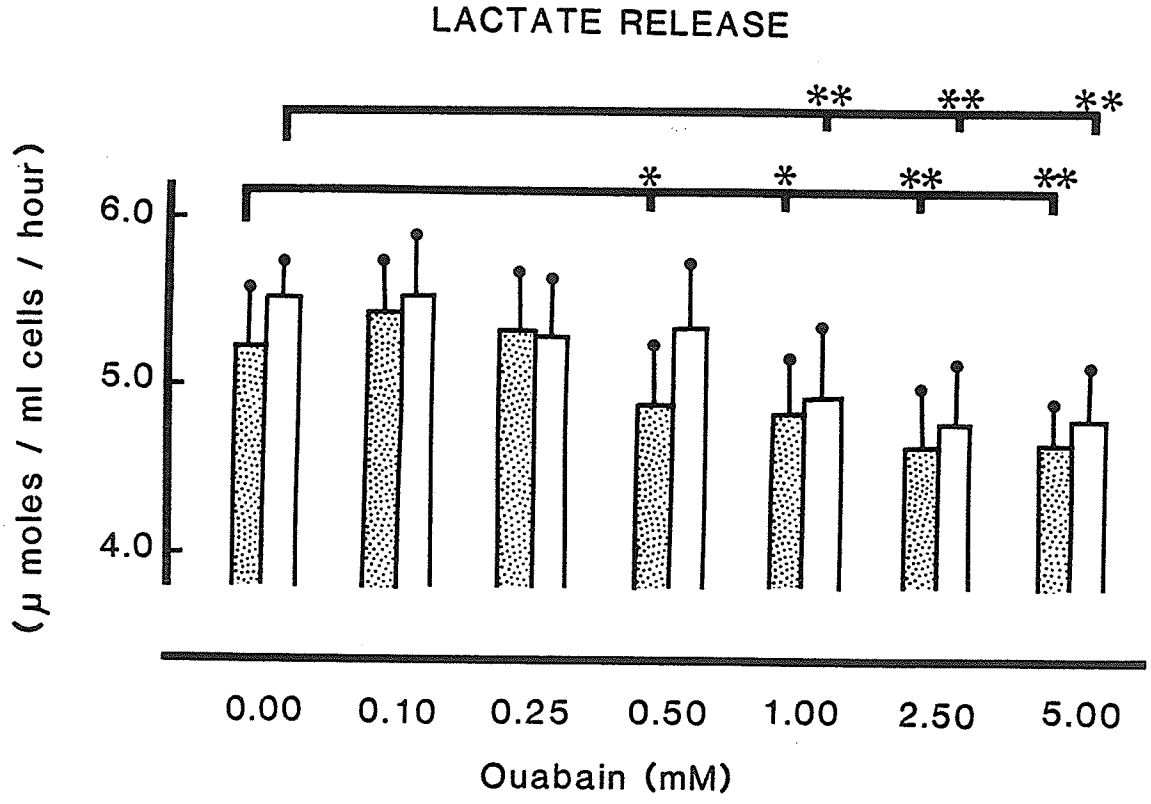
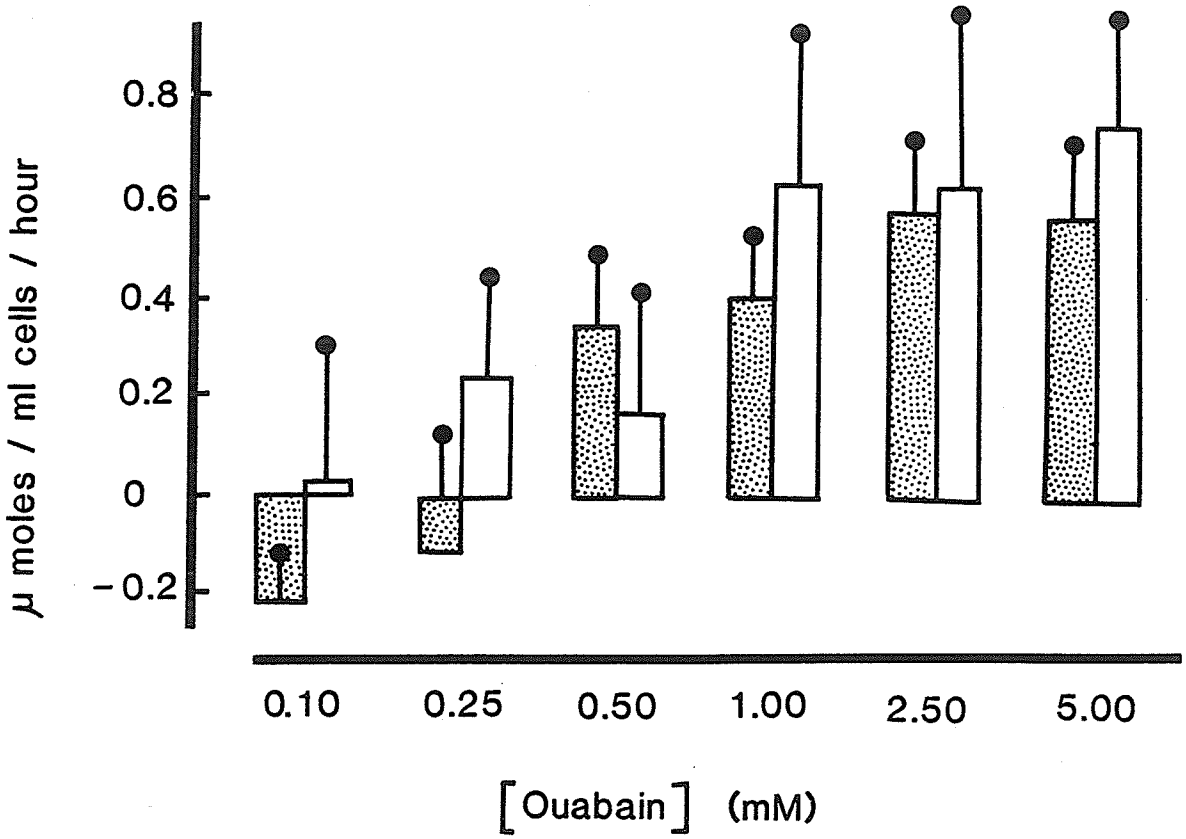

LACTATE RELEASE
**
**
**
*
*
**
**
6.0
5.0
4.0
(μ moles / ml cells / hour)
0.00
0.10
0.25
0.50
1.00
2.50
5.00
Ouabain (mM)

Figure 12. Inhibition of the rates of lactate by cells incubating in Krebs-Henseleit medium with the addition of the indicated concentration of ouabain. (WKY- filled; SHR- open)


INHIBITION OF LACTATE RELEASE
μ moles / ml cells / hour
0.8
0.6
0.4
0.2
0
-0.2
0.10
0.25
0.50
1.00
2.50
5.00
[Ouabain] (mM)

effects of ouabain on WKY and SHR cells with Students' unpaired t-test. Significance was not observed at any of the concentrations tested.

Figures 13 and 14 respectively illustrate the effect of ouabain in the presence of 0.10mM and 1.00mM furosemide. Significance is indicated where the rates have been statistically determined to differ from the basal rates. The maximal inhibition of lactate release is achieved with 2.5mM ouabain in the presence of either 0.10mM or 1.00mM furosemide, for both WKY and SHR cells.

The ouabain concentrations necessary for 50% maximal inhibition have been estimated from Eadie-Hofstee plots (not shown). The changes in lactate release rates, resulting from the addition of ouabain have been calculated and WKY, SHR statistical comparisons have been made. Only 5mM ouabain in the presence of 1.00mM furosemide was found to be significantly different in the statistical comparisons of WKY to SHR.

The values representing the maximal effect due to ouabain and the concentration of ouabain which produces 50% of the maximal inhibition are summarized in Table IV.

The effects of furosemide are illustrated in Figure 15. Each of the points plotted represents the lactate release rate by the cells in the presence of the indicated concentration of furosemide and are derived from the means of eight experiments. The variation is expressed as standard error of the mean. Significance is indicated where values were found to differ statistically from the basal values. These basal values were determined to be significantly different by Students' paired t-test. The standard error of the mean differences was 0.37. For each concentration of furosemide, Students' paired t-test was used for statistical comparisons of SHR cells to WKY rat cells where detected significance was denoted at the top of the figure.

Figure 13. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium containing furosemide (0.1mM) with the addition of ouabain (0.0-5.0mM) Bars represent the means and SEM of 5 experiments. (WKY- filled; SHR- open)

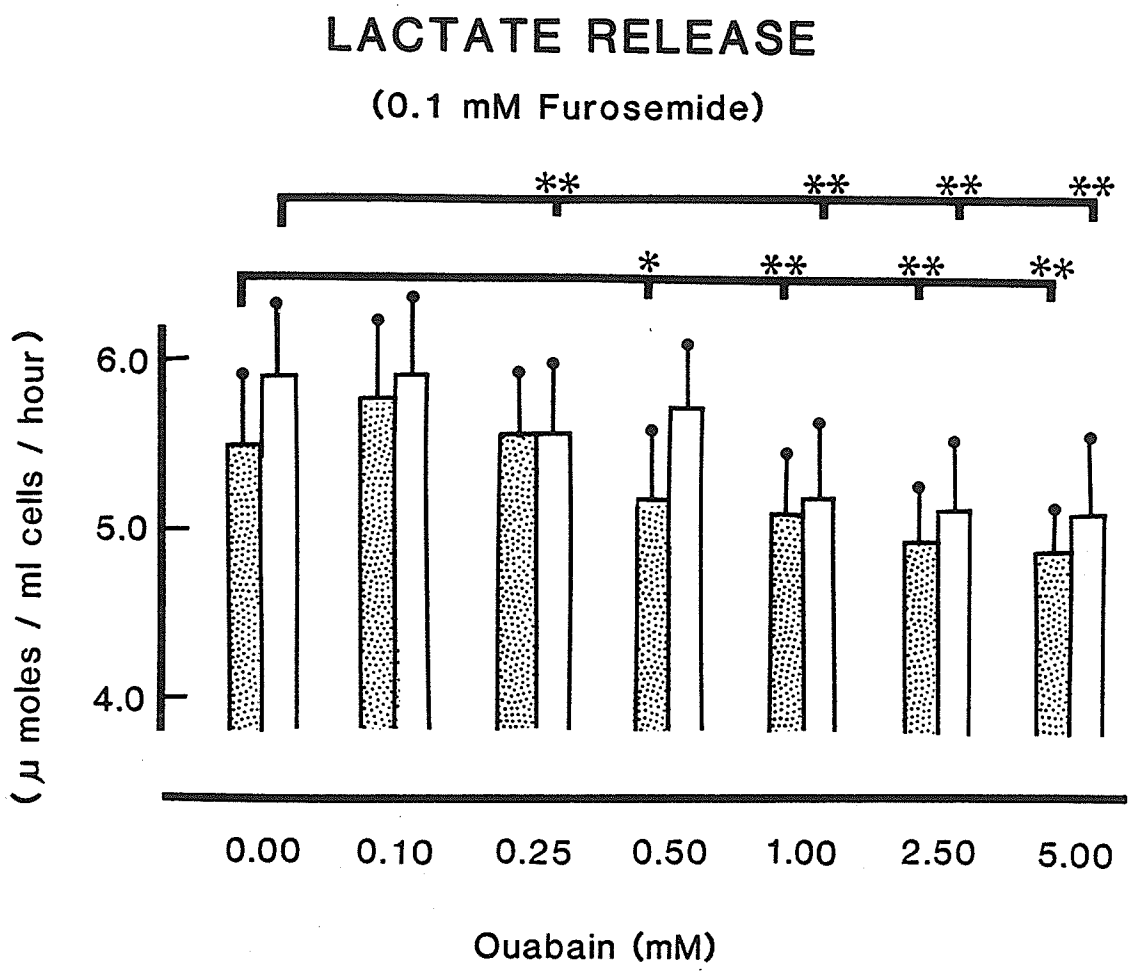

LACTATE RELEASE
(0.1 mM Furosemide)
**
**
**
**
*
**
**
**
6.0
5.0
4.0
(μ moles / ml cells / hour)
0.00
0.10
0.25
0.50
1.00
2.50
5.00
Ouabain (mM)

Figure 14. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium containing furosemide (1.0mM) with the addition of ouabain (0.0-5.0mM). Bars represent the means and SEM of 5 experiments. (WKY- filled; SHR- open)

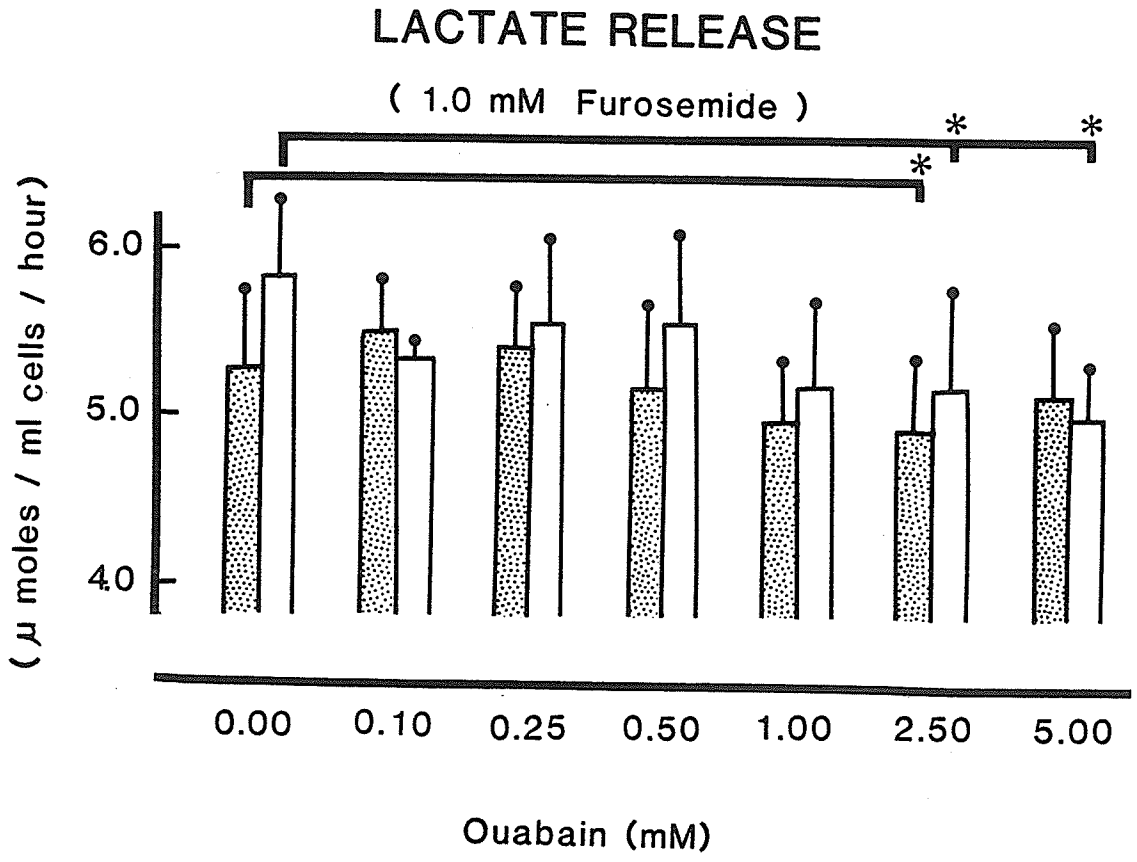

LACTATE RELEASE
(1.0 mM Furosemide)
*
*
*
(μ moles / ml cells / hour)
6.0
5.0
4.0
0.00
0.10
0.25
0.50
1.00
2.50
5.00
Ouabain (mM)

Table IV. Summary of the maximal inhibition of lactate release by ouabain and the sensitivities of the lactate release to inhibition by ouabain. Values are calculated from Eadie-Hofstee plots of the data presented in figures 11, 13 and 14.

	MAXIMAL INHIBITION	50% MAXIMAL INHIBITION
FUROSEMIDE 0.0 mM		
WKY	2.56 (11.1%)	0.49 mM
SHR	3.45 (14.1%)	0.52 mM
FUROSEMIDE 0.1 mM		
WKY	2.91 (12.0%)	0.65 mM
SHR	3.76 (14.4%)	0.39 mM
FUROSEMIDE 1.0 mM		
WKY	1.57 (6.7%)	0.65 mM
SHR	3.66 (14.3%)	0.63 mM

In this experiment an effect on lactate release by furosemide was not observed. Statistically, with one exception, none of the tested concentrations of furosemide altered the rate of lactate release from the basal value. With up to 0.100mM furosemide there appeared to be a slight increase in the rate of lactate release. Concentrations above 0.500mM furosemide reversed this effect. This trend was similar for both WKY rat and SHR cells. The difference in the rates of lactate release between SHR cells and WKY rat cells was not altered by furosemide.

Furosemide concentration response studies conducted with 0.50mM ouabain included in the incubation medium, resulted in significantly different responses between the SHR and WKY rat red blood cells. These results are presented in Figure 16. The basal values, on the left of the figure, are decreased 17.3% and 12.6%, for WKY and SHR respectively, from the conditions without ouabain (Figure 15).

The rate of lactate release by SHR cells was not affected by up to 0.500mM furosemide. None of these values were found to differ statistically from the basal rates. Furosemide (1.00mM) resulted in a significant increase from the basal rate. In contrast to the lack of effect of furosemide on the SHR cells, the rate of release by WKY rat cells was increased significantly by furosemide. Concentrations of 0.050mM to 1.00mM furosemide resulted in rates of lactate release which were statistically different from the basal rate. The slopes of the lines produced, between 0.050 and 1.00mM furosemide, in Figure 16 were found to differ significantly by Comparison of Regression Slopes Analysis. This indicated a differentiating effect of furosemide on the lactate release of WKY rat and SHR red blood cells.

From the data of these last two experiments (Figures 15 and 16), ouabain sensitive furosemide stimulation can be calculated. In other

Figure 15. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium with the addition of Furosemide (0.0-1.0mM). Bars represent the means and SEM of 8 experiments. (SHR - open symbols; WKY - solid symbols)

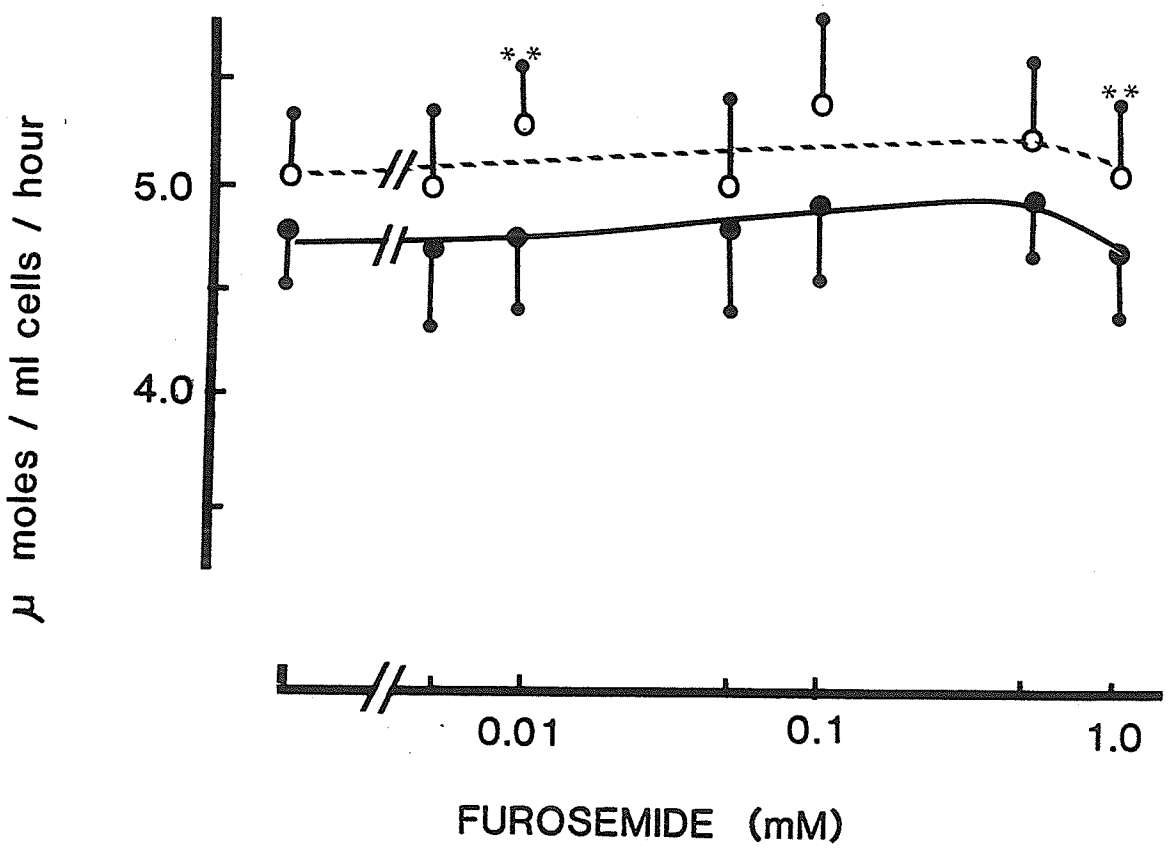
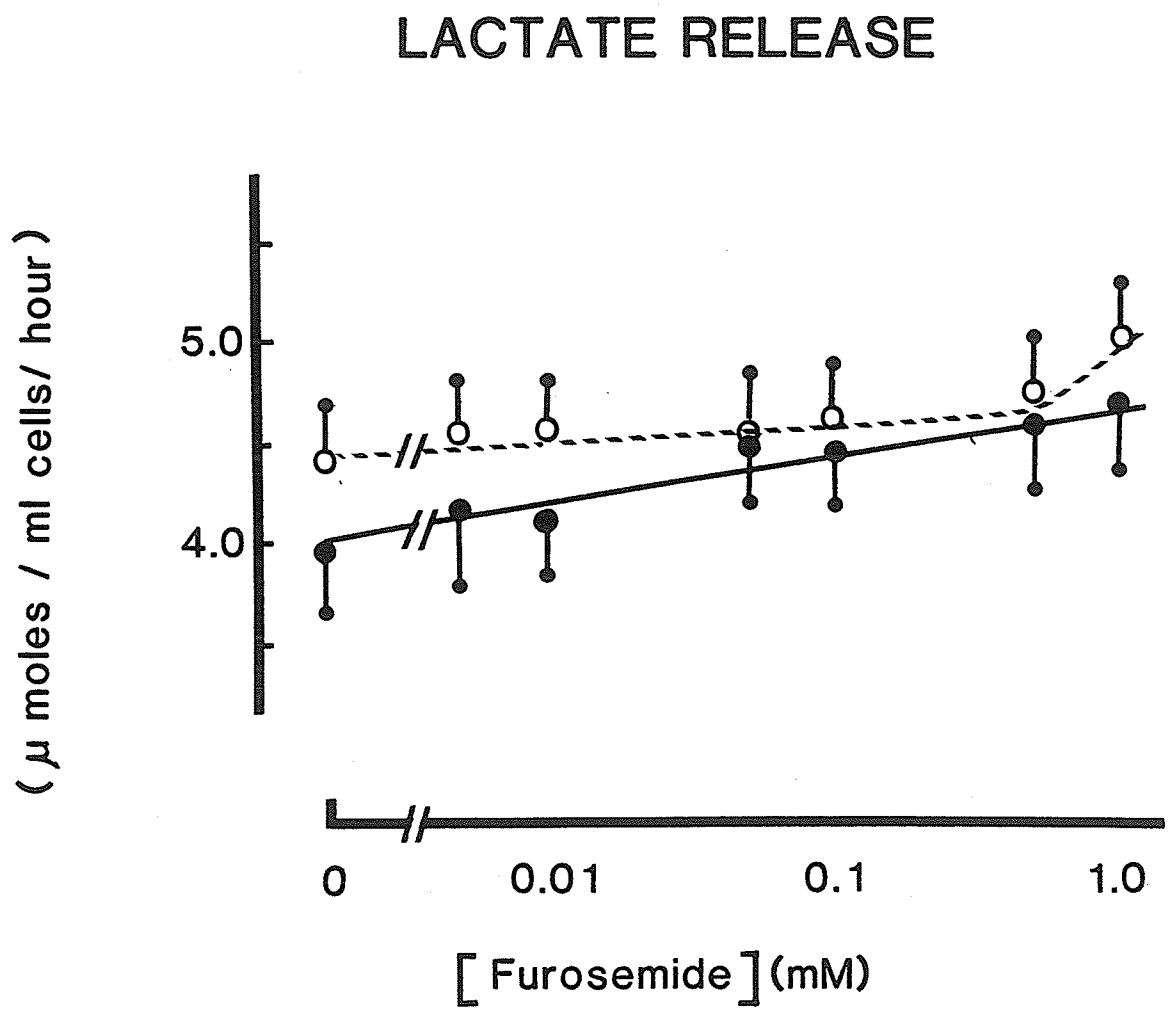

LACTATE RELEASE
μ moles / ml cells / hour
5.0
4.0
**
**
0.01
0.1
1.0
FUROSEMIDE (mM)

Figure 16. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium containing ouabain (0.5mM) with the addition of furosemide (0.0-1.0mM). Bars represent the means and SEM of 8 experiments. (SHR - open symbols; WKY - solid symbols)


LACTATE RELEASE
(μ moles / ml cells/ hour)
5.0
4.0
0
0.01
0.1
1.0
[Furosemide] (mM)

words the furosemide stimulated increase in lactate release which is probably due to stimulation of the sodium potassium ATPase pump. This calculation is performed simply by subtracting the values of Figure 16 from the corresponding values of Figure 15. The results are presented in Figure 17. For SHR cells these values tend to be positive suggesting that furosemide may be causing indirect stimulation of the sodium, potassium ATPase. For WKY rat cells the values are negative indicating that the furosemide stimulation of lactate release is dependent on the presence of ouabain. Comparisons of WKY to SHR have been made by Students' paired t-test, the results of which also appear on Figure 17. Although only two of the comparisons were statistically significant, many had produced p values close to the 0.05 level.

iv. THE EFFECT OF MANGANESE CHLORIDE

Manganese is representative of a group of cations to which smooth muscle of SHR is sensitive but that of WKY is not (Shibata et al., 1973). The mechanism for this effect is unknown but may be related to differences in the calcium ion handling by these tissues and manganese's ability to affect calcium activity. Dose response curves displaying lactate release rates in the presence of manganese chloride are illustrated in Figure 18. These data represent the means of four experiments. In this study, basal rates of lactate release were estimated at 4.85 ± 0.32 micromoles per ml cells per hour for WKY rat cells and 5.31 ± 0.58 micromoles per ml cells per hour for SHR cells. The continuing decline of the plotted lines suggested that a maximum inhibitory effect was not achieved. The highest concentration tested, 5.0mM, inhibited the lactate release rates 1.01 micromoles per ml cells per hour (20.8%) and 1.16 micromoles per ml cells per hour (21.9%) in the WKY and SHR cells respectively. Significance is indicated on the

Figure 17. Change in the rates of lactate by cells incubating in Krebs-Henseleit medium with the addition of the indicated concentration of furosemide that is sensitive to ouabain (0.5mM). These values are calculated by subtracting the furosemide stimulated rates of lactate release of figure 16 from the furosemide stimulated rates of lactate release of figure 15. (WKY- filled; SHR- open)

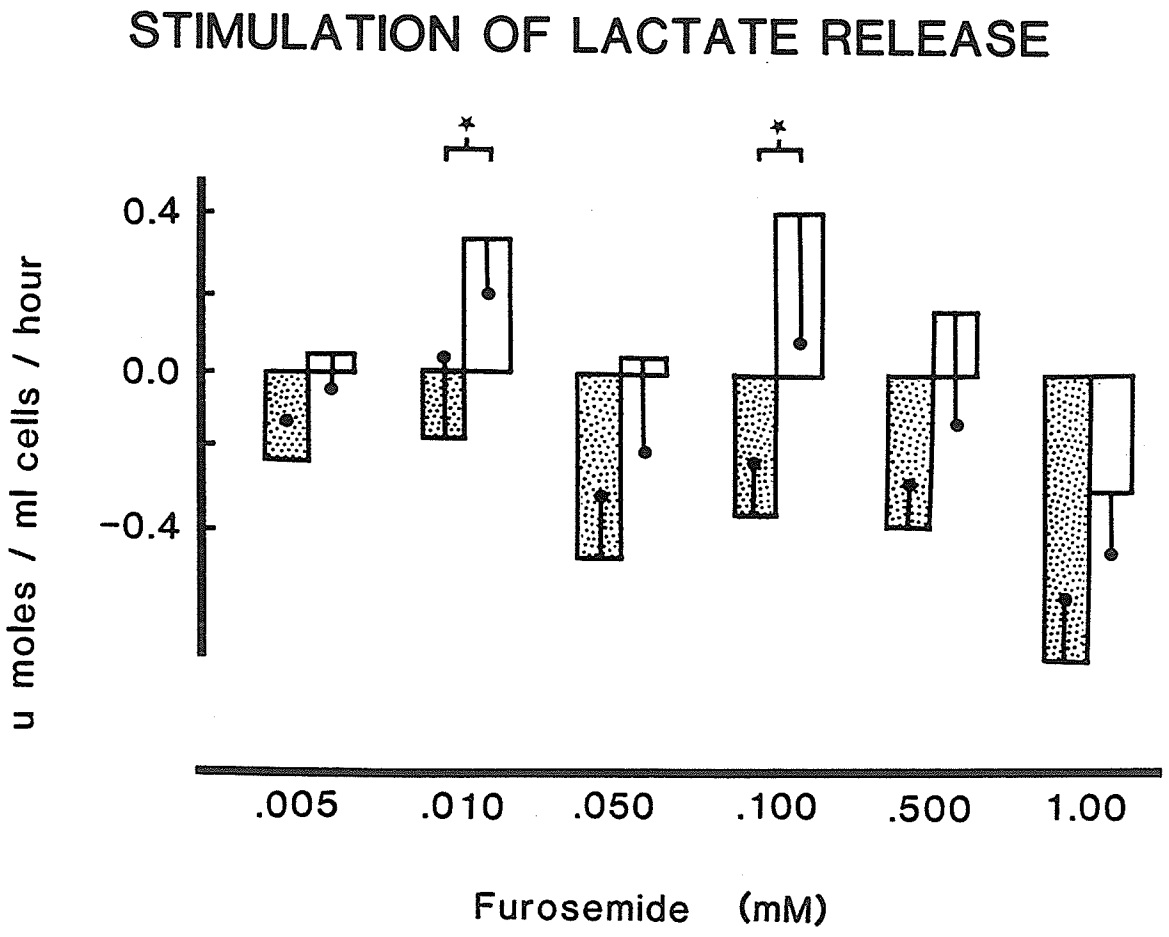

STIMULATION OF LACTATE RELEASE
u moles / ml cells / hour
0.4
0.0
-0.4
.005
.010
.050
.100
.500
1.00
Furosemide (mM)

Figure 18. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium containing manganese chloride (0.0 - 5.0mM). Symbols represent the means and SEM of 4 experiments. (SHR - open symbols; WKY - solid symbols)

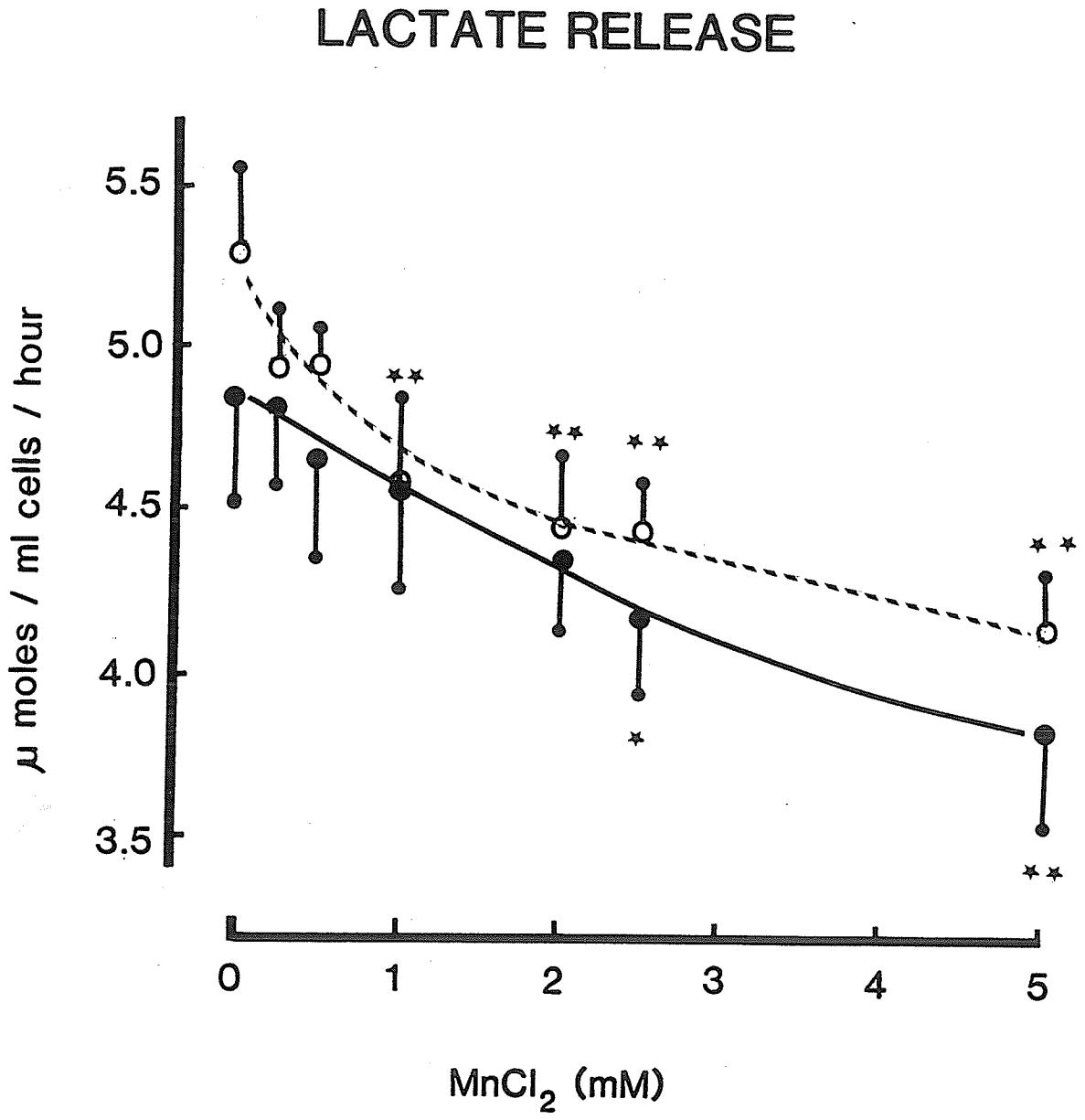

LACTATE RELEASE
5.5
5.0
4.5
4.0
3.5
μ moles / ml cells / hour
0
1
2
3
4
5
MnCl2 (mM)

figure where release values at a given concentration of manganese have been found statistically different from the basal rates. Statistical comparisons were also made between WKY rat and SHR at all concentrations of manganese, and none of the differences were found to be significant.

Inhibition of lactate release by manganese was calculated and converted to the Eadie-Hofstee plot in Figure 19. From this figure the concentrations of manganese, which produced half of the inhibitory effect, were estimated to be 6.66mM and 0.55mM for WKY rat and SHR cells respectively. Thus these results indicated a greater sensitivity to the effects of manganese by the cells from the hypertensive rats. Maximal inhibition estimated from the plot was 4.90 (21%) for SHR and 10.22 (48%) for WKY rats.

v. THE EFFECT OF EXTERNAL CALCIUM

Studies were conducted in which the extent to which alteration of external calcium affects lactate release was examined. For these experiments, cells were suspended in calcium-free Krebs-Henseleit and then appropriate amounts of calcium were added back to the suspensions. Figure 20 is a representation of a typical time study showing that while incubating in calcium-free Krebs-Henseleit medium, red blood cells release lactate at a linear rate for at least 60 minutes. In this particular experiment the coefficient of determination (R^2) values were 0.999 and 0.997 for WKY rat and SHR cells respectively.

Figure 21 illustrates dose responses of lactate release rates to external calcium. The graph indicates that adding back low amounts of calcium to the media (0.0-0.5mM) increased the rates of lactate release while a further increase in external calcium decreased the rates of lactate release. The highest concentration of calcium tested, 6.0mM, reduced lactate release rates 16.9% in WKY rat cells and 11.9mM in SHR.

Figure 19. Eadie-Hofstee plot of the inhibition of lactate release by manganese, from which the sensitivities of the rates of lactate release to manganese have been estimated. The rate of lactate release which was inhibited by manganese is abreviated "Inhibition". (SHR - open symbols; WKY - solid symbols)

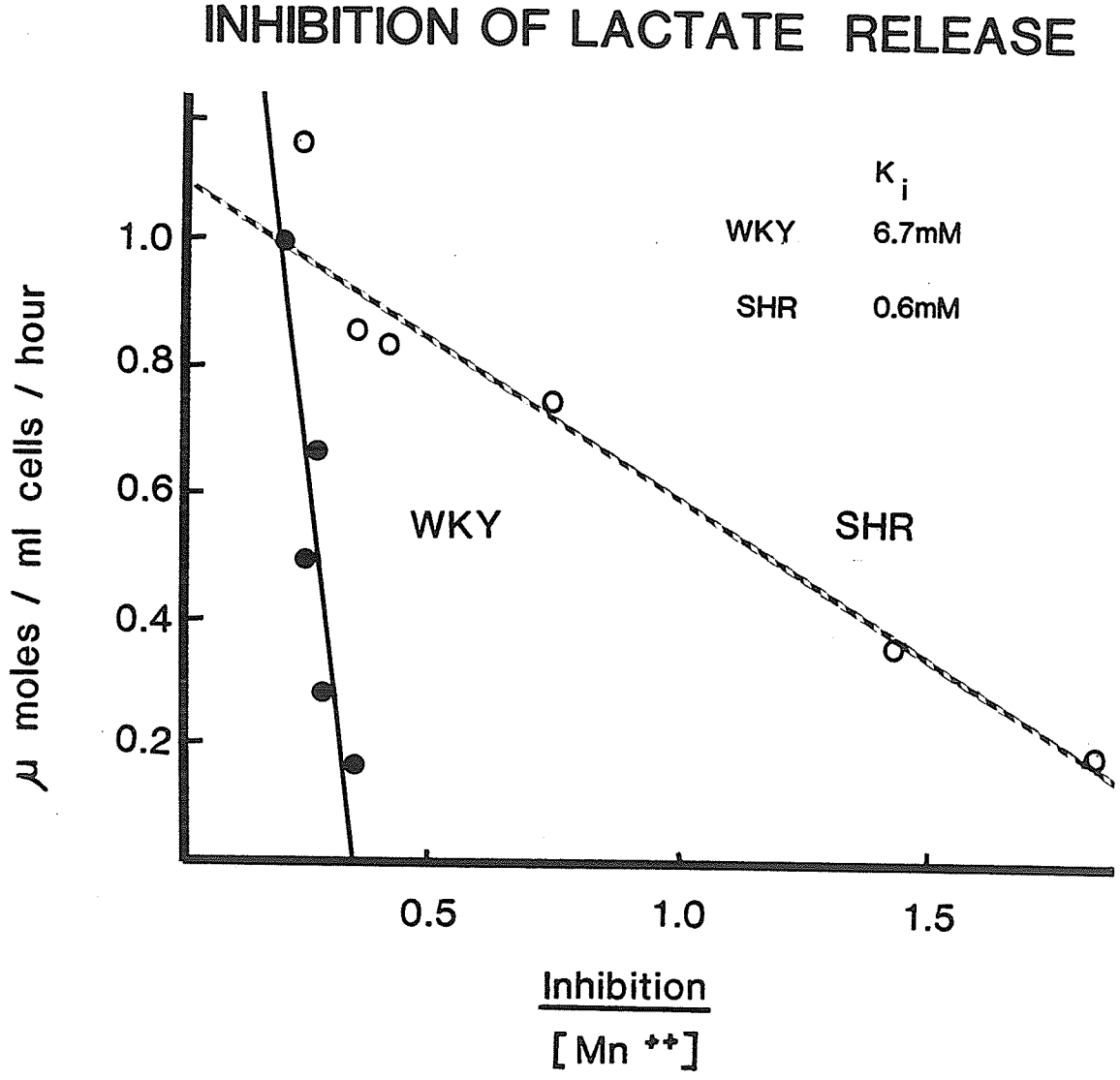

INHIBITION OF LACTATE RELEASE
μ moles / ml cells / hour
1.0
0.8
0.6
0.4
0.2
0.5
1.0
1.5
Inhibition
[Mn ++]
K i
WKY 6.7mM
SHR 0.6mM
WKY
SHR

Figure 20. Time study of lactate release from red blood cells incubating in low calcium Krebs-Henseleit medium. (SHR - open symbols; WKY - solid symbols)

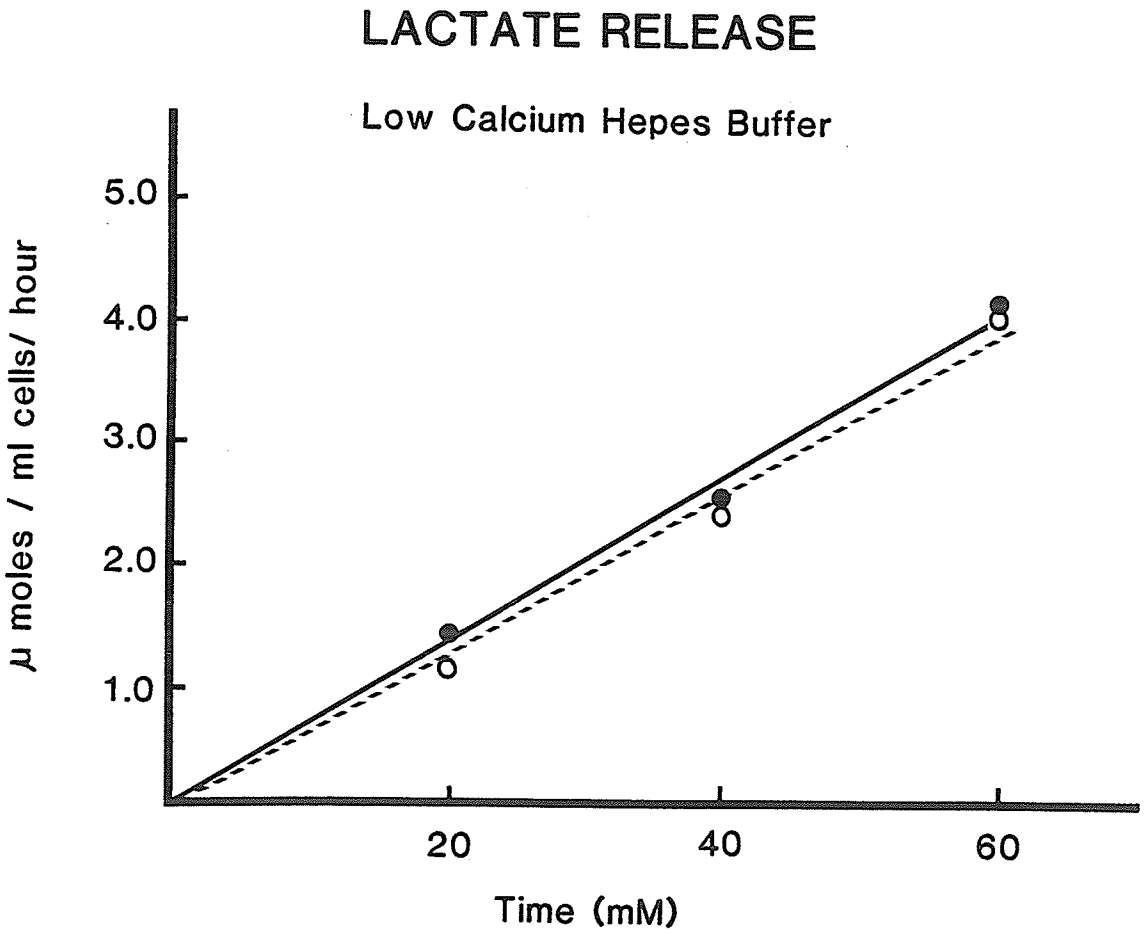
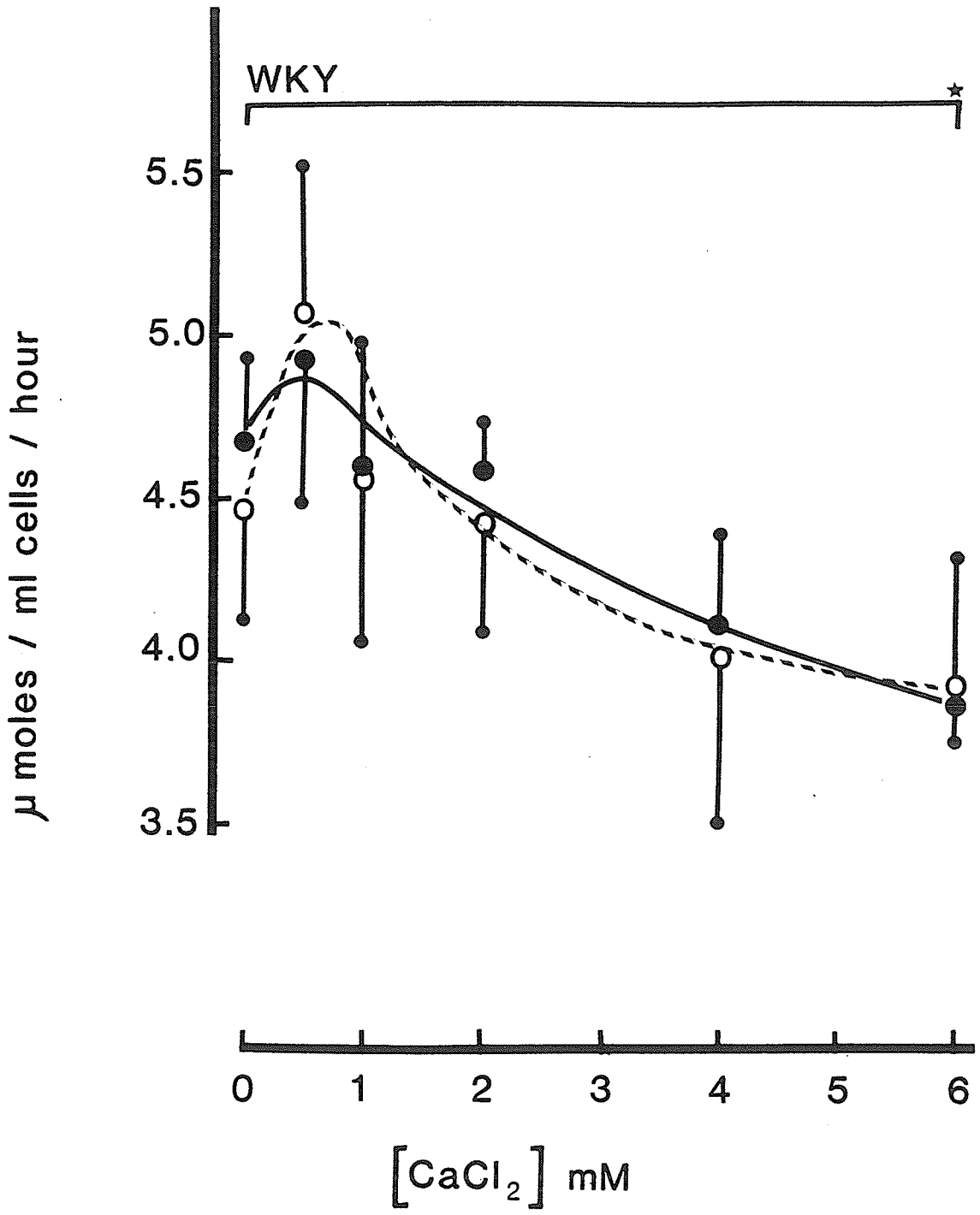

LACTATE RELEASE
Low Calcium Hepes Buffer
μ moles / ml cells/ hour
5.0
4.0
3.0
2.0
1.0
20
40
60
Time (mM)

Figure 21. Rates of lactate release of red blood cells incubating in Krebs-Henseleit medium in which the external calcium chloride concentration was varied from 0.0 mM. (SHR - open symbols; WKY - solid symbols)

LACTATE RELEASE



It should be pointed out however, that in 6mM $CaCl_2$, WKY rat and SHR rates differ by only 0.04 micromoles per ml cells per hour (1.09%), while in 0.0mM $CaCl_2$, the rate of SHR is 0.21 micromoles per ml cells per hour (4.58%). Although the resolution of this graph does not distinguish the optimal concentration, it is apparent that deviation from the approximate physiological concentration of calcium resulted in decreased rates of lactate release. Thus, while there is a calcium-dependent source of ATP utilization, excessive calcium inhibited ATP utilization from unknowm processes.

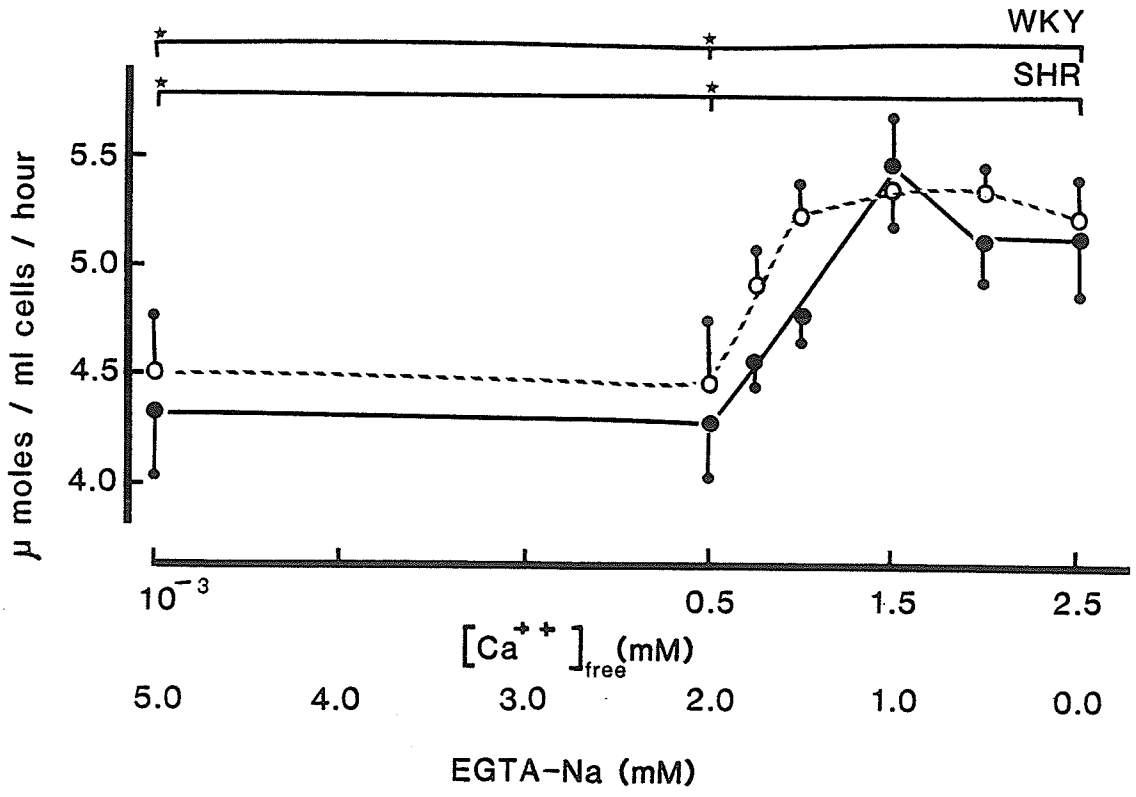
In this experiment comparisons between WKY rat and SHR failed to show significance. Statistical evaluation by Duncan's Multiple comparisons test found that only in 6.0mM calcium for WKY rat cells was the resulting lactate release significantly different from cells incubating in 0.0mM calcium.

vi. THE EFFECT OF EGTA-Na

In order to further elucidate the role of calcium in the differential responses observed in these cells, the effect of EGTA-Na was studied. EGTA is a specific chelator of the calcium ion and was used as a calcium buffer in order to ensure that the desired concentration of calcium was maintained. Figure 22 illustrates the rates of lactate release at varying concentrations of EGTA-Na. The calculated concentrations of free calcium are also included on the figure. Basal rates without added EGTA-Na are 5.14 ± 0.26 and 5.25 ± 0.19 micromoles per ml cells per hour for WKY rat and SHR cells respectively. The values are determined from four experiments.

Significance is indicated where differences were found between SHR and WKY values by Students' paired t-test. At the top of the figure significance is indicated where values differ from those determined

Figure 22. Rates of lactate release by red blood cells incubating in Krebs-Henseleit medium in which the external calcium chloride concentration was decreased by the addition of EGTA (0.0 - 5.0mM). Symbols represent the means of 4 experiments. (SHR - open symbols; WKY - solid symbols)


LACTATE RELEASE
WKY
SHR
μ moles / ml cells / hour
5.5
5.0
4.5
4.0
10⁻³
0.5
1.5
2.5
[Ca++]free (mM)
5.0
4.0
3.0
2.0
1.0
0.0
EGTA-Na (mM)

under basal conditions. An increase in free calcium from 0.5mM to 1.5mM increased the rates of lactate release while a further increase in free calcium up to 2.5mM resulted in a slight decrease in the rates of lactate release.

From this figure the data suggest that the increase in lactate release which occurred between 2.0mM EGTA-Na (0.5mM calcium) and 1.0mM EGTA-Na (1.5mM calcium) displayed a different sensitivities between the two strains.

2. DISCUSSION

As seen in Figure 10, there is a greater lactate release rate from the red blood cells of the SHR when compared to the control strain, the WKY rats. This difference in the rate of release of lactate represents a change to energy utilizing processes which lead to a greater requirement for ATP. The changes in lactate release, in response to pharmacological interventions, were studied in order to isolate the energy requiring process(es) responsible for the difference in lactate release rates and perhaps involved in the development of hypertension.

The ouabain sensitive (Na^+-K^+)-ATPase is the main mechanism for sodium extrusion and normally accounts for 10-15% of the lactate released. There is a general consensus that cells from hypertensives have a greater leakiness to sodium (Hilton, 1985). We therefore tested the hypothesis that the SHR cells utilize more ATP in order to support a greater rate of activity of the sodium potassium pump in order to maintain a proper concentration of intracellular sodium. A test for the validity of this postulate would be to look at the changes in lactate release due to inhibition of the ouabain sensitive pump. In Figure 11, the difference between the rates of lactate release from cells of the SHR and WKY rat strains was not blocked. The lactate release rate of SHR cells remained greater than that of the WKY rat cells even in the presence of maximally inhibiting concentrations of ouabain. Table IV indicates no difference in the sensitivities of rates of lactate release to ouabain. Furthermore, no differences were found in direct comparisons between ouabain-inhibited lactate release (Figure 12).

We considered the possibility that inhibition of the sodium potassium pump may lead to an activation of sodium potassium cotransport

as a secondary mechanism for sodium extrusion. Thus the net changes in lactate release in response to the addition of ouabain, would result from the combination of effects on the two activities of the two transporters. Studies of the ouabain effect performed in the presence of furosemide (0.1mM and 1.0mM) (Figures 13 and 14) also did not reveal any difference in sensitivity or maximal inhibition of lactate release between the two strains.

These data therefore support a conclusion that under basal conditions, the (Na^+-K^+)-ATPase activity of red blood cells remains the same in both WKY and SHR.

Garay and his co-workers have characterized a correlation between furosemide sensitive ion transport activity and essential hypertension. Their study, however, was of the maximal transport activity. Therefore, the physiological significance of this finding remains unknown since the role of the cotransporter in red blood cells from normal and hypertensives remains undefined. As an estimate of the fundamental importance of cotransport activity, we determined the extent to which ATP utilization was affected by furosemide. As observed in Figure 15 there was no significant effect of furosemide on the rates of lactate release in either SHR or WKY rat red blood cells. This observation would thus suggest that basal activity of cotransport is minimal under physiological conditions.

As illustrated in Figure 16, the rate of lactate release of the WKY rat cells, but not the SHR cells was significantly increased by furosemide when ouabain was added to the incubation medium. The response of the WKY cells can be explained as follows; In the presence of 0.5mM ouabain, the (Na^+-K^+)-ATPase is partially inhibited (Akera et al., 1969). In this situation an extra sodium load would be presented to the

cotransport protein, resulting in its activation. Addition of furosemide would block the extrusion of the excessive sodium load through the cotransporter, hence increase the intracellular levels further. The relatively high ratio of intracellular to extracellular sodium would cause a stimulation of the (Na^+-K^+)-ATPase inspite of the presence of ouabain (Beauge and Ortiz, 1973). A rise in intracellular sodium may also indirectly increase lactate release rates by causing a rise in the intracellular calcium through a mechanism such as sodium-calcium exchange.

In SHR cells, with ouabain present, the change in lactate release seen, with up to 0.5mM furosemide, was no different than the change observed in the absence of ouabain. Therefore it appears that partial inhibition of the sodium potassium pump has led to the activation of the sodium potassium cotransport in the WKY but not the SHR. These results suggest that in the SHR there is no further activation of the cotransport mechanism by the increased sodium load. This supports the proposal by Garay and his co-workers that there exists a defective sodium potassium cotransport system associated with essential hypertension.

Our data further indicate that under normal basal conditions the sodium potassium cotransporter has a minor role in the maintenance of the Na^+ and K^+ gradients in red blood cells. However, in the situations where the (Na^+-K^+)-ATPase activity is inhibited (such as in the presence of circulating endogenous inhibitors), the cotransporter is able to assume a more significant role in sodium extrusion in cells of WKY rats but not SHR.

An alternative explanation could be that the cotransporter is normal in the SHR cells and that the disparity in the responses is a

result of a greater sensitivity of the (Na^+-K^+)-ATPase of SHR cells to ouabain. This is unlikely since we did not observe a difference in the effects of ouabain.

Manganese decreased lactate release, presumably by an effect of decreasing calcium pump activity and hence ATP utilization. Manganese is commonly thought of as a calcium influx blocker (Keene et al., 1972), however, more recently it has been reported to directly inhibit the calcium pump (Enyedi et al., 1982). The results presented in Figure 19 indicate different sensitivities of the SHR and WKY red blood cells to the effect of manganese. Assuming that the action of manganese is a result of that cation's ability to displace calcium, this data indicates a weaker affinity of the calcium binding sites in the SHR red blood cells.

Experiments were designed to investigate the relationship between Ca^{2+} and the alterations to the rates of release of lactate. Varying the extracellular concentration of calcium had a dual effect on the lactate release rates (Figure 21). We attributed the rise in rates of lactate release to calcium activation of the $(Ca^{2+}-Mg^{2+})$-ATPase (Vincenzi and Hinds, 1980) and the decline due to inhibition of the (Na^+-K^+)-ATPase (Dunham and Glynn, 1961). Furtheremore, when an EGTA-Na buffer was employed to study the effect of lower concentrations of calcium on lactate release, the results indicated differences in the sensitivities of the lactate release rates to the inhibition by EGTA-Na. To explain the results of Figure 22 we propose that as the EGTA-Na chelates calcium and inhibits the calcium pump, it is also stripping calcium from the membrane sites which inhibit the (Na^+-K^+)-ATPase therefore resulting in an increase of its activity. This finding would therefore be consistent with the postulate that in the SHR cells there

exists a lower affinity for calcium binding at the site at which calcium inhibits the (Na^+-K^+)-ATPase activity. Hence, there is an apparent shift in the sensitivity of lactate release to inhibition by EGTA-Na due to a greater stimulation of the (Na^+-K^+)-ATPase. Other investigators have reported alterations in calcium binding to membrane sites, which are supportive of this postulate (Devynck et al., 1981; Postnov et al., 1979).

In the search for the primary pathogenic factor which is manifest as essential hypertension we are beginning to realize the importance of calcium in the regulation of many membrane activities. From our data, we have formulated the following hypothesis. A defective $(Ca^{2+}-Mg^{2+})$-ATPase exists in membranes of the red blood cells from the hypertensive rats. This defective calcium pump is the cause of the decreased calcium binding affinity by the SHR cell membranes. The observed alterations in sodium ion transport result from the abnormal calcium handling characteristics of the cell membrane.

STUDIES OF $^{86}Rb^{+}$ UPTAKE BY RED BLOOD CELLS

1. RESULTS

Our studies of the rates of lactate release by SHR and WKY rat red blood cells, particularly with the agent manganese, support a postulate that cells from hypertensive rats possess a basic defect in calcium ion handling by the cell membrane. Considering the inconsistencies amongst the results of a large number of studies on ouabain sensitive pump activity in hypertension, we thought that the calcium regulation of this pump may be an important factor.

i. EFFECT OF OUABAIN

Ouabain is recognized as an inhibitior of the ubiquitious sodium potassium pump protein. Although its potency may vary with species, its inhibitory effect has been observed in all tissues tested (Akera et al., 1969) and is attributed to its indirect binding to the transport protein. In addition to the inhibitory effect of ouabain, at low concentrations this agent has been observed to increase the activity of the sodium potassium pump (Ghysel-Burton and Godfraind, 1979), however, the mechanism by which the stimulatory action occurs remains uncertain. A proposal by Godfraind to explain this phenomenon suggests that the stimulatory action is actually a derepression, not a true stimulation. Godfraind hypothesized the existence of an active and a repressed conformation of the pump and that low concentrations of ouabain convert the repressed state to an active conformation, hence derepression.

As an indicator of the activity of the (Na^+-K^+)-ATPase pump, we measured accumulation of the isotope $^{86}Rb^+$ inside healthy, intact red blood cells. The Na^+, K^+ pump protein has been characterized to exchange internal Na^+ for external K^+. The radioactive tracer $^{86}Rb^+$ has been observed to be transported across the plasma membrane by the Na^+, K^+ pump as a substitute for K^+ (Beauge and Adragna, 1971)

Figure 23 illustrates the accumulation of the $^{86}Rb^{+}$ in the red blood cells from the SHR and WKY rats. In the absence of ouabain, the basal uptake of the isotope is the same for SHR as it is for WKY rat cells. From this figure it can be approximated that between 10^{-5}-10^{-4}M of ouabain are required for an inhibition. The maximal inhibitory effect is achieved with 10^{-3}M of ouabain. These characteristics of the inhibition of red blood cell $^{86}Rb^{+}$ uptake by ouabain were indistinguishable between the cells of SHR and WKY rats.

Figure 23 also displays an effect of ouabain which differs between the SHR and WKY red blood cells. At low ouabain concentrations, 10^{-8}-10^{-6}M, there is an increase in the amount of $^{86}Rb^{+}$ accumulated by the WKY rat red blood cells, which was not observed in the SHR red blood cells. The $^{86}Rb^{+}$ accumulation, greater than that observed in the absence of ouabain is an indication of stimulation of the Na^{+}, -K^{+} pump.

ii. EFFECT OF CALCIUM-FREE MEDIUM

In parallel experiments, aliquots of the red blood cells were studied under the same conditions, with the exception that no calcium was added to the medium. In this low calcium medium, sensitivity of the pump to ouabain was increased (Lelievre et al., 1979). Furthermore, Davis and Vincenzi (1971) have demonstrated an inverse relationship between the calcium ion concentration and the total sodium potassium pump activity. The mechanism by which calcium regulates the sodium potassium pump activity is also unknown, however evidence suggests that calcium binding sites on the internal side of the membrane are important (Lelievre et al., 1979).

The results of representative experiments are presented in Figure 24, in which comparisons to the studies performed in the presence of calcium are illustrated. Figure 25 summarizes the results of 8

Figure 23. A representative experiment showing rubidium uptake by red blood cells incubating in HEPES buffer. (SHR - open symbols; WKY - solid symbols)

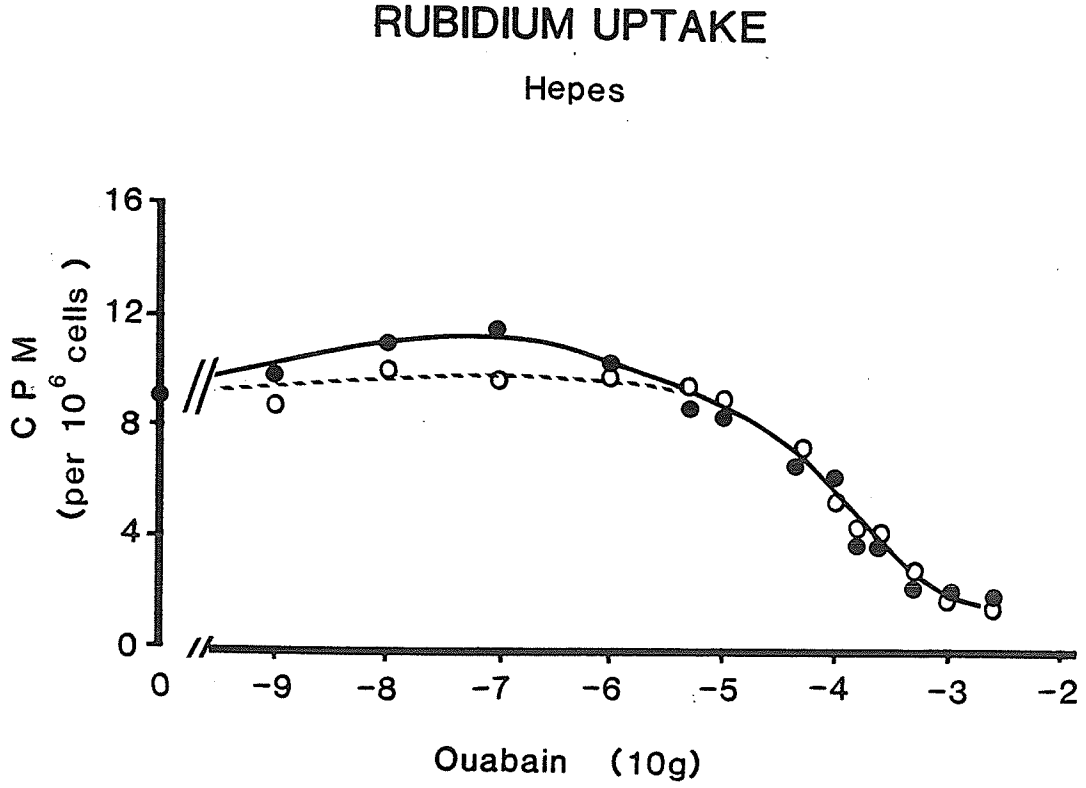

RUBIDIUM UPTAKE
Hepes
C P M
(per 10^6 cells)
16
12
8
4
0
0
-9
-8
-7
-6
-5
-4
-3
-2
Ouabain (10g)

Figure 24. Representative experiments showing the effect of low calcium buffer on rubidium uptake by red blood cells incubationg in HEPES medium. Panel A illustrates the results for WKY rat and panel B those for the SHR values. (SHR - open symbols; WKY - solid symbols: normal calcium - square; low calcium - triangle)

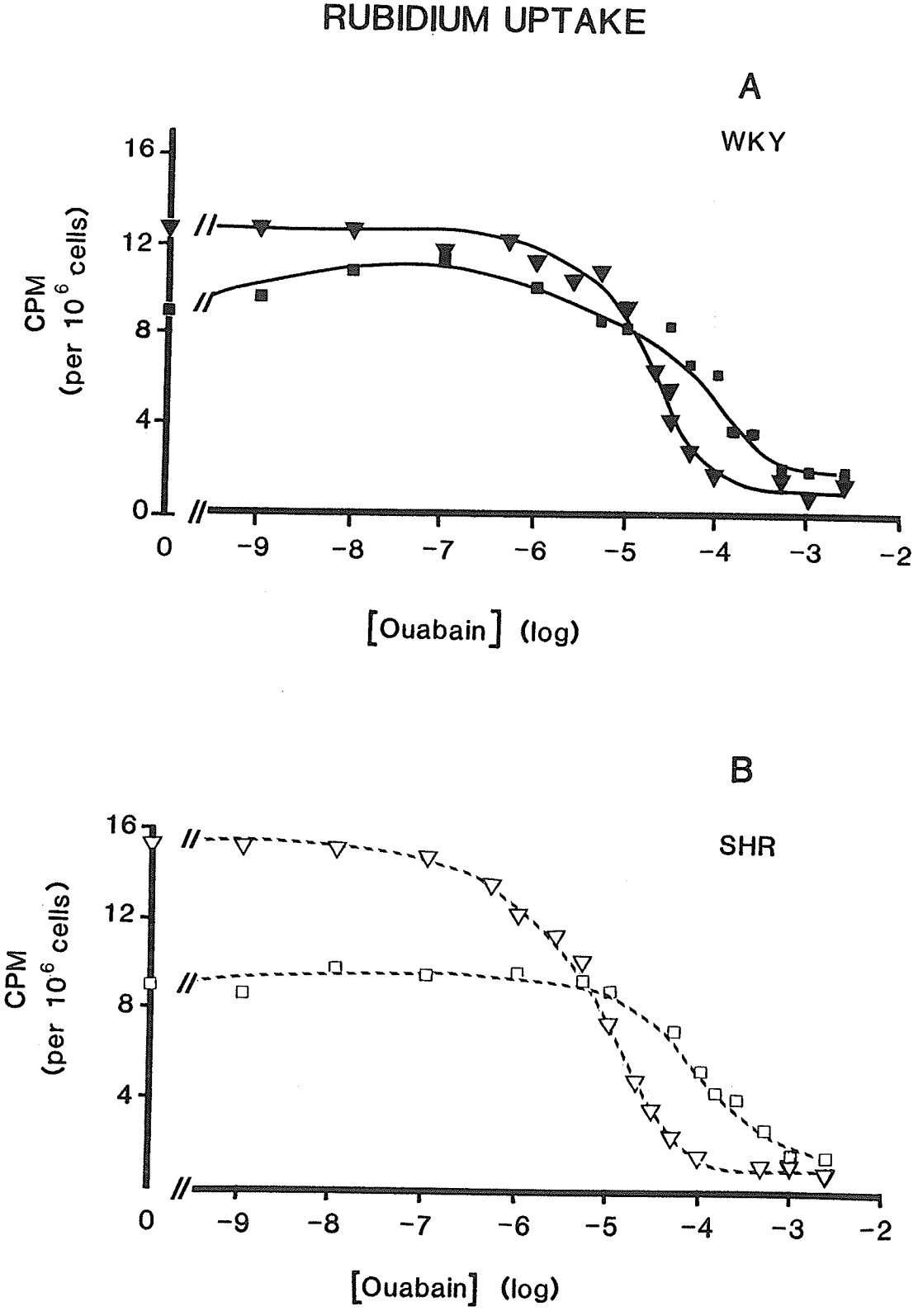
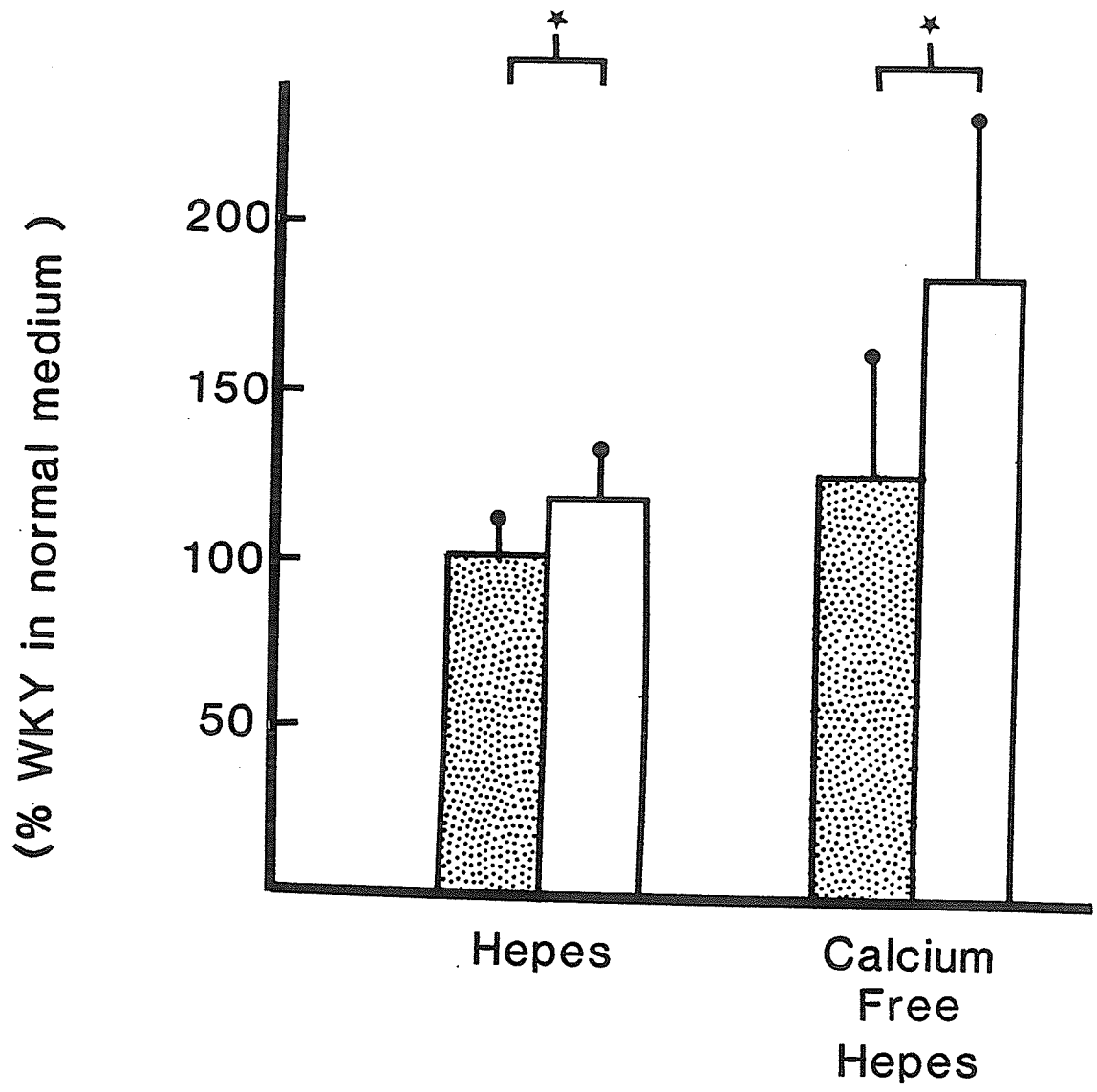

RUBIDIUM UPTAKE
A
WKY
CPM
(per 10^6 cells)
16
12
8
4
0
0
-9
-8
-7
-6
-5
-4
-3
-2
[Ouabain] (log)
B
SHR
CPM
(per 10^6 cells)
16
12
8
4
0
-9
-8
-7
-6
-5
-4
-3
-2
[Ouabain] (log)

Figure 25. Ouabain-sensitive rubidium uptake by red blood cells incubating in HEPES medium and low calcium HEPES medium expressed as the percent of the WKY cells incubated in the normal calcium HEPES medium. Bars represent the mean and SEM of 8 experiments. (WKY- filled; SHR- open)

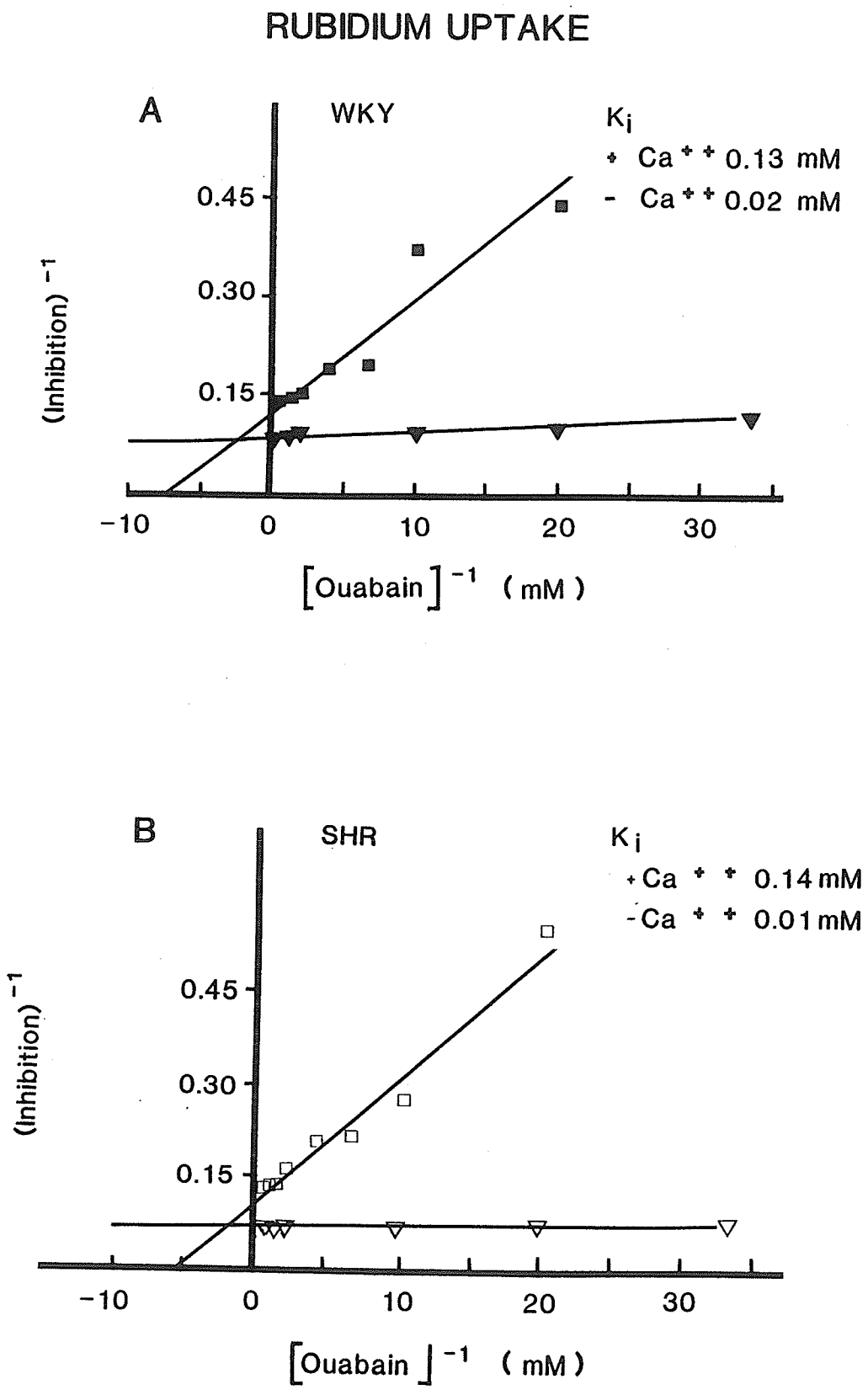
OUABAIN-SENSITIVE RUBIDIUM UPTAKE



experiments. From Figures 25 and 24A it is apparent that the removal of extracelluar calcium from WKY cells resulted in an increase in the basal levels of $^{86}Rb^{+}$ uptake. The increase in the rate of uptake seen in a low calcium medium was similar to that observed by the addition of a low concentration of ouabain in the presence of calcium. For SHR cells, (Figures 25 and 24B), the basal level of $^{86}Rb^{+}$ uptake was also increased. This increase was greater than the increase observed in the WKY cells.

Lineweaver-Burk plots of the inhibitory effect of ouabain indicate that in a low calcium medium the sensitivity of the $^{86}Rb^{+}$ uptake to inhibition by ouabain ($10^{-6}M$-$10^{-3}M$) has been increased (Figure 26). Sensitivity to ouabain inhibition was not found to differ between the WKY rats and SHR in either the low calcium or normal calcium media.

Figure 26. Lineweaver-Burk plots of the inhibitory effect of ouabain, calculated from the values in figure 24. Panel A illustrates values of WKY rat and panel B illustrates values of SHR rat. HEPES medium is represented by the squares and low calcium HEPES medium is represented by triangles.


RUBIDIUM UPTAKE
A
WKY
Ki
+ Ca++ 0.13 mM
- Ca++ 0.02 mM
0.45
0.30
0.15
(Inhibition)-1
-10
0
10
20
30
[Ouabain]-1 (mM)
B
SHR
Ki
+Ca++ 0.14 mM
-Ca++ 0.01 mM
0.45
0.30
0.15
(Inhibition)-1
-10
0
10
20
30
[Ouabain]-1 (mM)

2. DISCUSSION

These results add some insight into the question of the sodium potassium pump activity in SHR. Currently, reports in the literature do not agree on whether the sodium potassium pump is increased, decreased or unchanged in hypertension. Our data indicated that the pump activity is dependent on a pool of calcium which is bound to the membrane, with differing affinities in the SHR and WKY rat red blood cells. Therefore differences in how the cells were handled and washed before the assay would have a major effect on the outcome of the experiment. In a medium with 2.5mM calcium, the pump of the SHR cells is slightly more active than that of the WKY rat. (Although this was not apparent in the representative study, significance was observed by the statistical comparison of the pooled data from 8 experiments). Removing calcium from the medium causes a depletion of membrane bound calcium and hence a stimulation of the pump.

The higher basal level of sodium potassium pump activity and the greater increase of this activity seen in low calcium medium support the hypothesis that the affinity of the membrane for the pool of calcium which inhibits the sodium potassium pump activity is weak in the SHR.

In addition, we observed low concentrations of ouabain to stimulate the pump in WKY but not SHR, when incubated in a calcium free medium. In a low calcium medium, where basal levels of pump activity are higher, no further increase by low concentrations of ouabain was evident. Godfraind's proposal of repressed and derepressed states of the pump may be similar to the two levels of activity observed with and with out calcium. Therefore, both of these phenomena may be due to a common mechanism. Removal of calcium does not affect the inhibitory phase of ouabain, thus a direct interaction between calcium and ouabain is unlikely.

STUDIES OF ^{45}Ca UPTAKE BY RED BLOOD CELLS

1. RESULTS

The previous studies of lactate release rates from red blood cells revealed a difference between SHR and WKY rats in their sensitivities to the effect of manganese (Figure 19). Bohr (1974) and Shibata et al., (1973) observed that manganese induced a contraction in the vascular smooth muscle of SHR but not WKY rats. These observations suggest that the site of action of manganese may be involved in the hypertensive process. In an attempt to elucidate the mechanism of action of manganese we looked specifically at the effect that manganese had on the accumulation of $^{45}Ca^{2+}$ within the cells. Figure 27 illustrates a representative study of the effect of 1mM manganese on $^{45}Ca^{2+}$ accumulation. From this figure it can be seen that there was no observed difference in basal amounts of $^{45}Ca^{2+}$ accumulated betweem SHR and WKY rats. Manganese increased the net uptake of $^{45}Ca^{2+}$ and this increase was greater in the SHR than WKY. Also shown in this figure is the dependence on sodium of the $^{45}Ca^{2+}$ accumulation. Prior to this experiment the cells were washed in a sodium-free, calcium free medium to deplete intracellular sodium. The assay was then performed in the absence of sodium. Under these conditions, minimal $^{45}Ca^{2+}$ uptake was observed in the presence or absence of 1mM $MnCl_2$.

Statistical comparisons of the effect of manganese on $^{45}Ca^{2+}$ accumulation between the WKY rat and SHR are presented in Figure 28. The results are presented as the percent of $^{45}Ca^{2+}$ uptake in the absence of manganese and are the means of 5 and 6 experiments for WKY and SHR respectively. The basal values were not significantly different from each other.

A concentration-response study of the effect of manganese on net $^{45}Ca^{2+}$ uptake indicates a biphasic response (Figure 29). A stimulation

Figure 27. Time study of ^{45}Ca uptake by red blood cells incubating in HEPES Krebs-Henseleit medium (top panel) and the effect of manganese chloride (1mM) (bottom panel). Also shown in this figure is the dependency of 45-Ca accumulation on sodium in which the cells were washed with sodium free, low calcium buffer prior to the incubation. The WKY rat is represented by the squares and solid lines, the SHR is represented by triangles and broken lines. The experiments conducted in the absence of sodium are illustrated with open symbols.

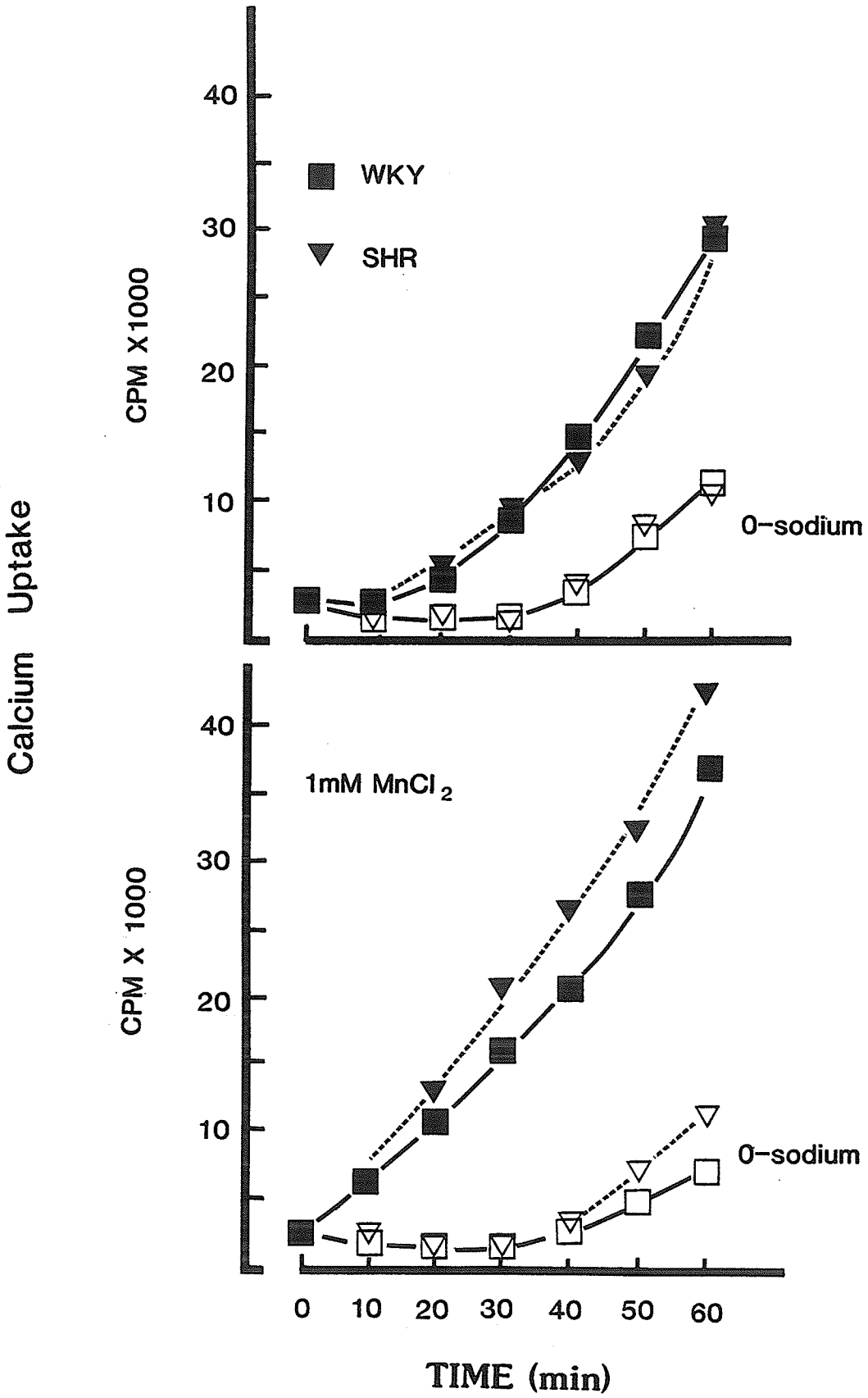

Calcium Uptake
CPM X1000
40
30
20
10
WKY
SHR
0-sodium
1mM MnCl 2
CPM X 1000
40
30
20
10
0-sodium
0
10
20
30
40
50
60
TIME (min)

Figure 28. The effect of manganese (1.0mM) on ^{45}Ca accumulation by red blood cells incubationg in HEPES Krebs-Henseleit medium. The basal levels of 45-Ca uptake were 1084+248CPM and 812+96CPM for the WKY and SHR respectively, and these values were not significantly different. Bars represent the means and SEM of 5 and 6 experiments for WKY and SHR repectively. Experiments performed with manganese (1mM) are indicated by the hatched bars.

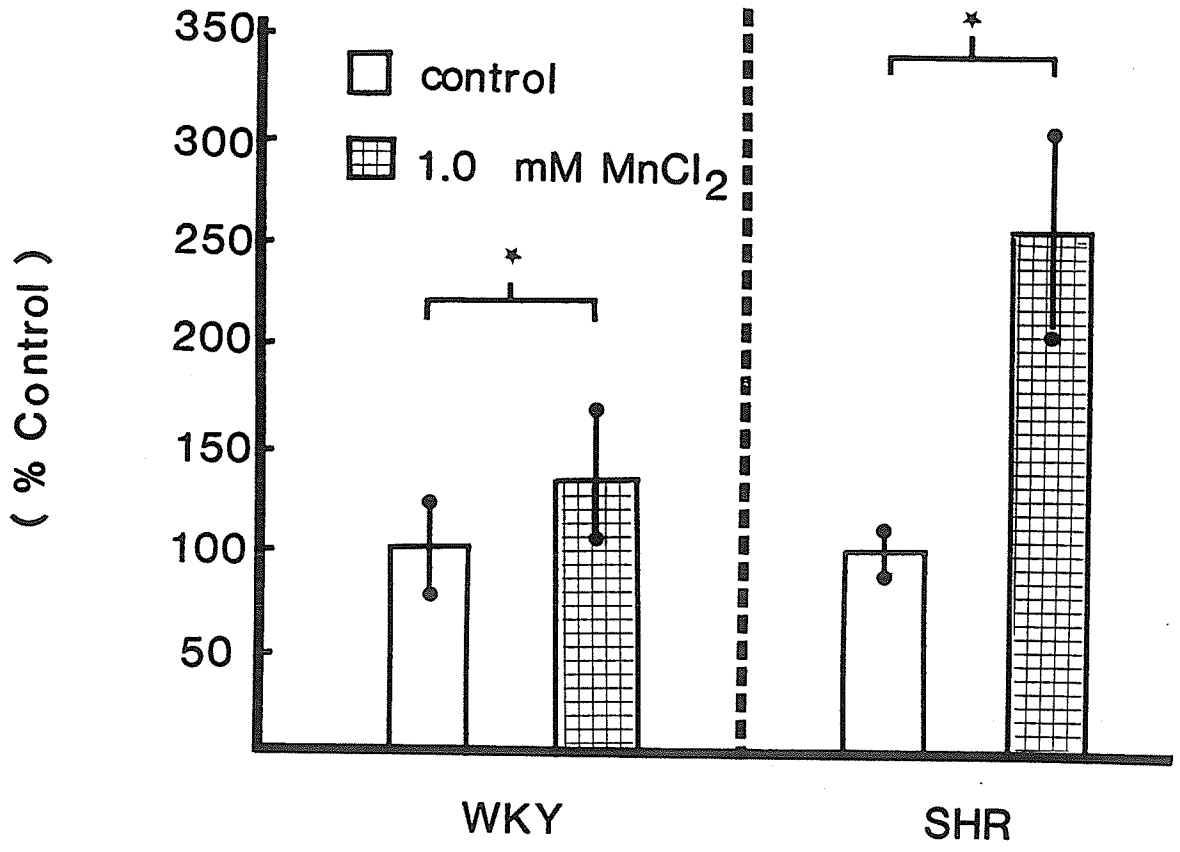
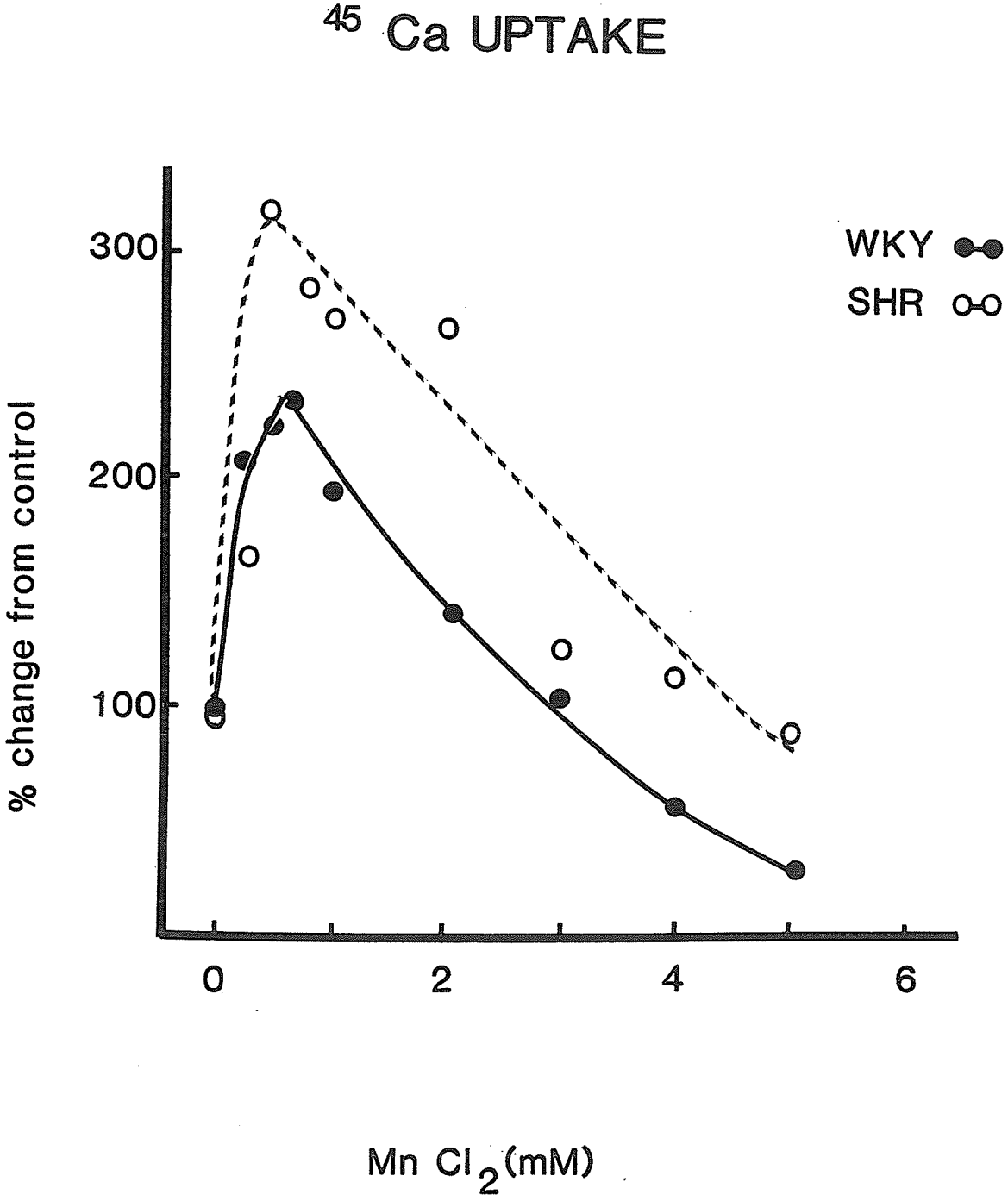

45 Ca UPTAKE
(% Control)
350
300
250
200
150
100
50
control
1.0 mM MnCl2
WKY
SHR

Figure 29. A representative experiment illustrating the effect of manganese (0.0-5.0mM) on the rates of 45-Ca accumulation by red blood cells incubation in HEPES medium. (WKY- filled; SHR- open)


^{45} Ca UPTAKE
WKY
SHR
% change from control
300
200
100
0
2
4
6
Mn Cl_2(mM)

of $^{45}Ca^{2+}$ accumulation is seen optimally with approximately 0.5mM Mn^{2+}. With higher concentrations, the increase in net $^{45}Ca^{2+}$ uptake is reduced, hence the effect of blocking Ca^{2+} influx becomes dominant.

To supplement these experiments we looked at the direct effect of manganese on the (Ca^{2+}-Mg^{2+})-ATPase. This experiment was performed on saponin-permeabilized red blood cells. The method for permeabilization of the cells was taken from the method employed by Burgess et al. (1983) in a study with hepatocytes. When the cells were prepared by this procedure 2uM exogenously added calmodulin did not further stimulate the (Ca^{2+}-Mg^{2+})-ATPase, indicating that this preparation did not lose its endogenous calmodulin. As seen in Figures 30 and 31, manganese significantly inhibited the (Ca^{2+}-Mg^{2+})-ATPase activity. Basal Mg^{2+}-ATPase activity was approximately 1% of the total ATPase activity in both SHR and WKY cells. The K_m for inhibition was calculated from Eadie-Hofstee plots (not shown) and found to be 0.66$\pm$.18mM Mn^{2+} and 0.40$\pm$0.9mM Mn^{2+} for SHR and WKY respectively. These values were not significantly different.

Figure 30. EGTA-Mg ATPase activities (panel B) and calcium stimulated magnesium ATPase (panel C) of WKY and SHR saponin-permeabilized red blood cells in the absence and presence of 1mM manganese. (WKY - filled; SHR - open) At the top of the figure (panel A) is the calmodulin content of SHR and WKY red blood cells, determined by radioimmunoassay (AMERSHAM)

CALMODULIN CONTENT of RBCs

μM

SHR 1.62 ± 0.19

WKY 1.55 ± 0.3

A

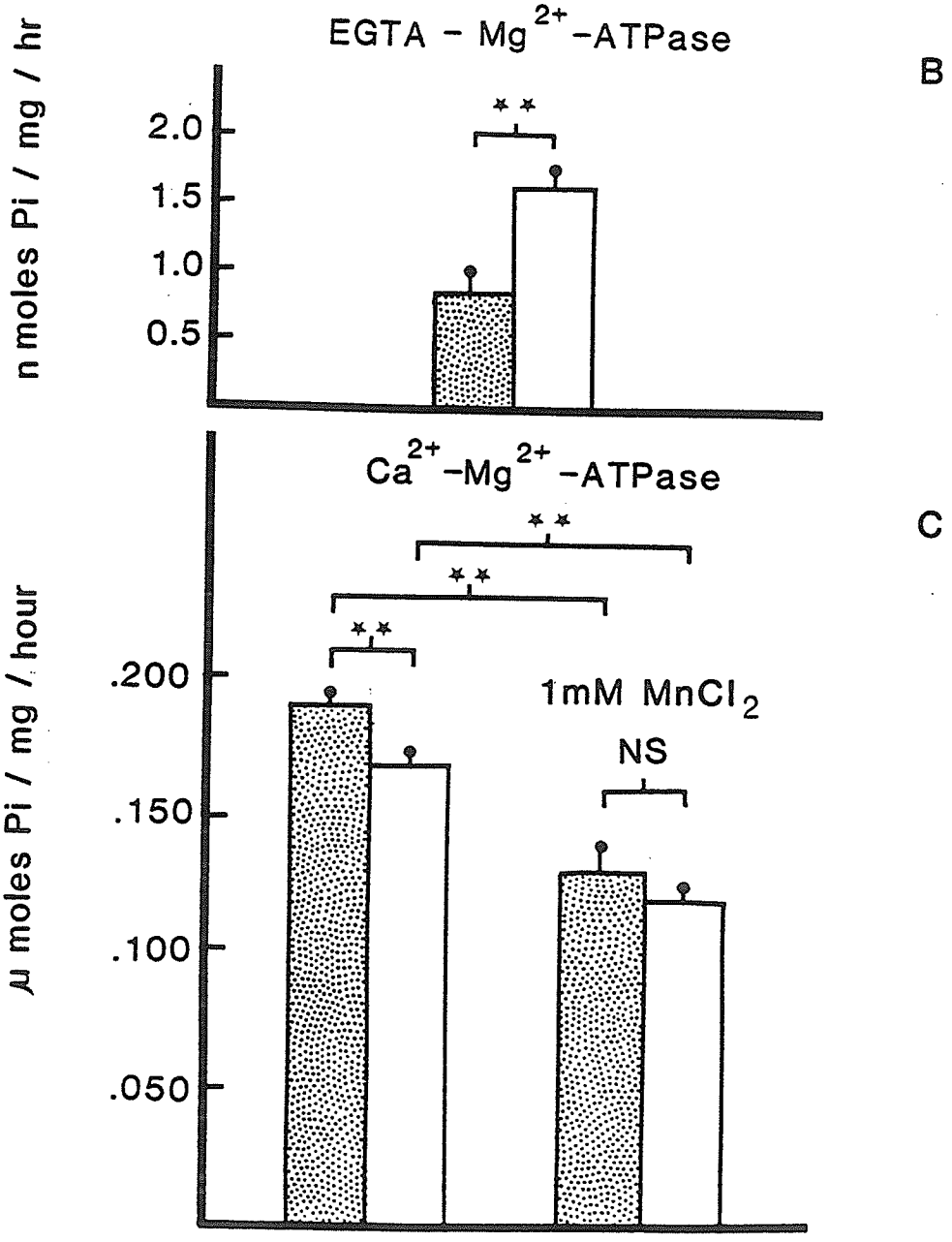
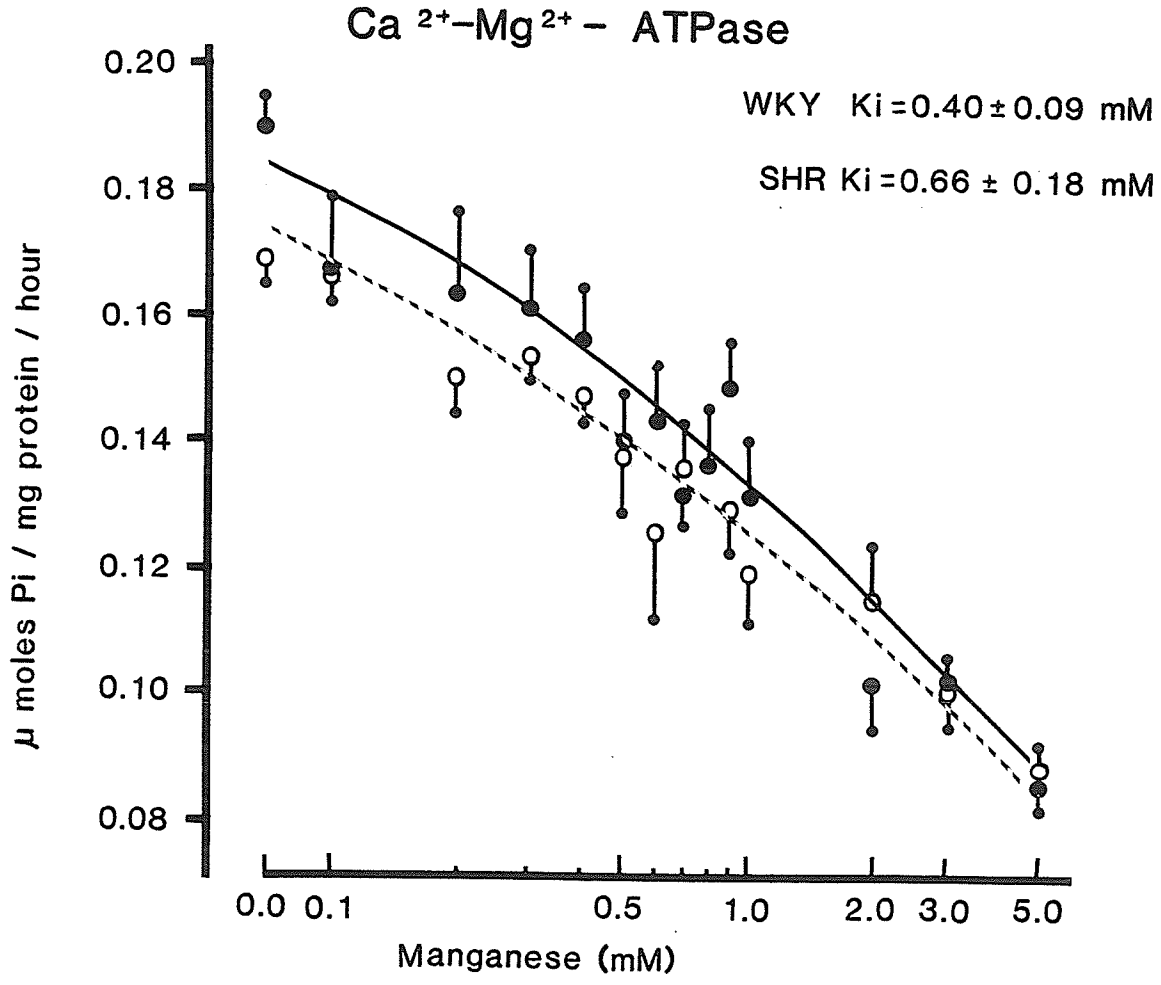


Figure 31. Effect of manganese on calcium ATPase of saponin-permeabilized cells. Assay mixture contained saturating concentrations of calmodulin (2uM) and calcium (200uM) for maximal activation. (WKY - filled; SHR - open)


Ca 2+–Mg 2+ – ATPase
WKY Ki = 0.40 ± 0.09 mM
SHR Ki = 0.66 ± 0.18 mM
μ moles Pi / mg protein / hour
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.0 0.1 0.5 1.0 2.0 3.0 5.0
Manganese (mM)

2. DISCUSSION

Because of our observations, and those of Bohr, and Shibata and his co-workers, it appears that the elucidation of the action of manganese may provide important information into the pathogenesis of essential hypertension. Previous evidence has indicated an inhibiting effect of manganese on calcium permeability of the membrane (Keene et al., 1972). Enyedi et al. (1982) have characterized two opposing effects of manganese on the calcium pump. Low concentrations stimulated pump activity by forming required metal ATP complexes. Higher concentrations were observed to inhibit the pump possibly by competing with calcium for binding sites on the pump.

These studies examined the relationship of the action of manganese on calcium flux, to the hypertensive process. The observation that manganese increased $^{45}Ca^{2+}$ accumulation to a greater extent in SHR than WKY, provides an explanation for the greater reactivity of SHR tissue to manganese than WKY tissue, as previously reported. Our data show a biphasic effect of manganese on $^{45}Ca^{2+}$ accumulation, which is similar to the effect shown in Bohr's results on the reactivity in SHR vascular smooth muscle. His data showed that 1mM manganese was an optimal concentration for manganese induced contraction. We observed 0.5mM manganese to be optimal for the stimulation of $^{45}Ca^{2+}$ accumulation.

In the absence of sodium, $^{45}Ca^{2+}$ uptake was reduced, indicating a requirement of sodium for calcium influx. A possible explanation could be that Ca^{2+} entry is primarily through sodium - calcium exchange. Sodium - calcium exchange has not yet been reported in rat red blood cells and further studies are required in this area.

This experiment does not distinguish between the effect of increased calcium influx or a decreased pump activity, either of which would lead to the same result. In an attempt to answer this question we studied the effect of manganese and calcium ionophore A23187 on $^{45}Ca^{2+}$ accumulation. We expected to determine whether or not a combination of manganese and A23187 would result in a greater rate of net $^{45}Ca^{2+}$ uptake than A23187 alone. This would confirm that manganese was inhibiting calcium efflux, however, the manganese actually reduced the total $^{45}Ca^{2+}$ uptake in the presence of A23187. This was due to the ability of 1mM Mn^{2+} to compete with the 2.5mM Ca^{2+} for entry into the cells through the ionophore.

The study of (Ca^{2+}-Mg^{2+})-ATPase activity of saponin-permeabilized cells did not reveal any significant differences between WKY and SHR in terms of sensitivity to manganese. To explain the greater sensitivity of $^{45}Ca^{2+}$ accumulation of SHR cells, it appears that in the SHR cells there is a greater passive influx of calcium. To maintain the proper intracellular calcium concentration, the calcium pump would be operating at a higher level in these cells. Therefore addition of manganese would cause a greater decrease in calcium extrusion activity, even though the percent inhibition of pump activity remains the same. However, the possiblity cannot be excluded that the pump efficiency has been altered, hence although the ATPase activity is similar there would be less calcium extrusion from the cells.

Chan et al. (1983) reported both (Ca^{2+}-Mg^{2+})-ATPase and Mg^{2+}-ATPase activity of SHR red blood cells to be greater than the activity of the WKY cells. The discrepancy between their results and ours may be in the technique for the preparation of the red cells for the assay. The procedure of Chan and co-workers required hemolysis of

the cells in hypotonic sodium chloride solutions. This treatment may cause a loss of loosely bound membrane proteins preferentially from the SHR cells. The procedure of saponin treatment to permeabilize the cells did not appear to cause a drastic loss of intracellular bound proteins. This was evident by the fact that addition of exogenous calmodulin did not cause significant increases in ATPase activity. A low Mg^{2+}-ATPase indicated a more natural state of the pump.

For the $^{45}Ca^{2+}$ uptake studies we adapted the procedure of Godfraind (1976) which employs an ice cold lanthanum containing solution for displacement of extracellular calcium and sealing of the membrane. This method is superior to washing the cells with a EDTA solution as it ensures that none of the $^{45}Ca^{2+}$ leaks out of the cells, and displaces the extracellular $^{45}Ca^{2+}$.

STUDIES OF PHOSPHORYLATION OF PROTEINS FROM RED BLOOD CELL MEMBRANES

1. RESULTS

i. PROTEIN PROFILES

Sodium-dodecyl Sulphate (SDS) polyacrylamide gel (PAG) electrophoresis was the technique employed for the separation of the membrane proteins. In the presence of SDS and mercaptoethanol, which cleaves sulfhydryl bonds, all proteins are completely denatured and of equal charge to mass ratio. Movement of the proteins by an electrical field then results in the separation of the proteins according to their molecular weight. The lighter proteins will travel at a faster rate, and hence a greater distance, compared to the heavier proteins.

Figure 32 is a reproduction of the gels resulting from the separation of proteins of membranes from red blood cells of WKY rat and SHR strains by SDS-PAG electrophoresis (pH 8.8), and stained with coomassie blue. Molecular weight markers and their respective molecular weights have been included on the illustration for reference. They are: 1) phosphorylase B 2) bovine serum albumin 3) ovalbumin 4) carbonic anhydrase 5) soybean trypsin inhibitor. Densitometric traces are also illustrated in Figure 31. No significant differences were observed between the protein profiles of the SHR and WKY rat red blood cell membranes. The silver staining method for the identification of proteins produced similar results. The gels consisted of 8% acrylamide and contained 0.1% SDS.

ii. BASAL CONDITIONS

For these studies, ATP substituted with either [^{32}P] or [^{35}S] in the terminal phosphate position was used as a phosphate donor for phosphorylation. Both [^{32}P]-ATP and [^{35}S]-ATP are acceptable substrates for kinase reactions, however once attached to a protein, only the [^{32}P] but not the [^{35}S] can be removed by phosphatase enzymes. Protein

incorporation of the radiolabel has been visualized by autoradiography. Only freshly isolated ghosts were used since membranes which were previously stored in a frozen state showed different patterns of incorporation of the [^{32}P]. Many more bands of radioactivity were observed and differences between SHR and WKY were not detectable. A typical experiment is illustrated in Figure 33 which shows the autoradiograms indicating [^{32}P] labelled proteins. Figure 34 is a similar experiment in which [^{35}S]-ATP was used for labelling. In addition to being a poor substrate for the phosphatase, it was apparent that the thiophosphorylated ATP was a poor substrate for some kinases and for autophosphorylation of proteins. Hence with thiophosphorylation, only a few protein bands were observed to incorporate radioactivity, even after prolonged incubation (up to 40 minutes) and prolonged exposure of the X-ray film (up to 30 days).

In the autoradiograms of phosphorylated and thiophosphorylated proteins it was consistently observed that a protein of high molecular weight, greater than 92,500 daltons, showed a greater degree of radioactivity incorporation in the SHR strain than the WKY strain. Quantitation of the extent of phosphorylation is indicated in the densitometric trace of Figure 33, and quantitation of thiophosphorylation is indicated in Figure 34.

The molecular weight of the protein exhibiting altered phosphorylation characteristics, as determined on a 6% SDS gel, has been estimated at 140,000 $\pm$ 15,000 daltons (Figure 35). The relative mobility of the unknown protein was compared with that of the known molecular weight markers. In addition to the molecular weight standards described in Figure 32, Myosin (200,000 daltons) and B-galactosidase (116,250 daltons) are also present. The molecular weight of the unknown protein

Figure 32. Separation of red blood cell membrane proteins on sodium dodecyl sulphate (SDS) gel electrophoresis (pH 8.8) on 8% acrylamide gels containing 0.1% SDS. Densitometric tracings were obtained after staining with coomassie blue.

PROTEIN STAIN

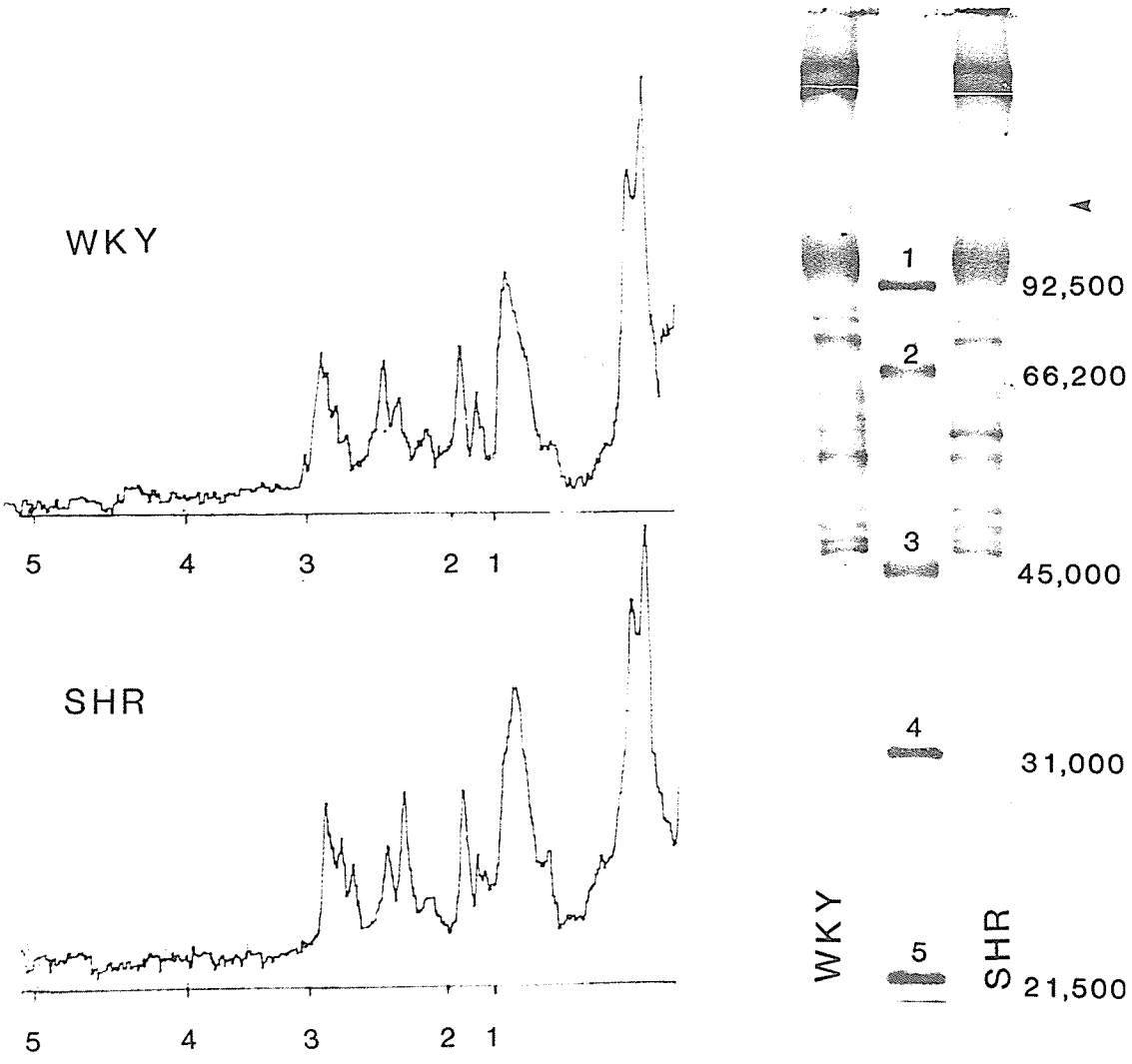


Figure 33. The procedure for separation of red blood cell membrane proteins was similar to that described in figure 32. The stained and dried slab gel was put in contact with X-ray film and exposed for different time periods in order to obtain optimum intensities of various bands. The densitometric trace was obtained from the X-ray film. Each slot had 27ug of either SHR or WKY red blood cell membrane proteins.

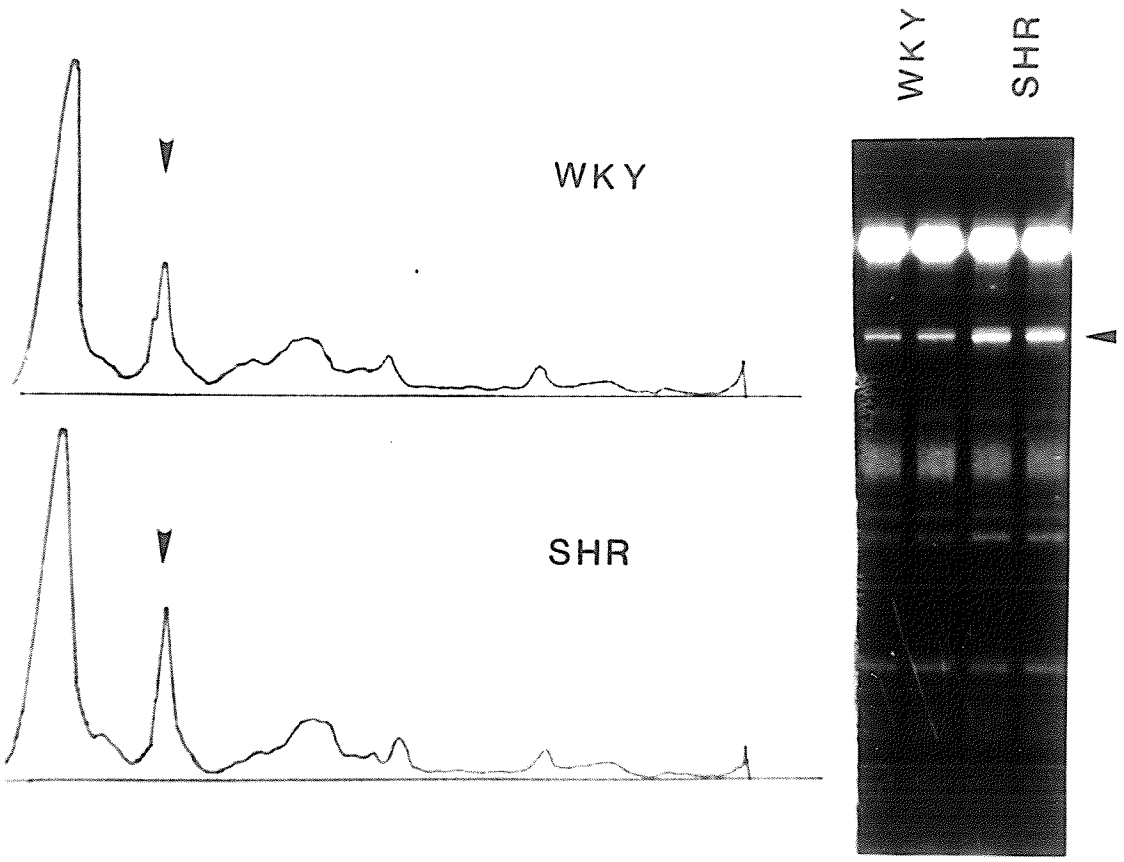
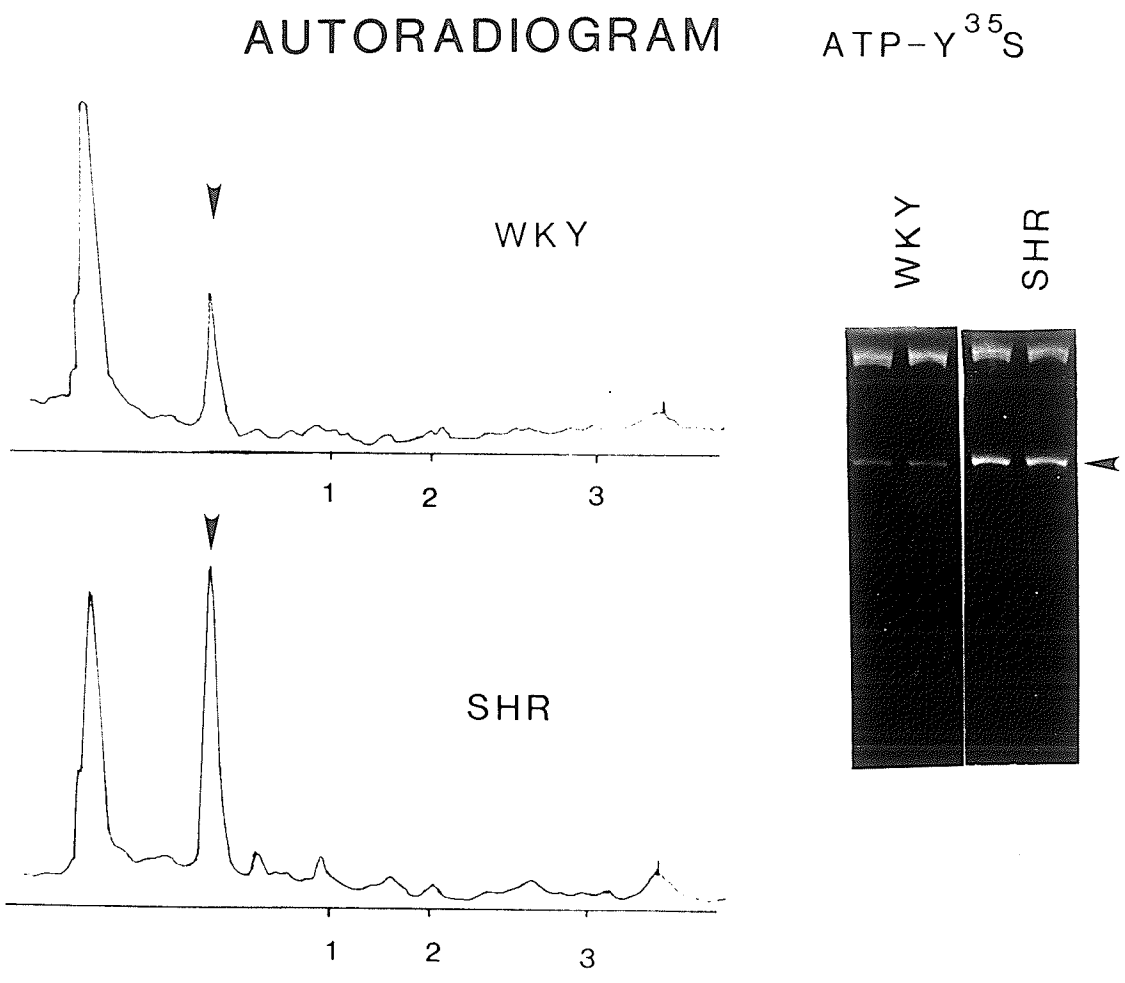

AUTORADIOGRAM ATP-Y^{32}P
WKY
SHR
WKY
SHR

Figure 34. Thio-phosphorylated red cell membrane proteins (16ug per slot) from SHR and WKY ghosts were separated on 6% acrylamide SDS gels. 20uCi of ATP-gammma 35-S was present in 100ul of the incubation mixture instead of the ATP-gamma 32-P.


AUTORADIOGRAM
ATP-Y^{35}S
WKY
SHR
1
2
3
WKY
SHR
1
2
3

was estimated as the mean of five similar experiments. In Figure 35 this protein is indicated by a star.

For statistical evaluation the amount of radiolabel incorporated was quantitated by measuring the peak height in the densitometric trace. A protein of 100,000 daltons, which did not differ in phosphorylation between WKY and SHR was used as an internal standard. For comparison of [^{35}S] thio-phosphorylation, spectrin (200,000 daltons) was employed as the internal standard since the 100,000 dalton protein was not labelled with [^{35}S]-ATP. This method of quantitation allowed comparison of results obtained following incubation in different environments.

Figure 36 indicates the results of experiments conducted in calcium free media. Maximal phosphorylation of both the 100,000 dalton and the 140,000 dalton proteins was achieved within three minutes at 37^{o}C hence the results reported here represent maximal phosphorylation of the substrate and not the rate of the kinase reaction. The top and middle panels represent phosphorylation and thio-phosphorylation of the affected protein relative to spectrin. Thiophosphorylation experiments, although longer in duration (due to longer exposure times needed for X-ray films), brought out greater differences between the WKY and SHR membranes. The thiophosphorylated substrates are poorly utilized by the phosphoprotein phosphatases, therefore these results suggested that the abnormality was in the substrate or the kinase. Significant differences in the amounts of [^{35}S] incorporated were detected between the WKY rat and SHR strains from the means of six experiments. The bottom panel illustrates the significant difference which was observed in [^{32}P] incorporation of the 140,000 dalton protein when the 100,000 dalton protein was the internal standard ($p \tilde{} 0.01$).

Figure 35. Determination of molecular weight of the unknown protein on 6% acrylamide slab gels containing 0.1% SDS and tris buffer at pH 8.8. The molecular weight standards contained myosin, (200,000 daltons) and B-galactosidase (116,250 daltons) in addition to those indicated in figure 32. Position of the affected unknown protein is marked with a asterisc and the phosphorylated protein used for an internal reference is indicated by an open square. From this graph the molecular weight of the unknown protein has been estimated at 140,000 daltons.

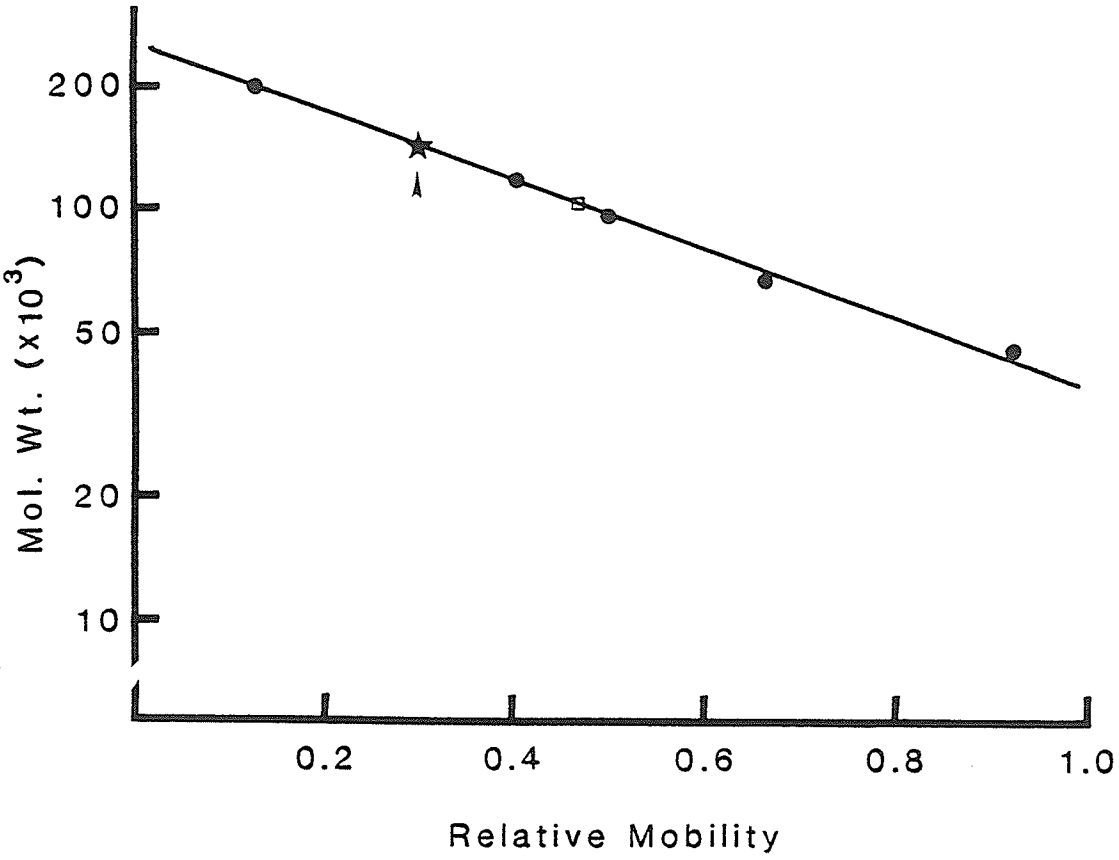
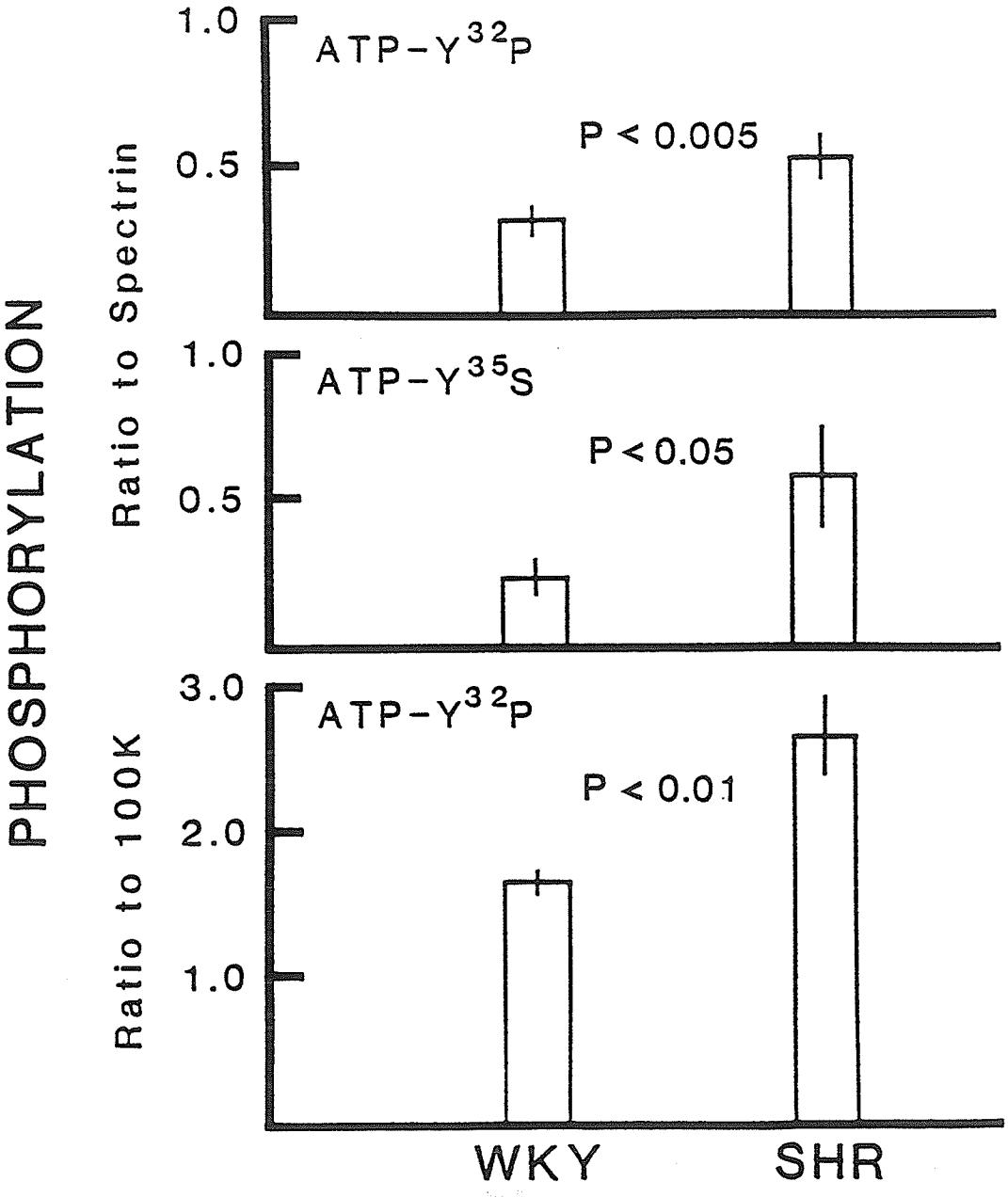

200
100
50
20
10
Mol. Wt. (x10^3)
0.2
0.4
0.6
0.8
1.0
Relative Mobility

Figure 36. Quantitation of phosphorylation of the affected protein obtained from red blood cells. In the top (phosphorylation) and middle (thio-phosphorylation) panels the incorporated radioactivity of the affected protein, measured as a peak in the densitometric trace, compared with the spectrin peak of the same trace (used as a reference) is shown. In the bottom panel phosphorylation of the 100,000 dalton protein was used as a reference for comparing phosphorylation of the affected protein. Bars represent the means and SEM of 6 experiments.


PHOSPHORYLATION
Ratio to Spectrin
Ratio to 100K
1.0
0.5
ATP-Y32P
P < 0.005
1.0
0.5
ATP-Y35S
P < 0.05
3.0
2.0
1.0
ATP-Y32P
P < 0.01
WKY
SHR

iii. EFFECT OF CALCIUM

Calcium is an important regulator of phosphorylation. Typical autoradiograms of experiments performed in the presence of low calcium, (0.1mM EGTA), or 0.1mM calcium are presented in Figure 37. The gels were 8% acrylamide. Phosphorylation of the unknown protein is expressed as a ratio to the 100,000 dalton protein, whose phosphorylation was not affected by calcium. Values are the means of 5 experiments. The blood pressures were not significanlty different between the males and females of either strain.

The greatest differences in phosphorylation of the 140,000 dalton protein between the SHR and WKY were observed when the phosphorylation was in the EGTA containing medium. Calcium in the incubation medium greatly increased the phosphorylation of the 140,000 dalton and 100,000 dalton proteins, and also of several other (at least three) low molecular weight proteins. It is unlikely that some of these are proteolytic fragments of the 140,000 dalton protein, since inclusion of a mixture of protease inhibitors, (para methyl sulfonyl fluoride, pepstatin, and tosyl-lysine chloromethyl ketone), at all stages after hemolysis of the red cells, did not change the results. The difference between WKY and SHR was only significant in the absence of calcium.

iv. EFFECT OF TRANSPORT INHIBITIORS

The ion transport inhibitiors, manganese (1mM), furosemide (0.1mM) and ouabain (1mM) were evaluated for their effect on phosphorylation of the 140,000 dalton protein. The results from four experiments were summarized and presented in Figure 38. The phosphorylation of proteins previously used as internal standards were found to be affected by furosemide. Therefore the degree of phosphorylation was expressed relative to the level of phosphorylation

Figure 37. Effect of calcium on the phosphorylation of the 140,000 dalton protein. A typical autoradiogram from one pair of animals is shown on the right. Red blood cells were processed identically and run on the same 8% acrylamide slab gel containing 0.1% SDS. Blood pressures of the rats are illustrated in the upper panel. In the lower panel phosphorylation of the affected protein is expressed as a ratio of phosphorylation of the 100,000 dalton protein whose phosphorylation was not affected by removal of calcium.The bars represent means and SEM of 5 experiments.

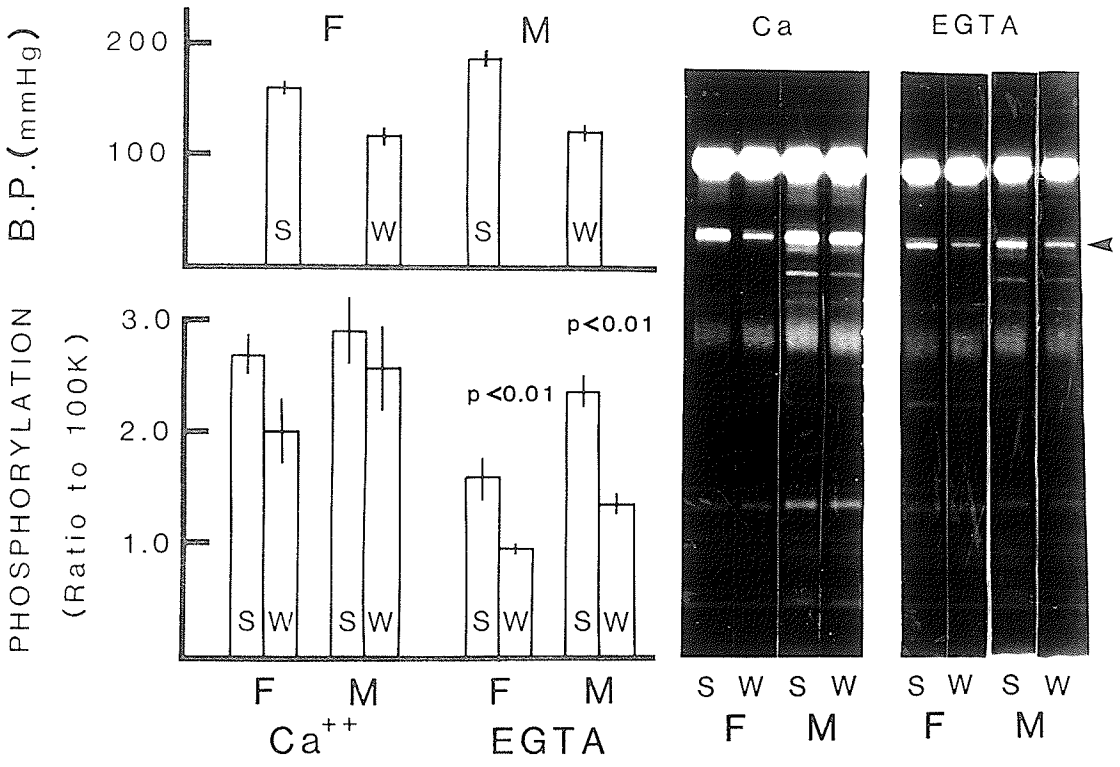
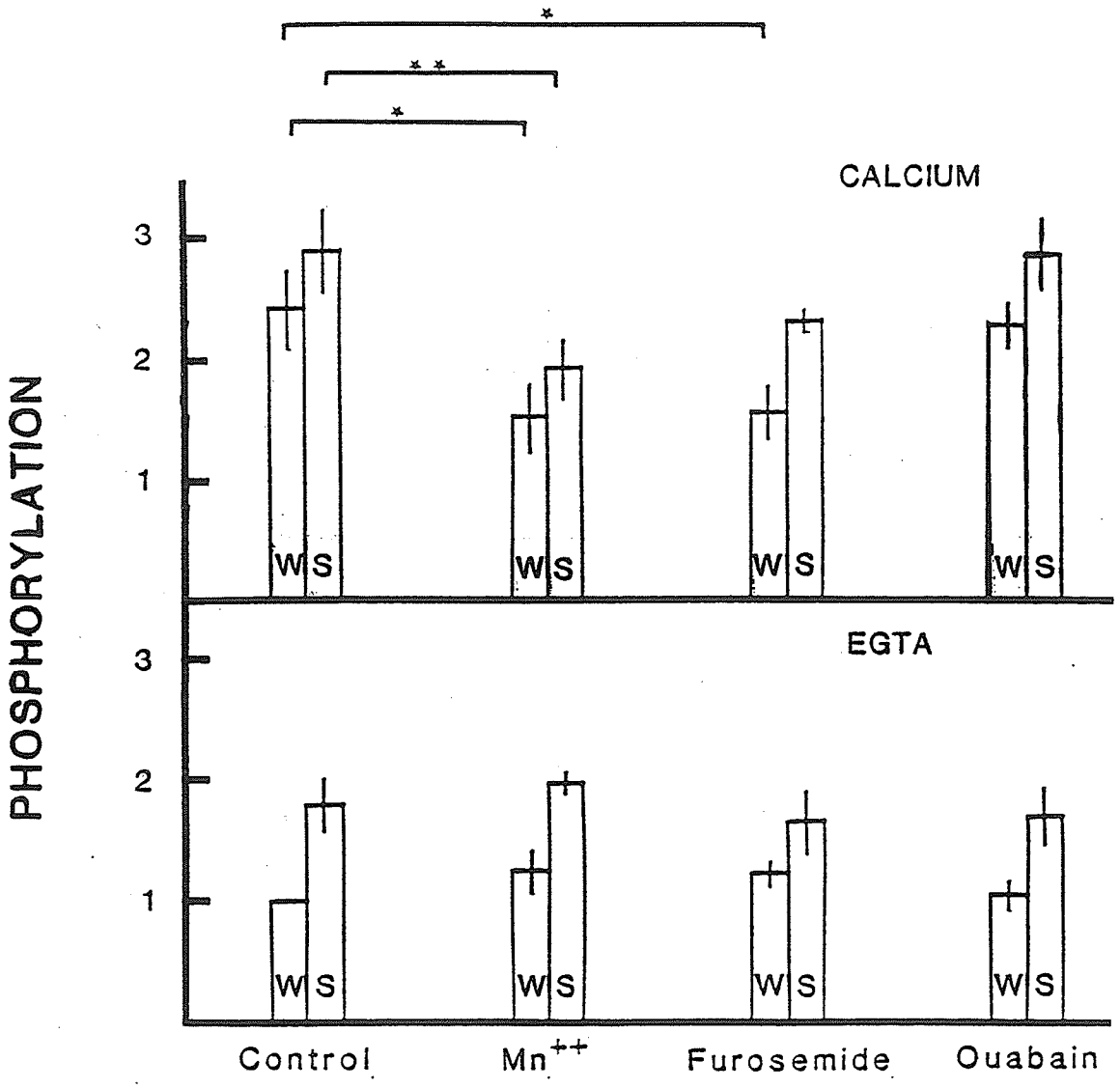

B.P.(mmHg)
200
100
F
M
S
W
S
W
PHOSPHORYLATION
(Ratio to 100K)
3.0
2.0
1.0
p<0.01
p<0.01
S W S W S W S W
F M F M
Ca++
EGTA
Ca
EGTA
S W S W S W S W
F M F M

Figure 38. Effect of manganese (1mM), furosemide (0.1mM) and ouabain (1mM) in medium containing either 5mM EGTA or 0.1mM calcium. The level of phosphorylation of the 140,000 dalton protein is expressed as the ratio to the level of phoshorylation in EGTA of the 140,000 dalton protein from WKY rats because the phosphorylation of other proteins previously used as internal standards were affected by furosemide. The bars represent the means and SEM of 4 experiments.


*
**
*
CALCIUM
EGTA
PHOSPHORYLATION
3
2
1
W
S
Control
Mn++
Furosemide
Ouabain

in EGTA of the WKY rat strain 140,000 dalton protein. In EGTA none of the agents tested affected the phosphorylation of the protein. In the calcium containing medium, manganese was found to produce statistically significant inhibition of the phosphorylation of the 140,000 dalton protein. Furosemide significantly depressed the phosphorylation in the WKY membranes. Ouabain was ineffective.

In order to demonstrate that the difference observed in phosphorylation is not due to inherent differences in these highly inbred strains but is in fact related to the hypertensive process, we studied the red blood cell membrane proteins from Wistar, Sprague-Dawley and Long Evans rats. In all of these normotensive rats the values for the ratio of the phosphorylation of the 140,000 dalton protein to the 100,000 dalton protein were closer to the value observed in WKY rats than to that of the SHR. This suggests a correlation with blood pressure (Table V).

The difference in phosphorylation of the 140,000 dalton protein was already apparent in four week old rats. The values for the ratios were 2.3 ± 0.3 for SHR and 1.6 ± 0.4 for WKY ($p \sim 0.05$, $n=4$). Blood pressures of SHR and WKY rats are not different at this age. Most of the rats used in these experiments were 16 to 24 weeks old. A few experiments were performed with 8 and 12 month old rats and age was not found to influence the ratio of phosphorylation.

Table V. Red blood cell membrane proteins from male rats of five different strains were treated identically and run on the same gel for comparison. Data shown here are the means from three rats of each strain. Phosphorylation was performed in EGTA containing medium.

STRAIN	PHOSPHORYLATION (Ratio to 100K)	B. P.
WISTAR	1.74	95
SPRAGUE-DAWLEY	1.68	110
LONG EVANS	1.8	115
WISTAR-KYOTO (WKY)	1.44	80
SPONTANEOUSLY HYPERTENSIVE (SHR)	2.42	170

2. DISCUSSION

Kinase dependent phosphorylation is a cellular mechanism for protein regulation. Although the regulatory role of phosphorylation is recognized for many proteins (Greengard, 1978), the role of phosphorylation in the regulation of many membrane proteins is not known. In the study of altered membrane processes in hypertension, we were considerably interested in determining whether or not the phosphorylation - dephosphorylation process of a membrane ion transport protein has a role in the hypertensive process.

The finding of a protein in the membrane of the SHR with altered phosphorylation characteristics may be an indication of the location of the primary defect in essential hypertension. Although we do not know the function of this protein, its molecular weight (140,000 daltons) suggests the possibility that it may be the (Ca^{2+}-Mg^{2+})ATPase. This finding lends further support to the postulate that it is a primary defect in calcium handling by the cell membranes which results in essential hypertension.

When studied in the presence of calcium, phosphorylation of the 140,000 dalton protein is decreased by manganese to the level observed in the presence of EGTA. This observation is consistent with our hypothesis that manganese interacts with calcium regulation of ($Ca^{2+}Mg^{2+}$)-ATPase activity.

The protein does not appear to be the sodium pump because most of the reported values for the molecular weight of that protein are closer to 100,000 daltons and also the phosphorylation of the protein was not affected by ouabain. The Na^{+}, K^{+} cotransport has not been fully characterized and it remains possible that this protein is a subunit of the transport mechanism.

SECTION IV

GENERAL DISCUSSION AND CONCLUSIONS

A. ION TRANSPORT AND LACTATE PRODUCTION

Theoretically any change in activity of an ATP utilizing mechanism should result in alterations to the cellular rate of ATP production. Since the rate of lactate production can be used as an indirect indication of ATP production, we should therefore expect to observe alterations in lactate production in response to alterations in active transport activities. Many investigators have studied lactate release in response to perturbations in active, ouabain sensitive, ion transport.

Murphy (1963) observed in human red blood cells, a 15% decrease in lactate production when the (Na^+-K^+)-ATPase was inhibited with ouabain. Whittam and co-workers also observed the same decrease in total lactate production in human red blood cells when the Na^+, K^+ pump was inhibited, independent of the method of inhibition employed (Whittam et al., 1964a; Whittam et al., 1964b). Furthermore they demonstrated a stimulation of lactate production by internal sodium and external potassium that was sensitive to ouabain (Whittam and Ager, 1965). Minakami and Yoshikawa (1966) studied this phenomenon more closely and confirmed that the inhibition of lactate release by ouabain was an indirect consequence of cessation of active transport. The ATP sparing effect of ouabain increased the [ATP]/[ATP][Pi] ratio, which inhibits the phosphofructokinase enzyme activity.

Parker and Hoffman (1961) and Proverbio and Hoffman (1977) suggested that the association of (Na^+-K^+)-ATPase activity to glycolysis was primarily a result of common pools of ATP and ADP between the ATPase and the phosphoglycerate kinase enzyme. (Na^+-K^+)-ATPase activity provides ADP substrate for conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. The resulting ATP produced becomes substrate for hydrolysis by the (Na^+-K^+)-ATPase.

The association of lactate production to active ion transport has been extended to tissues capable of oxidative metabolism. Paul et al. (1979) demonstrated that in vascular smooth muscle the rate at which lactate was released by the tissues was specifically coupled to Na^+ and K^+ transport processes and independent of the contractile energy requirements.

In our study we have found the rate of lactate release from SHR red blood cells to be approximately 10% greater than the corresponding rate from WKY rat cells. This result suggested a derangement in energy utilizing processes of the cell, possibly ion transport. With addition of ouabain, we have observed a decrease in the rates of lactate release, presumably as a result of inhibition of the (Na^+-K^+)-ATPase. In the comparison of SHR to WKY rat cells, we were not able to detect any difference in the sensitivities of the rates of lactate release to ouabain. Ouabain-sensitive release of lactate continued to show a difference between cells of SHR and WKY. We therefore concluded that activity of the (Na^+-K^+)-ATPase does not account for the alteration to energy utilization in SHR cells.

When added in the presence of ouabain, furosemide caused an increase in the rate of lactate of WKY cells. The furosemide-sensitive cotransport is not known to be an active transport mechanism, therefore, the change in lactate release must be a secondary effect probably resulting from a rise in intracellular sodium and sodium, calcium exchange would lead to an increase in ATP utilization by activation of the calcium pump. This response was only observed in the cells of WKY, indicating a possible defect of the (Na^+-K^+) cotransport mechanism in sodium ion regulation of the SHR cells.

B. CALCIUM HANDLING IN THE RED BLOOD CELLS OF SHR

1. INTERACTION WITH MANGANESE

This thesis was a study of membrane processes in the hypertensive state. In summary, we have looked at cellular rates of lactate release as an indicator for the cellular ATP requirements. The most significant result from these studies was that observed with the effect of manganese. In the magnesium-sucrose medium, manganese stimulated the rates of lactate release while in Krebs-Henseleit medium the rates of lactate release were decreased by this cation. Since lactate response is a summation of responses from a variety of energy utilizing processes, manganese may be affecting more than one process. We have identified one possible site of action within the red blood cell.

Our data indicated that manganese caused a direct inhibition of the $(Ca^{2+}\text{-}Mg^{2+})$-ATPase. In the physiological Krebs-Henseleit medium, manganese was observed to decrease the rates of lactate release from the red blood cells. This medium, unlike the magnesium-sucrose medium, contains calcium, and hence $(Ca^{2+}\text{-}Mg^{2+})$-ATPase activity would normally be occurring. We also observed manganese to increase the net $^{45}Ca^{2+}$ accumulation by intact red blood cells and to inhibit the $(Ca^{2+}\text{-}Mg^{2+})$-ATPase activity of saponin permeabilized red blood cells. Considering these results we propose that manganese (at 1mM concentration) primarily inhibits calcium extrusion from the red blood cells through the calcium pump. Differences in the sensitivities of the SHR and WKY rat cells to the effects of manganese were noted in both the rates of lactate release and the net accumulation of $^{45}Ca^{2+}$. However the red blood cells of the two strains of rats appeared to be equally sensitive to the effect of manganese on the $(Ca^{2+}\text{-}Mg^{2+})$-ATPase activity,

which was measured in the presence of 0.1mM calcium. These experiments support the hypothesis of an abnormal calcium handling defect of a greater passive inward leak of the cells to calcium which results in higher basal rates of calcium pumping activity. It would be because of the faster rate of turnover of the calcium pump that the cells of SHR show increased responsiveness to the action of manganese.

Manganese, in the magnesium-sucrose medium, caused a ouabain sensitive increase in lactate release which indicated a further action of manganese on the activity of the (Na^+-K^+)-ATPase. As proposed by Davis and Vincenzi (1971) the (Na^+-K^+)-ATPase is regulated by a pool of calcium and we suspect that it is through an interaction with this pool of calcium by which manganese can stimulate the Na^+, K^+ pump activity. This hypothesis is supported by the experiments in which removal of calcium from the medium stimulated ^{86}Rb uptake by the cells. This stimulation of uptake activity was to a much greater extent in the cells of the SHR than those of the WKY rat.

The studies of membrane protein phosphorylation have indicated that there exists at least one protein within the membrane which is altered in SHR cells compared to WKY rat cells. Neither the identity of this protein, nor the significance of this finding are known at this time. Such a project would require considerably more work but would be an important contribution to the study of essential hypertension. From its molecular weight, the calcium pump is a very likely candidate for the identity of this protein (Niggli et al., 1981b). Recently Hofmann et al. (1986) have isolated the calcium channel protein from cardiac sarcolemma and have identified its molecular weight to be 140,000 dalton.

Our study on net $^{45}Ca^{2+}$ uptake revealed that manganese increased accumulation by SHR cells to a greater extent than accumulation by WKY cells. This effect most likely resulted from manganese inhibition of the calcium pump. At higher concentrations of manganese total uptake of $^{45}Ca^{2+}$ was inhibited however a concentration of 1mM manganese predominantly blocked efflux over influx. In support of this site of action for manganese we have demonstrated that manganese decreased $(Ca^{2+}-Mg^{2+})$-ATPase activity. We attempted to measure $^{45}Ca^{2+}$ efflux from intact red blood cells in order to elucidate more clearly the role of the calcium pump in the responses to manganese. Red blood cells however have a very low permeability to calcium which made the $^{45}Ca^{2+}$ loading procedure difficult and created considerable amounts of variability within the experiment. As a result, these experiments were inconclusive.

A reduced binding affinity of calcium to the membrane has been reported (Postnov et al., 1979; Devynck et al., 1981). It has been shown that calcium can affect the activity of the sodium, potassium pump and may in fact have a physiological role its regulation (Davis and Vincenzi, 1971). In our study of $^{45}Ca^{2+}$ accumulation we have observed a dependence of calcium uptake on the presence of sodium suggesting that a sodium-calcium exchange may be involved in the passive influx of calcium. Hence the sodium ion handling appears to be linked to the calcium handling and therefore observed abnormalities in sodium and potassium ion transport may also be secondary to a defect in calcium handling.

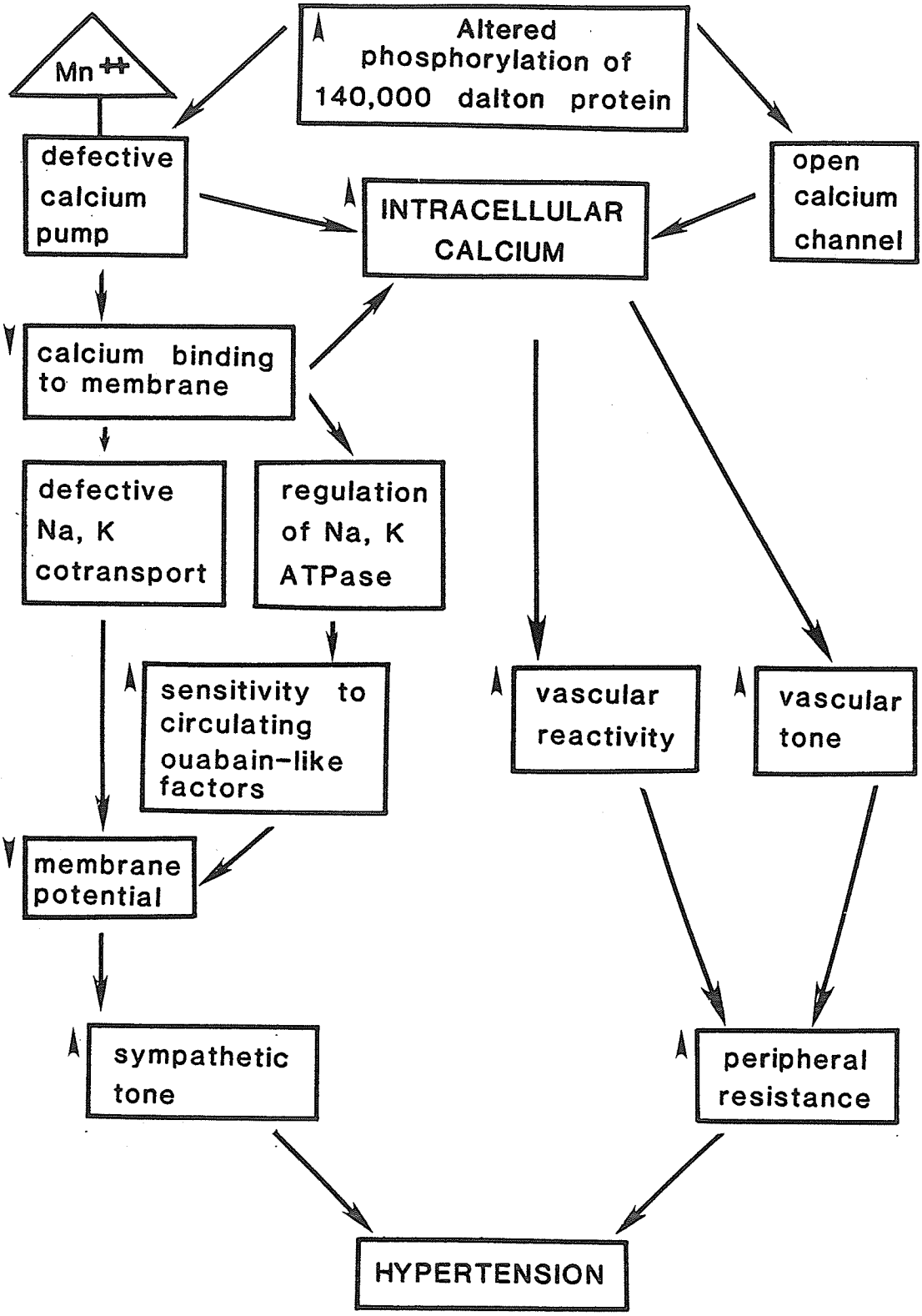
Other actions of manganese, which have not been explored in this thesis have been reported. Buck et al. (1985) observed manganese to stimulate ^{3}H-inositol incorporation into the membrane phospholipid component of dissociated aortic smooth muscle cells from SHR and WKY

rats. The rate of ^{3}H-inositol incorporation in the cells of WKY rats was twice the rate in the cells of the SHR. Inositol triphosphate turnover is now recognized as an important second messenger in cellular metabolism and regulation of calcium handling (Berridge, 1984). The relationship of this alteration to the results reported here and its possible significance as a primary defect remain to be worked out. Braun et al. (1982) reported a biphasic effect of manganese on adenylate cyclase in turkey erythrocytes. Concentrations less that 0.1mM activate the adenylate cyclase, while higher concentrations are inhibiting. It is unlikely that this site of action is responsible for the observed effects on lactate release by manganese since there was no effect of forskolin.

2. HYPOTHESIS FOR A DEFECT IN ESSENTIAL HYPERTENSION

From the data of this thesis, together with information already published, a flowchart depicting a possible mechanism of the development of essential hypertension has been formed (Figure 39). The underlying cause of essential hypertension is genetically transmitted defective gene(s). This gene is ultimately expressed as an altered protein and should be found in all cells, regardless of the cells function in blood pressure regulation. We have identified an unknown membrane protein with a molecular weight of 140,000 daltons that is altered with respect to kinase-dependent phosphorylation. Membrane proteins of 140,000 daltons which have been identified are the calcium pump (Niggli et al., 1981b) and recently the calcium channel (Hofmann et al., 1986). Both of these proteins are integral components in the regulation of calcium handling by cells. A defect in the calcium extrusion ability of a cell, or excessively leaky calcium channels can both contribute to an increase in the intracellular levels of calcium. Increased influx of calcium through

Figure 39. Flowchart depicting a possible mechanism of the development of hypertension. The proposed site of action of manganese is indicated with a triangle. See text for explanation.


Mn++
Altered phosphorylation of 140,000 dalton protein
defective calcium pump
INTRACELLULAR CALCIUM
open calcium channel
calcium binding to membrane
defective Na, K cotransport
regulation of Na, K ATPase
sensitivity to circulating ouabain-like factors
vascular reactivity
vascular tone
membrane potential
sympathetic tone
peripheral resistance
HYPERTENSION

calcium channels has been demonstrated in other cell types (Carafoli, 1984). This part of the hypothesis is supported by the data of Zidek et al. (1982) who, using ion selective electrodes, measured higher intracellular calcium in red blood cells from essentially hypertensive patients. Raised intracellular calcium ion activity was not observed by Wehling et al. (1983) in red blood cells from hypertensive patients under basal conditions but he did observe that when the cells were metabolically depleted, the increase in intracellular Ca^{2+} was at a faster rate and to higher levels in the cells of the hypertensives. Decreased binding of calcium to the inside of the red blood cell membrane has been observed previously (Devynch et al., 1981; Postnov et al., 1979), and could be a consequence of altered calcium pump activity and would also contribute to a higher concentration of intracellular calcium.

Using $^{45}Ca^{2+}$ we have observed greater rates of net $^{45}Ca^{2+}$ accumulation by red blood cells from the SHR. We were not able to distinguish between an increased influx or a decreased efflux. Chan and co-workers (Chan et al., 1983) have presented evidence to support the hypothesis that in red blood cells the rate of passive calcium influx is increased and the changes in pump activity are secondary. We have shown that manganese increased the rate of $^{45}Ca^{2+}$ accumulation in both SHR and WKY cells but that the effect is greater in the SHR. Furthermore, we directly demonstrated that manganese decreased calcium stimulated, magnesium dependent ATPase ((Ca^{2+}-Mg^{2+})-ATPase) activity in both WKY rat and SHR cells but we did not detect any difference in their sensitivities as judged the Ki's for manganese inhibition. This finding of no difference in the sensitivities of the (Ca^{2+}-Mg^{2+})-ATPase to manganese could suggest a normal calcium pump but an increased passive

calcium influx. However it remains possible that a defect to the calcium pump decreased the efficiency of the pump (ie. ratio of Ca^{2+} pumped per ATP hydrolysed) and hence could not be detected by a measure of ATPase activity.

An alteration to a cells ability to properly regulate calcium may lead to the development of hypertension by several possible mechanisms. In vascular smooth muscle cells these defects could result in greater vascular tone and reactivity, both of which are contributors to increased peripheral resistance.

In earlier studies of cellular abnormalities in essential hypertension, much attention has been focused on the role of sodium. More recent hypotheses state that an alteration to sodium handling is secondary to abnormal calcium handling (Sprenger, 1985). From our study of the effect of furosemide on lactate release we have indirect evidence supporting the claim of Garay and his co-workers of a defective (Na^+-K^+) cotransport activity. We have recently observed that furosemide increased $^{45}Ca^{2+}$ accumulation (data not shown), suggesting a link between the two transport mechanisms. Garay (1982) has demonstrated a regulating role of intracellular calcium on the (Na^+-K^+) cotransport. Furthermore activity of the (Na^+-K^+)-ATPase protein is regulated by calcium (Davis and Vincenzi, 1971) and we have demonstrated differences in (Na^+-K^+)-ATPase activity between SHR and WKY rat cells, which is calcium dependent. Removal of calcium also increased the sensitivity of the (Na^+-K^+)-ATPase to ouabain, hence it is conceivable that in cells of SHR, where the membrane binding of calcium is decreased, the (Na^+-K^+)-ATPase may have an increased sensitivity to circulating ouabain-like factors.

In neuronal cells, these alterations would lead to a decrease in membrane potential, a condition which facilitates the release of neurotransmitters and may potentiate the consequence of altered calcium handling in vascular smooth muscle cells.

The results of the work performed in this thesis are consistent with the hypothesis that a primary alteration to membrane ion transport in hypertension is located in the calcium ion handling mechanisms. The intracellular sodium is affected by the altered calcium handling of the cell and together these factors initiate the pathogenic hypertensive process.

SECTION V

REFERENCES

Abboud, F.M.
The sympathetic system in hypertension
Hypertension 4(suppl II): II 208-II 225, 1982.

Adragna, N.C., M.L. Canessa, H. Solomon, E. Slater and D.C. Tosteson
Red cell lithium-sodium countertransport and sodium-potassium cotransport in patients with essential hypertension.
Hypertension 4: 795-804, 1982.

Adragna, N.C., C.M. Perkins and P.K. Lauf
Furosemide-sensitive Na^+-K^+ cotransport and cellular metabolism in human erythrocytes
Biocim. et Biophys. Acta. 812: 293-296, 1985

Akagawa, K., N. Hara and Y. Tsukada
Partial purification and properties of the inhibitors of Na, K-ATPase and ouabain-binding in bovine central nervous system.
J. Neurochem. 42: 775-780, 1984.

Akera, T., F.S. Larsen and T.M. Brody
The effect of ouabain on sodium and potassium activated adenosine triphosphatase from the hearts of several mammalian species.
The Journal of Pharmacology and Experimental Therapeutics 170: 17-26, 1969.

Alper, S.L., K.G. Beam and P. Greengard
Hormonal control of Na^+-K^+ cotransport in turkey erythrocytes.
J. Biol. Chem. 255: 4864-4871, 1980a.

Alper, S.L., H.C. Palfrey, S.A. DeRiemer and P. Greengard
Hormonal control of protein phosphorylation in turkey erythrocytes.
J. Biol. Chem. 255: 11029-11039, 1980b.

Aoki, K., N. Ikeda, K. Yamashita, K. Tazumi, I. Sato and K. Hotta
Cardiovascular contraction in spontaneously hypertensive rats: Ca^{2+} interaction of myofibrils and subcellular membrane of heart and arterial smooth muscle.
Jap. Circ. J. 38: 1115-1119, 1974.

Armando-Hardy, M., J.C. Ellory, H.G. Ferreira, S. Fleminger and V.L. Lew
Inhibition of the calcium-induced increase in the potassium permeability of human red blood cells by quinine.
J. Physiol. 250: 32P, 1975.

Aronson, P.S.
Red cell sodium-lithium countertransport and essential hypertension.
N. Engl. J. Med. 307: 317, 1982.

Baskin, G.S. and R.G. Langdon
A spectrin-dependent ATPase of the human erythrocyte membrane
J. Biol. Chem. 256: 5428-5435, 1981.

Beauge, L.A. and O. Ortiz
Na fluxes in rat red blood cells in K-free solutions.
J. Memb. Biol. 13: 165-184, 1973.

Beauge, L.
Non-pumped sodium fluxes in human red blood cells. Evidence for facilitated diffusion.
Biochem. et Biophys. Acta 401: 95-108, 1975.

Beauge, L.A. and N. Adragna
The kinetics of ouabain inhibition and the partition of rubidium influx in human red blood cells.
J. Gen. Physiol. 57: 576-592, 1971.

Beauge, L.A. and Campos
Calcium inhibition of the ATPase and phosphatase activities of (Na^+-K^+)-ATPase
Biochim. et Biophys. Acta. 729: 137-149, 1983

Ben-Ishay, D., A. Auriham and R. Viskoper
Increased erythrocytes sodium efflux in genetic hypertensive rats of the Hebrew University strain.
Experientia 31: 660-662, 1975.

Bennett, V. and P.J. Stenbuck
The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes
Nature 280: 468-473, 1979.

Berlund, G., L. Sigstrom, S. Lundin, B.E. Karlberg and H. Herlitz
Intra-erythrocytic sodium and (Na^+,K^+-activated)-ATPase concentration and urinary aldosterone exretion in Spontaneously Hypertensive rats
Clinical Science 60: 229-232, 1981.

Berridge, M.J.
Inositol trisphosphate and diacylglyarol as second messengers.
Biochem. J. 220: 345-360, 1984.

Blaustein, M.P.
Sodium ions, calcium ions, blood pressure regulation and hypertension: A reassessment and a hypothesis.
Am. J. Physiol. 232: C165-C173, 1977.

Blaustein, M.P., J.M. Hamlyn
Role of natriuretic factor in essential hypertension.
Ann. Int. Med. 98(Suppl): 785-792, 1983.

Bloom, D.S., M.G. Stein and C. Rosendorff
Effects of hypertensive plasma on the responses of an isolated artery preparation to noradrenaline.
Cardiovasc. Res. 10: 268-274, 1976.

Blum, R.M. and J.F. Hoffman
The membrane locus of Ca-stimulated K transport in energy depleted human red blood cells.
J. Memb. Biol. 6: 315-328, 1971.

Bohr, D.F.
Reactivity of vascular smooth muscle from normal and hypertensive rats: effect of several cations.
Fed. Proc. 33: 127-132, 1974.

Boivin, P., M. Garbarz, D. Dhermy and C. Galand
In vitro phosphorylation of the red blood cell cytoskeleton complex by cyclic AMP-dependent protein kinase from erythrocyte membranes.
Biochem. Biophys. Acta. 647: 1-6, 1981.

Bonting, S.L., L.L. Caravaggio, N.M. Hawkins
Studies on sodium-potassium-activated adenosinetriphosphatase. IV. Correlation with cation transport sensitive to cardiac glycosides.
Archives Biochemistry and Biophysics 98: 413-419, 1962.

Braun, S., H. Arad and A. Levitzki
The interaction of Mn^{2+} with turkey erythrocyte adenylate cyclase.
Biochimica et Biophysica Acta 705: 55-62, 1982.

Brewer, G.J.
Red cell metabolism and function, in: The Red Blood Cell.
Academic Press, New York, Ed. D. MacN. Surgenor,
Vol. 1, 2nd ed., p.480, 1974.

Brock, T.A., J.B. Smith, H.W. Overbeck
Relationship of vascular sodium-potassium pump activity to intracellular sodium in hypertensive rats.
Hypertension 4(Suppl. II): II-43-II-48, 1982.

Brotherus, J.R., P.C. Jost, O.H. Griffith, J.F.W. Keana and L.E. Hokin
Charge selectivity at the lipid-protein interface of membranous Na, K-ATPase
Proc. Nat. Acad. Sci. 77: 272-276, 1980.

Brugnara, C., R. Corrocher, L. Foroni, M. Steinmayr, F. Bonfanti and G. DeSandre
Lithium-sodium countertransport in erythrocytes of normal and hypertensive subjects.
Hypertension 5: 529-534, 1984.

Buck, S.H., T. Nabika and W. Lovenberg
Mn^{++}-stimulated ^{3}H-Inositol incorporation in cultured vascular smooth muscle cells: Deficiency in cells from spontaneously hypertensive rats.
Clin. and Exper. Theory and Practice A7(7), 1033-1041, 1985.

Burgess, G.M., J.S. McKinney, A. Fabiato, B.A. Leslie and J.W. Putney Jr.
Calcium pools in saponin-permeabilized guinea pig hepatocytes
J. Biol. Chem. 258: 15336-15345, 1983

Cabantchik, Z.I. and A. Rothstein
The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives.
J. Membrane Biol. 10: 311-330, 1972.

Cabantchik, Z.I. and A. Rothstein
Membrane proteins related to anion permeability of human red blood cells.
J. Membrane Biol. 15: 207-226, 1974.

Canessa, M., N. Adragna, H.S. Solomon, T.M. Connolly and D.C. Tosteson
Increased sodium-lithium countertransport in red cells of patients with essential hypertension.
N. Engl. J. Med. 302: 772-776, 1980.

Canessa, M., I. Bize, N. Adragna and D.C. Tosteson
Cotransport of lithium and potassium in human red cells.
J. Gen. Physiol. 80: 149-168, 1982.

Carafoli, E.
Calcium-transporting systems of plasma membranes, with special attention to their regulation
Advances in Cyclic Nucleotide and Protein Phosphorylation Research
17: 543-549, 1984.

Caroni, P. and E. Carafoli
An ATP-dependent Ca^{+}-pumping system in dog heart sarcolemma
Nature 283:765-767, 1980.

Caroni, P. and E. Carafoli
The Ca^{+}-pumping ATPase of heart sarcolemma
J. Biol. Chem. 256: 3263-3270, 1981.

Chan, T.C.K., D.V. Godin and M.C. Sutter
Erythrocyte membrane abnormalities in hypertension: A comparison between two animal models
Clin. and Exper. Hyper. A5(5): 691-719, 1983

Chipperfield, A.R.
Chloride dependence of frusemide and phloretin-sensitive passive and sodium and potassium fluxes in human red cells.
J. Physiol. 312: 435-444, 1981.

Cole, C.H.
Erythrocyte membrane sodium transport in patients with treated and untreated essential hypertension.
Circulation 68: 17-22, 1983.

Coleman, T.G., R.E. Samar and W.R. Murphy
Autoregulation versus other vasoconstrictors in hypertension.
Hypertension 1: 324-330, 1979.

Cusi, D., C. Barlassina, M. Ferrandi, R. Brevi, A. Vazzola and G. Bianchi
Erythrocyte membrane transport systems as possible markers for essential hypertension.
Clin. Science 63: 575-595, 1982.

D'Amico, G.
Red cell Na and K in congestive heart failure, essential hypertension and myocardial infarction.
Am. J. Med. Sci. 236: 156-161, 1958.

Dagher, G. and R.P. Garay
A Na^+, K^+ co-transport assay for essential hypertension.
Can. J. Biochem. 58: 1069-1074, 1980.

Dahl, L.K., K.D. Knudsen and J. Iwai
Humoral transmission of hypertension: Evidence from parabiosis.
Circ. Res. 1(Suppl. 24;25): 21-33, 1969.

Dalmark, M.
Effects of halides and bicarbonate on chloride transport in human red blood cells.
J. Gen. Physiol. 67: 223-234, 1976.

Davidson, J.S., L.H. Opie and B. Keding
Sodium-potassium cotransport activity as genetic marker in essential hypertension.
Br. Med. J. 284: 539-541, 1982.

Davis, P.W. and F.F. Vincenzi
Ca-ATPase activation and Na,K-ATPase inhibition as a function of calcium concentration in human red cell membranes.
Life Sciences 10: 401-406, 1971.

De Mendonca, M., A. Knorr, M.L. Grichois, D. Ben-Ishay, R.P. Garay and P. Meyer
Erythrocytic sodium ion transport systems in primary and secondary hypertension of the rat.
Kidney International. 21(Suppl. 11): 569-575, 1982.

De Pont, J.J.H.H.M., A. Van Prooijen-Van Eeden and S.L. Bonting
Role of negatively charged phospholipids in highly purified $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla
Biochem. Biophys. Acta. 508: 464-477, 1978.

Deuticke, B., E. Beyer and B. Forst
Discrimination of three parallel pathways of lactate transport in the human erythrocyte membrane by inhibitors and kinetic properties.
B.B.A. 684: 96-110, 1982.

Devynck, M.A., M.G. Pernollet, A.M. Nunez and P. Meyer
Analysis of calcium handling in erythrocyte membranes of genetically hypertensive rats.
Hypertension 3: 397-403, 1981.

Dissing, S. and J.F. Hoffman
Ouabain-insensitive Na efflux from human red blood cells stimulated by outside H, Na or Li ions (abstr.).
J. Gen. Physiol. 80: 15a, 1982.

Drickamer, L.K.
Fractionation of the band 3 polypeptide from human erythrocyte membranes. Identification of regions likely to interact with the lipid bilayer
J. Biol. Chem. 252: 6909-6917, 1977.

Duhm, J. and B.F. Becker
Studies on the lithium transport across the red cell membrane. II. Characterization of ouabain sensitive and ouabain insensitive Li^+ transport. Effects of Bicarbonate and dipyridamole.
Pfugers Arch. 367: 211-219, 1977a.

Duhm, J. and B.F. Becker
Studies on the lithium transport across the red cell membrane. III. Factors contributing to the intra individual variablity of the in Vitro Li^+ distribution across the human red cell membrane
Pflugers Arch. 368: 203-208, 1977b.

Duhm, J. and B.F. Becker
Studies on the lithium transport across the red cell membrane. IV. Interindividual variations in the Na^+-dependent Li^+ countertransport system of human erythrocytes.
Pfluger. Arch. 370: 211-219, 1977c.

Duhm, J., F. Eisenried, B.F. Becker and W. Greil
Studies on the lithium transport across the red cell membrane. I. Li^+ uphill transport by the Na^+-dependent Li^+ countertransport system of human erythrocytes.
Pfugers Arch. 364: 147-155, 1976.

Duhm, J., B.O. Gobel, R. Lorenz and P.C. Weber
Sodium-lithium exchange and sodium-potassium cotransport in human erythrocytes. II. A simple uptake test applied to normotensive and essential hypertensive individuals.
Hypertension 4: 477-482, 1982.

Duhm, J., B.O. Gobel and F.Y. Beck
Sodium and potassium ion transport accelerations in erythrocytes of DOC, DOC-salt, two-kidney, one clip, and spontaneously hypertensive rats.
Hypertension 5: 642-652, 1983.

Duhm, J. and B.O. Gobel
Role of the furosemide-sensitive Na^+/K^+ transport system in determining the steady-state Na^+ and K^+ content and volume of human erythrocytes in vitro and in vivo.
J. Memb. Biol. 77: 243-254, 1984.

Duhm, J. and B.O. Gobel
Na^+-K^+ transport and volume of rat erythrocytes under dietary K^+ deficiency.
Am. J. Physiol. 246 (Cell Physiol. 15): C20-C29, 1984.

Dunham, E.T. and I.M. Glynn
Adenosinetriphosphatase activity and the active movements of alkali metal ions.
J. Physiol. 156: 274-293, 1961.

Dunham, P.B., G.W. Stewart and J.C. Ellory
Chloride-activated passive potassium transport in human erythrocytes.
Proc. Natl. Acad. Sci. 77: 1711-1715, 1980.

Ellory, J.C. and G.W. Stewart
The human erythrocyte Cl-dependent Na-K cotransport system as a possible model for studying the action of loop diuretics.
Br. J. Pharmac. 75: 183-188, 1982.

Ellory, J.C., P.W. Flatman and G.W. Stewart
Inhibition of human red cell sodium and potassium transport by divalent cations.
J. Physiol. 340: 1-17, 1983.

Enyedi, A., B. Sarkadi, A. Nyers and G. Gardos
Effects of divalent metal ions on the calcium pump and membrane phosphorylation in human red cells.
Biochimica et Biophysica Acta 690: 41-44, 1982.

Erdmann, E., K. Werdan, R. Hegelberger and M. Pruchiewski
Determination of the number of (Na^+-K^+) ATPase their enzymatic activity and the active Na^+/K-transport in human erythrocytes on hypokalaemia and hypertension.
In: Intracellular Electrolytes and Arterial Hypertension, ed. H. Zumkley and H. Losse. Stuttgart: Thieme, p. 164-170, 1980.

Fadeke Aderounmu, A. and L.A. Salako
Abnormal normal cation composition and transport in erythrocytes from hypertensive patients.
Eur. J. Clin. Invest. 9: 369-375, 1979.

Fairbanks, G., J. Avruch, J.E. Dino and V.P. Patel
Phosphorylation and dephosphorylation of spectrin.
J. Supramol. Struct. 9: 97-112, 1978.

Fairbanks, G., T.L. Steck and D.F.H. Wallach
Electrophoretic analysis of the major polypeptides of the human erythrocyte membranes.
Biochemistry 10: 2602-2617, 1971.

Feig, P.U., P.P. Mitchell and J.W. Boylan
Erythrocyte membrane transport in hypertensive humans and rats. Effect of sodium depletion and excess.
Hypertension 7: 423-429, 1985.

Fitzgibbon, W.R., T.O. Morgan and J.B. Meyers
Erythrocyte ^{22}Na efflux and urinary sodium excretion in essential hypertension.
Clin. Sci. 59: 1955-1975, 1980.

Freifelder, D.M.
Physical Biochemistry, pg. 216-220, 1976.
San Francisco: W.H. Freeman and Company

Friedman, S.M.
An ion exchange approach to the problem of intracellular sodium in the hypertensive process.
Circ. Res. (Suppl.)34-35: I-123-I-128, 1974

Friedman, S.M., M. Nakashima and R.A. McIndoe
Glass electrode measurement of net Na^+ and K^+ fluxes in erythrocytes of the spontaneously hypertensive rat.
Can. J. Physiol. Pharmacol. 55: 1302-1310, 1977

Friedman, S.M. and C.L. Friedman
Cell permeability, sodium transport, and the hypertensive process in the rat.
Circ. Res. 39: 433-441, 1976.

Friedman, S.M., M. Nakashima, R.A. McIndoe and C.L. Friedman
Increased erythrocyte permeability to Li and Na in the spontaneously hypertensive rat.
Experentia 32: 476-478, 1976.

Friedman, S.M.
Sodium in blood vesels.
Blood Vessels 16: 2-16, 1979.

Frizzell, R.A., M.J. Koch and S.G. Schultz
Ion transport by rabbit colon. I. Active and passive components.
J. Memb. Biol. 27: 297-316, 1976.

Frizzell, R.A.
Active chloride secretion by rabbit colon: Calcium-dependent stimulation by ionophore A23187.
J. Memb. Biol. 35: 175-187, 1977.

Frizzell, R.A., M. Field and S.G. Schultz
Sodium-coupled chloride transport by epithelial tissues.
Am. J. Physiol. 236: F1-F8, 1979.

Funder, J., D.C. Tosteson and J.O. Wieth
Effects of bicarbonate on lithium transport in human red cells.
J. Gen. Physiol. 71: 721-746, 1978.

Furthmayr, H. and V.T. Marchesi
Subunit structure of human erythrocyte glycophorin A.
Biochemistry 15: 1137-1144, 1976.

Garay, R.P. and P. Meyer
A new test showing abnormal net Na^+ and K^+ fluxes in erythrocytes of essential hypertensive patients.
Lancet 1: 349-353, 1979.

Garay, R.P., G. Dagher, M.G. Pernollet, M.A. Devynck and P. Meyer
Inherited defect in a (Na + K) co-transport system in erythrocytes from essential hypertensive patients.
Nature (London) 284: 281-283, 1980.

Garay, R.P., G. Dagher and P. Meyer
An inherited sodium ion-potassium ion cotransport defect in essential hypertension.
Clin. Sci. 59: 1915-1935, 1980a.

Garay, R.P., G. Dagher, M.G. Pernollet, M.A. Devynck and P. Meyer
Inherited defect in a Na^+,K^+ co-transport system in erythrocytes from essential hypertensive patients.
Nature 284: 281-283, 1980b.

Garay R.P., J.L. Elghozi, G. Dagher and P. Meyer
Laboratory distinction between essential and secondary hypertension by measurement of erythrocyte cation fluxes.
N. Engl. J. Med. 302: 769-771, 1980c.

Garay, R., N. Adragna, M. Canessa and D. Tosteson
Outward sodium and potassium cotransport in human red cells.
J. Memb. Biol. 62: 169-174, 1981.

Garay, R.P.
Inhibition of the Na^+/K^+ cotransport system by cyclic AMP and intracellular Ca^{2+} in human red cells.
B.B.A. 688: 786-792, 1982.

Garay, R.P., C. Nazaret, P. Hannaert and M. Price
Abnormal Na^+,K^+ cotransport function in a group of patients with essential hypertension.
Eur. J. Clin. Invest. 13: 311-320, 1983b.

Garay, R., C. Nazaret, J. Diez, G. Dagher, P. Hannaert and P. Braquet
The effect of cyclic nucleotides and icosanoids on Na^+ and K^+ transport in human red cells.
Biomed. Biochim. Acta 42: 553-557, 1983a.

Gardner, J.D., G.D. Aurbach, A.M. Spiegel and E.M. Brown
Receptor function and ion transport in turkey erythrocytes.
Recent. Prog. Horm. Res. 32: 567-595, 1976.

Gardos, G.
The role of calcium in the potassium permeability of human erythrocytes.
Acta Physiologica Academiae Scientiarum Hungaricae 15: 121-125, 1959.

Garrahan, P.J. and I.M. Glynn
The sensitivity of the sodium pump to external sodium.
J. Physiol., London 192: 175-188, 1967a.

Garrahan, P.J. and I.M. Glynn
Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalyzed by the sodium pump.
J. Physiol., London 192: 189-216, 1967b.

Garrahan, P.J. and Rega, A.F.
Cation loading of red blood cells
J. Physiol. 193: 459-466, 1967.

Ghysel-Burton, J. and T. Godfraind
Stimulation and inhibition of the sodium pump by cardioactive steriods in relation to their binding sites and their inotropic effect on Guinea-Pig isolated atria
Br. J. Pharmacol. 66: 175-184, 1979.

Glynn, I.M.
Sodium and potassium movements in human red cells.
J. Physiol. 134: 278-310, 1956.

Glynn, I.M.
The action of cardiac glycosides on sodium and potassium movements in human red cells.
J. Physiol. 136: 148-173, 1957.

Glynn, I.M. and J.F. Hoffman
Nucleotide requirements for sodium:sodium exchange catalyzed by the sodium pump in human red cells.
J. Physiol., London 218: 239-256, 1971.

Glynn, I.M. and Karlish
ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: evidence for allosteric effects of intracellular ATP and extracellular sodium.
J. Physiol., London 256: 465-496, 1976.

Godfraind T.
Calcium exchange in vascular smooth mescle. Action of noradrenaline and lanthanum
J. Physiol. 260: 21-35, 1976.

Gomori, C.J.
A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter
J. Lab. Clin. Med. 27: 955-960, 1941.

Goodman, S.R., J. Yu, C.F. Whitfield, E.N. Culp and E.J. Posnak
Erythrocyte membrane skeletal protein bands 4.1a and b are sequence-related phosphoproteins.
J. Biol. Chem. 257:4564-4569, 1982.

Gopinath, R.M. and F.F. Vincenzi
Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+}-Mg^{2+}) ATPase.
Biochem. Biophys. Res. Commun. 77: 1203-1209, 1977.

Greengard, P.
Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters.
Nature 260: 101-108, 1976.

Greengard, P.
Cyclic nucleotides, phosphorylated proteins, and the nervous system.
Fed. Proc. 38: 2208-2217, 1979.

Greengard, P.
Phosphorylated proteins as physiological effectors.
Science 199: 146-152, 1978.

Greenquist, A.C., S.B. Shohet and S.E. Bernstein
Marked reduction of spectrin in hereditary spherocytosis in the common house mouse.
Blood 51: 1149-1155, 1978.

Guyton, A.C., J.E. Hall, T.E. Lohmeier, T.E. Jackson and P.R. Kastner
Blood pressure regulation: basic concepts.
Federation Proc. 40: 2252-2256, 1981.

Haas, M., J. Schooler and D.C. Tosteson
Coupling of lithium to sodium transport in human red cells.
Nature 258: 425-427, 1975.

Haase, W. and H. Koepsell
Substructure of membrane-bound Na^+-K^+-ATPase protein.
Pflugers Arch. 381: 127-135, 1979.

Haddy, F.J.
Abnormalities of membrane transport in hypertension.
Hypertension 5(Supp. V): V66-V72, 1983.

Haley, B.E. and J.F. Hoffman
Interactions of a photo-affinity ATP analog with cation-stimulated adenosine triphosphatases of human red cell membranes.
Proc. Nat. Acad. Sci. 71: 3367-3371, 1974.

Hamet, P., D.J. Franks, S. Adnot and J.F. Coquil
Cyclic nucleotides in hypertension.
Adv. in Cyc. Nucleotide Research 12: 11-23, 1980.

Hamlyn, J.M., R. Ringel, J. Schaeffer, P.D. Levinson, B.P. Hamilton, A.A. Kowarski and M.P. Blaustein
A circulating inhibitor of (Na^+ + K^+) ATPase associated with essential hypertension.
Nature 300: 650-652, 1982.

Harder, D.R. and K. Hermsmeyer
Membrane mechanisms in arterial hypertension.
Hypertension 5: 404-408, 1983.

Harris, E.J. and B.C. Pressman
Obligate cation exchanges in red cells.
Nature 216: 918-920, 1967.

Hart, M.M., D.D. Heistad and M.J. Brody
Effect of chronic hypertension and sympathetic denervation on wall/lumen ratio of cerebral vessels.
Hypertension 2: 419-423, 1980.

Hasselbach, W., J. Suko, M.H. Stromer and R. The
The mechanism of calcium transport in sarcoplasmic reticulum.
Ann. N.Y. Acad. Sci. 264: 335-349, 1975.

Hastings, D.F. and J.A. Reynolds
Molecular weight of (Na^+, K^+)ATPase from shark rectal gland.
Biochemistry 18: 817-821, 1979.

Henningsen, N.C., S. Mattsson, B. Nosslin, D. Nelson and O. Ohlsson
Abnormal whole body and cellular (erythrocytes) turnover of ^{22}Na in normotensive relatives of probands with established essential hypertension.
Clin. Sci. 57: 321S-324S, 1979.

Hermsmeyer, K.
Cellular basis for incresed sensitivity of vascular smooth muscle in spontaneously hypertensive rats.
Circ. Res. 38(Supp. II): II53-II57, 1976a.

Hermsmeyer, K.
Electrogenesis of increased norepinephrine sensitivity of arterial vascular muscle in hypertension.
Circ. Res. 38: 362-367, 1976b.

Hilton, P.J.
Cellular sodium transport in essential hypertension.
New England Journal of Medicine 314: 222-229, 1985.

Hoffman, J.F. and F.M. Kregenow
The characterization of new energy dependent cation transport processes in red blood cells.
Ann. N.Y. Acad. Sci. 137: 566-576, 1966.

Hoffman, J.F.
The interaction between tritiated ouabain and the Na-K pump in red blood cells.
J. Gen. Physiol. 54: 343s-350s, 1969.

Hofmann F., B. Jung, H.-J. Oeken and V. Flockerzi
Biochemistry and physiology of cardiac calcium channels
Proc. I.U.P.S. 16: 557, 1986.

Hohorst, H.J.
in: Methods of Enzymatic Analysis,
eds. H.U. Bergmeyer, (Academic Press. Inc., New York and London)
p266, 1963.

Hokin, L.E., J.L. Dahl, J.D. Devpree, J.F. Dixon, J.F. Hackney and J.F. Perdue
Studies on the characterization of the sodium-potassium transport adenosine triphosphate.
J. Biol. Chem. 248: 2593-2605, 1973.

Hollander, W., D.M. Kramsch, M. Farmelant and I.M. Madoff
Arterial wall metabolism in experimental hypertension of coarctation of the aorta of short duration.
J. Clin. Invest. 47: 1221-1229, 1969.

Ibsen, K.K., H.A. Jensen, J.O. Wieth and J. Funder
Essential hypertension: Sodium-lithium countertransport in erythrocytes from patients and from children having one hypertensive parent.
Hypertension 4: 703-709, 1982.

Ives, H.E., V.J. Yee and D.G. Warnock
Mixed type inhibition of the renal Na^+/H antiporter by Li^+ and amiloride.
J. Biol. Chem. 258: 9710-9716, 1983.

Jarrett, H.W. and J.T. Penniston
Partial purification of the Ca^{2+}-Mg^{2+} ATPase activator from human erythrocytes its similarity to the activator of 3'-5' cyclic nucleotide phosphodiesterase.
Biochem. Biophys. Res. Commun. 77: 1210-1216, 1977.

Jean, D.H. and R.W. Albers
Molecular organization of subunits of electroplax (sodium plus potassium)- activated adenosine triphosphate.
J. Biol. Chem. 252: 2450-2451, 1977.

Jones, A.W.
Altered ion transport in vascular smooth muscle from spontaneously hypertensive rats.
Circ. Res. 33: 563-572, 1973.

Jones, A.W. and R.G. Hart
Altered ion transport in aortic smooth muscle during deoxycorticosterone acetate hypertension in the rat.
Circ. Res. 37: 333-341, 1975.

Jones, A.W.
Altered ion transport in large and small arteries from spontaneously hypertensive rats and the influence of calcium.
Circ. Res. (Suppl.) 34-35: i-117-i-112, 1974.

Jorgensen, P.L. and J. Petersen
Purification and characterization of $(Na^+ + K^+)$-ATPase vi. Differential tryptic modification of catalytic functions of the purified enzyme in the presence of NaCl and KCl.
Biochem. Biophys. Acta. 466: 97-108, 1977.

Karlish, S.J.D., J.C. Ellory and V.L. Lew
Evidence against Na^+-pump mediation of Ca^{2+}-activated K^+ transport and diuretic-sensitive (Na^+/K^+)-cotransport.
Biochem. Biophys. Acta. 646: 353-355, 1981.

Keene, J.J., C.L. Seidel and D.F. Bohr
Manganese on calcium flux and norepinephrine-induced tension in arterial smooth muscle.
Proc. Soc. Exptl. Biol. Med. 139: 1083-1085, 1972.

Kinsella, J.L., P.S. Aronson
Interaction of NH_4^+ and Li^+ with the renal microvillus membrane Na^+-H^+ exchanger.
Am. J. Physiol. 241 (Cell Physiol. 10): C220-C226, 1981.

Kornfeld, R. and S. Kornfeld
The structure of a phytohemagglutinin receptor site from human erythrocytes.
J. Biol. Chem. 245:2536-2545, 1970.

Kott, M., E. Spitzer, J. Beer, J. Malur and K.R.H. Repke
Identification of the ouabain-binding peptide of (Na, K)-ATPase.
Acta. Biol. Med. Germ. 34: K19-K27, 1975.

Koutouzov, S., P. Marche, J.F. Cloix and P. Meyer
Phospholipid phosphorylation in erythrocyte of spontaneously hypertensive rats.
Am. J. Physiol. 243: H590-H597, 1982.

Koutouzov, S., P. Marche, A. Girard, P. Meyer
Altered turnover of polyphosphoinositides in the erythrocyte membrane of the spontaneously hypertensive rat.
Hypertension 5: 409-414, 1983

Kregenow, F.M.
Osmoregulatory salt transporting mechanisms. Control of cell volume in anisotonic medicine.
Ann. Rev. Physiol. 43: 493-505, 1981.

Kreye, V.A.W., P.K. Bauer and I. Villhauer
Evidence for furosemide-sensitive active chloride transport in vascular smooth muscle.
European J. Pharmacol. 73: 91-95, 1981.

Lackington, I. and F. Orrega
Inhibition of calcium-activated potassium conductance of human erythrocytes by calmodulin inhibitory drugs.
FEBS Lett. 133: 103-106, 1981.

Lee, S.W., A. Schwartz, R.J. Adams, Y. Yamori, K. Whitmer, L.K. Lane and E.T. Wallick
Decreased in Na^+,K^+-ATPase activity and [^{3}H]ouabain binding sites in sarcolemma prepared from hearts of spontaneously hypertensive rats.
Hypertension 5: 682-683, 1983.

Lehninger, A.L.
Biochemistry 2nd edition, Worth Publishers Inc., New York, New York, Chapter 16, 1975.

Lelievre, L., A. Zachowski, D. Charlemagne, P. Laget and A. Paraf
Inhibition of (Na^++K^+)-ATPase by ouabain: Involvement of calcium and membrane proteins
Biocim. et Biophys. Acta. 557: 399-408, 1979.

Levy, R., E. Paran, A. Keynan and A. Liune
Essential hypertension: Improved differentiation by the temperature dependence of Li efflux in erythrocytes.
Hypertension 5: 821-827, 1983.

Lew, V.L.
Effect of ouabain on the Ca^+-dependent increase in K^+ permeability in depleted Guinea-pig red cells.
Biochem. Biophys. Acta. 249: 236-239, 1971.

Lew, V.L. and L.A. Beauge
Passive cation fluxes in red cell membranes
In: Membrane transport in biology v.II
Giebisch, G., Tosteson, D.C. and Ussing, H.H. eds.
Berlin. Springer-Verlag: p81-115, 1979.

Limas, C.J. and J.N. Cohn
Defective calcium transport by cardiac sarcoplasmic reticulum of spontaneously hypertensive rats.
Circulation 54(Suppl. II): II58, 1976.

Liu, S.-C., J. Palek and J.T. Prchal
Defective spectrin dimer-dimer association in hereditary elliptocytosis.
Proc. Natl. Acad. Sci. 79: 2072-2076, 1982.

Losse, H., H. Wehmeyer and F. Wessels
Der Wasser-und Electrolytgehalt von Erythrocytes bei arterieller Hypertonie.
Klin. Worchenschr. 38: 393-402, 1960.

Losse, H., W. Zidek, H. Zumkley, F. Wessels and H. Vetter
Intracellular Na as a genetic marker of essential hypertension.
Clin. Exp. Hypertens. 3: 627-640, 1981.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall
Protein measurements with the folin phenol reagents
J. Biol. Chem. 193: 265-275, 1951.

Mahoney, J.R., N.L. Etkin, J.D. McSwigan and J.W. Eaton
Assessment of red cell sodium transport in essential hypertension.
Blood 59: 439-442, 1982.

Makinose, M. and W. Hasselbach
ATP synthesis by the reverse of the sarcoplasmic calcium pump.
FEBS Lett. 12: 271-272, 1971.

Marshall, P.J. and L.E. Hokin
Microheterogeneity of the glycoprotein subunit of the (sodium + potassium)- activated adenosine triphosphatase from the electroplax of Electrophorus Electricus.
Biochem. Biophys. Research Commun. 87: 476-482, 1979.

Martin, K.
Extracellular cations and the movement of choline across the erythrocyte membrane.
J. Physiol. 224: 207-230, 1972.

Martin, K.
Choline transport in red cells.
In: Membrane transport in red cells.
Ellory, J.C. and Lew, V.L. eds.
London. Academic Press: 101-113, 1977.

Mason, M.
A new method for ethanol measurement utilizing an immobilized enzyme
American Society of Brewing Chemists Journal 40: 78-79, 1982

Milanick, M.A. and J.F. Hoffman
Separate effects of internal and external pH on cation fluxes in human red blood cells as studied by means of a pH clamp (abstr.).
J. Gen. Physiol. 80: 20a, 1982.

Minakami, S. and H. Yoshikawa
Studies on erythrocyte glycolysis. III. The effects of active cation transport pH and inorganic phosphate concentration on erythrocyte glycolysis.
J. Biochem. 59: 145-150, 1966.

Munro-Faure, A.D., D.M. Hill and J. Anderson
Ethnic differences in human blood cell sodium concentration.
Nature, London 231: 457-458, 1971.

Murer, H., U. Hopfer and R. Kinne
Sodium/proton antiport in brush-border membrane vesicles isolated from rat small intestine and kidney.
Biochem. J. 154: 597-604, 1976.

Murphy, J.R.
Erythrocyte metabolism. V. Active cation trnsport and glycolysis.
J. Lab. Clin. Med. 61: 567-577, 1963.

Nakashima, K. and E. Beutler
Comparison of structure and function of human erythrocyte and human muscle actin.
Proc. Natl. Acad. Sci. 76: 935-938, 1979.

Nelson, M.J., D.L. Daleke and W.H. Huestis
Calmodulin-dependent spectrin kinase activity in resealed human erythrocyte ghosts.
Biochem. Biophys. Acta. 686: 182-188, 1982.

Niggli, V., J.T. Penniston and E. Carafoli
Purification of the (Ca^{2+}-Mg^{2+})-ATPase from human erythrocyte membranes using a calmodulin affinity column.
J. Biol. Chem. 254: 9955-9958, 1979a.

Niggli, V., P. Ronner, E. Carafoli and J.T. Penniston
Effects of calmodulin on the (Ca^{2+} + Mg^{2+})ATPase partially purified from erythrocyte membranes.
Arch. Biochem. Biophys. 198: 124-130, 1979b.

Niggli, V., E.S. Adunyah, J.T. Penniston and E. Carafoli
Purified (Ca^{2+} - Mg^{2+})-ATPase of the erythrocyte membrane.
J. Biol. Chem. 256: 395-401, 1981a.

Niggli, V., E.S. Adunyah and E. Carafoli
Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase.
J. Biol. Chem. 256: 8588-8592, 1981b.

Nishizuka, Y.
Turnover of inositol phospholipids and signal transduction.
Science 225: 1365-1370, 1984.

O'Farrell, P.H.
High resolution two-dimensional electrophoresis of proteins.
J. Biol. Chem. 250: 4007-4021, 1975.

Palfrey, H.C., P.W. Feit and P. Greengard
cAMP-stimulated cation cotransport in avian erythrocytes: inhibition by "loop" diuretics.
Am. J. Physiol. 238 (Cell Physiol. 7): C139-C148, 1980.

Palfrey, H.C. and P. Greengard
Hormone-sensitive ion transport systems in erythrocytes as models for epithelial ion pathways.
Ann. N.Y. Acad. Sci. 372: 291-308, 1981.

Pandey, G.N., B. Sarkadi, M. Haas, R.B. Gunn, J.M. Davis and D.C. Tosteson
Lithium transport pathways in human red blood cells.
J. Gen. Physiol. 72: 233-247, 1978.

Parker, J.C. and J.F. Hoffman
The role of membrane bound phosphoglycerate kinase in the control of glycolytic rate by active cation transport in human red blood cells.
J. Gen. Physiol. 50: 893-916, 1961.

Parker, J.C. and L.E. Berkowitz
Physiologically instructive genetic varients involving the human red cell membrane.
Physiological Reviews 63:261-313, 1983.

Passow, H.
Passive ion permeability of the erythrocyte membrane.
Prog. Biophys. Mol. Biol. 19, 425-467, 1969.

Paul, R.J., M. Bauer and W. Peae
Vascular smooth muscle: Aerobic glycolysis linked to sodium and potassium transport processes.
Science 206: 1414-1416, 1979.

Peters, W.H.M., J.J.H.H.M. De Pont, A. Koppers and S.L. Bonting
Studies on ($Na^+ + K^+$)-activated ATPase. Chemical composition, Molecular weight and molar ratio of the subunits of the enzyme from rabbit kidney outer medulla.
Biochem. Biophys. Acta. 641: 55-70, 1981.

Post, R.L., C. Hegyvary and S. Kume
Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase.
J. Biol. Chem. 247: 6530-6540, 1972.

Post, R.L., C.R. Merritt, C.R. Kinsolving and C.D. Albright
Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in human erythrocyte.
J. Biol. Chem. 235: 1796-1802, 1960.

Post, R.L., A.K. Sen and A.S. Rosenthal
A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes.
J. Biol. Chem. 240: 1437-1445, 1965.

Postnov, Y.V., S.N. Orlov, P. Gulak and A. Shevchenko
Altered permeability of the erythrocyte membrane for sodium and potassium ions in spontaneously hypertensive rats.
Pfluegers Arch. 365: 257-263, 1976.

Postnov, Y.V., S.N. Orlov, A. Schevchenko and A.M. Adler
Altered sodium permeability, calcium binding and Na-K-ATPase activity in the red blood cell membrane in essential hypertension.
Pflugers Arch. 371: 263-269, 1977.

Postnov, Y.V. and S.N. Orlov
Alteration of cell membranes in hypertension: Role of altered membrane control over intracellular calcium.
First Joint US-USSR Symposium on Hypertension, Sochi, USSR, June 20-23, 1978. NIH Pub. # 79-1272.

Postnov, Y.V., S.N. Orlov and N.I. Pokudin
Decrease of calcium binding by the red blood cell membrane in spontaneously hypertensive rats and in essential hypertension.
Pflugers Arch. 379: 191-195, 1979.

Postnov, Y.V. nd S.N. Orlov
Evidence of altered calcium accumulation and calcium binding by the membranes of adipocytes in spontaneously hypertensive rats.
Pflugers Arch. 385: 85-89, 1980.

Poston, L., R.B. Sewell, S.P. Wilkinson, P.J. Richardson, R. Williams, E.M. Clarkson, G.A. MacGregor and H.E. De Wardener
Evidence for a circulating sodium transport inhibitor in essential hypertension.
Br. Med. J. 282: 847-849, 1981.

Powis, D.A., T.A. Anderson, H. Jackson and G.D. Wattus
Stimulation of neuronal Na^+,K^+-ATPase by calcium.
Biochemical Pharmacology 32: 1219-1227, 1983.

Price, M., P. Hannaert, G. Dagher and R.P. Garay
Interaction of internal Na^+ and external K^+ with the erythrocyte Na^+, K^+ cotransport system in essential hypertension.
Hypertension 6: 352-359, 1984.

Proverbio, F. and J.F. Hoffman
Membrane compartmentalized ATP and its preferential use by the Na,K-ATPase of human red cell ghosts.
J. Gen. Physiol. 69: 605-632, 1977.

Rapaport, S. and J. Luebering
The formation of 2,3-diphosphoglycerate in rabbit erythrocytes: The existence of a diphosphoglycerate mutase.
J. Biol. Chem. 183: 507-516, 1950.

Reuter, H., M.P. Blaustein and G. Hausler
Na-Ca exchange and tension development in arterial smooth muscle.
Philos. Trans. R. Soc. Lond. (Biol.) 265: 87-94, 1973.

Richart, H.-W., G.F. Fuhrmann and P.A. Knauf
Dog red blood cells exhibit a Ca stimulated increase in K permeability in the absence of (Na^+ + K^+)-ATPase activity.
Nature 279: 248-250, 1979.

Rindler, M.J., L.M. Chuman, L. Shaffer and M.H. Saier Jr.
Retention of differentiated properties in an established dog kidney epithelial cell line (MDCK).
J. Cell Biol. 81: 635-648, 1979.

Rindler, M.J., J.A. McRoberts and M.H. Saier Jr.
(Na^+,K^+) cotransport in the Madin-Darby canine kidney cell line.
J. Biol. Chem. 257: 2254-2259, 1982.

Ronner, P., P. Gazzotti and E. Carafoli
A lipid requirement for the $(Ca^{2+} + Mg^{2+})$-activated ATPase of erythrocyte membranes.
Arch. Biochem. Biophys. 179: 578-583, 1977.

Rudolph, S.A. and P. Greengard
Regulation of protein phosphorylation and membrane permeability by beta-adrenergic agents and cyclic adenosine 3'-5'-monophosphate in the avian erythrocyte.
J. Biol. Chem. 249: 5684-5687, 1974.

Sachs, J.R.
Ouabain-insensitive Na movements in the human red blood cell.
J. Gen. Physiol. 57: 259-282, 1971.

Saier, M.H. and D.A. Boyden
Mechanisms, regulation and physiological significance of the loop diuretic-sensitive NaCl/KCl symport system in annual cells.
Mol. Cell. Biochem. 59: 11-32, 1984.

Sarkadi, B., Alifimoff, R.B. Gunn and D.C. Tosteson
Kinetics and stoichiometry of Na-dependent Li transport in human red blood cells.
J. Gen. Physiol. 72: 249-265, 1978.

Sarkadi, B., I. Szasz, A. Gerloczy and G. Gardos
Transport parameters and stoichiometry of active calcium ion extrusion in intact human red cells.
Biochem. Biophys. Acta. 464: 93-107, 1977.

Schatzmann, H.J.
ATP-dependent Ca^{++}-extrusion from human red cells.
Experientia 22: 364-365, 1966.

Schatzmann, H.J. and F.F. Vincenzi
Calcium movements across the membrane of human red cells.
J. Physiol. London. 201: 369-395, 1969.

Schroeder, E.
Relationship beteen plasma renin, plasma sodium and erythrocyte sodium in healthy persons and hypertensives.
Ger. Med. Mon. 13: 384-389, 1968.

Seamon, K.B. and J.W. Daley
Forskolin: a unique diterpene activator of cAMP generating systems.
J. Cyclic Nucleotide Res. 7: 201-224, 1981.

Sen, A.K. and R.L. Post
Stoichiometry and localization of adenosine triphosphate-dependent sodium and potassium transport in the erythrocyte.
J. Biol. Chem. 239: 345-352, 1964.

Shamoo, A.E. and M. Myers
Na^+- dependent ionophore as part of the small polypeptide of the ($Na^+ + K^+$) -ATPase from Eel Electroplax membrane.
J. Memb. Biol. 19: 163- 178, 1974.

Shapiro, D.L. and V.T. Marchesi
Phosphorylation in membranes of intact human erythrocytes.
J. Biol. Chem. 252: 508-517, 1977.

Sheppard, H. and C. Burghardt
The stimulation of adenyl cyclase of rat erythrocyte ghosts.
Mol. Pharacol. 6: 425-429, 1970.

Shibata, S., K. Kurashi and M. Kuchii
A possible etiology of contractility impairment of vascular smooth muscle from spontaneously hypertensive rats.
J. Pharmacology and Experimental Therapeutics 185: 406-417, 1973.

Shotton, D.M., B.E. Burke and D. Branton
The molecular structure of human erythrocyte spectrin.
J. Mol. Biol. 131: 303-329, 1979.

Siegel, D.L. and D. Branton
Human erythrocyte band 4.9 (abstr.).
J. Cell. Biol. 95: 265a, 1982.

Simons, T.J.B.
Potassium : Potassium exchange catalysed by the sodium pump in human red cells.
J. Physiol. London. 237: 123-155, 1974.

Simons, T.J.B.
The interaction of ATP-analogues possessing a blocked -phosphate group with the sodium pump in human red cells.
J. Physiol. London. 244: 731-739, 1975.

Smith, J.B., K. Owen Ash, S.C. Hunt, W.M. Hentschel, W. Sprowell, M.M. Dadone and R.R. Williams
Three red cell sodium transport systems in hypertensive and normotensive Utah adults.
Hypertension 6: 159-166, 1984.

Sprenger, K.B.G
Alterations of cellular calcium metabolism as the primary cause of hypertension
Clin. Physiol. Biochem. 3: 208- 220, 1985.

Steck, T.L., G. Fairbanks and D.F.H. Wallach
Disposition of the major proteins in the isolated erythrocyte membrane. Proteolytic dissection.
Biochemistry 10: 2617-2624, 1971.

Steck, T.L., B. Ramos and E. Strapazon
Proteolytic dissection of band 3, the predominant transmembrane polypeptide of the human erythrocyte membrane.
Biochemistry 15: 1154-1161, 1976.

Stessman, J., J. Mekler, R. Sharon and D. Ben-Ishay
Erythrocyte Na^+,K^+ cotransport and blood pressure in identical twins.
Clin. and Exp. Hyper. A5: 493-499, 1983.

Stokes, G.S., J.C. Monaghan and A.T. Middleton
Erythrocyte cation fluxes in normal and hypertensive human subjects.
Clin. Exp. Pharmacol. Physiol. 10: 279-282, 1983.

Swarts, H.G.P., S.L. Bonting, J.J.H.H.M. De Pont, F.M.A.H. Schuurmans Stekhoven, T.A. Thien and A. Vant Laar
Cation fluxes and Na^+-K^+-activated ATPase activity in erythrocytes of patients with essential hypertension.
Hypertension 3: 641-649, 1981.

Tada, M., M. Yamada, M. Kadoma, M Inui and F. Ohmori
Calcium transport by cardiac sarcoplasmic reticulum and phosphorylation of phospholamban.
Molec. Cell. Biochem. 46: 73-95, 1982.

Takayama, Y., K. Suzuki, A. Seki, K. Yamaoki and J. Fujii
Reduced number of erythrocyte sodium pump units in essential hypertension.
Tohoku J. exp. Med. 143: 383-384, 1984.

Tang, L.C., E. Schoomaker and W.P. Wiesmann
Cholinergic agonists stimulate calcium uptake and cGMP formation in human erythrocytes.
Biochimica et Biophysica Acta 772: 235-238, 1984.

Thomas, D.B. and R.J. Winzler
Structural studies on human erythrocyte glycoproteins.
J. Biol. Chem. 244: 5943-5946, 1969.

Tilney, L.G. and P. Detmers
Actin in erythrocyte ghosts and its association with spectrin.
J. Cell. Biol. 66: 508-520, 1975.

Tobian, L. Jr. and J.T. Binion
Tissue cations and water in arterial hypertension.
Circulation 5: 754-758, 1952.

Tomaselli, M.B., K.M. John and S.E. Lux
Elliptical erythrocyte membrane skeletons and heat-sensitive spectrin in hereditary elliptocytosis.
Proc. Natl. Acad. Sci. 78: 1911-1915, 1981.

Tosteson, D.C.
Cation countertransport and cotransport in human red cells.
Fed. Proc. 40: 1429-1433, 1981a.

Tosteson, D.C.
Lithium and mania.
Sci. Amer. 244: 164-174, 1981b.

Trevisan, M., R. Cooper, D. Ostrow, W. Miller, S. Sparks, Y. Leonas, A. Allen, M. Steinhauer and J. Stamler
Dietary sodium, erythrocyte sodium concentration, sodium-stimulated lithium efflux and blood pressure.
Clin. Sci. 61: 29s-32s, 1981.

Trevisan, M., D. Ostrow Jr., R. Cooper, K. Liu, S. Sparks, A. Okoner, E. Stevens, J. Marquardt and J. Stamler
Abnormal red blood cell ion transport and hypertension.
Hypertension 5: 363-367, 1983.

Urry, D.W., T.L. Trapane, K.S. Andrews, M.M. Long, H.W. Overbeck and S. Oparil
NMR observation of altered sodium interaction with human erythrocyte membranes of essential hypertensives.
Biochem. Biophys. Res. Commun. 96: 514-521, 1980.

Vincenzi F.F. and T.R. Hinds
Calmodulin and Plasma membrane calcium transport
Calcium and Cell Function 1: 127-165, 1980.

Walter, U. and A. Distler
Abnormal sodium efflux in erythrocytes of patients with essential hypertension.
Hypertension 4: 205-210, 1982.

Wambach, G., A. Helber, G. Bonner, W. Hummerich, A. Konrads and W. Kaufmann
Sodium-potassium dependent ATPase activity in erythrocyte ghosts of patients with primary and secondary hypertension.
Clin. Sci. 59: 183s-185s, 1980.

Wambach, G. and A. Helber
Na,K ATPase in erythrocyte ghosts is not a marker for primary hypertension.
Clin. Exp. Hypertension 3: 663-674, 1981.

de Wardener, H.E. and G.A. MacGregor
Dahl's hypothesis that a saluretic substance may be responsible for a sustained rise in arterial pressure: Its possible role in essential hypertension.
Kidney Int. 18: 1-9, 1980.

Weder, A.B., B.A. Torretti and S. Julius
Racial differences in erythrocyte cation transport.
Hypertension 6: 115-123, 1984.

Wehling N., W. Vetter, L. Neyses, H. Groth, H.-J., Boerlin, R. Locher, W. Siegenthaler and U. Kuhlmann
Altered calcium and sodium metabolism in red blood cells of hypertensive man: Assessment by ion-selective electrodes
J. Hypertension 1: 171: 176, 1983

Wei, J.W., R.A. Janis and E.D. Daniel
Calcium accumulation and enzymatic activities of subcellular fractions from aortas and ventricles of genetically hypertensive rats.
Circ. Res. 39: 133-140, 1976.

Weller, J.M.
The acid-base balance and sodium distribution of the blood in essential hypertension.
J. Lab. Clin. Med. 53: 553-556, 1959.

Whittam, R., M.E. Ager and J.S. Wiley
Control of lactate production by membrane adenosine triphosphatase activity in human erythrocytes.
Nature 202: 1111-1112, 1964a.

Whittam, R., K.P. Wheeler and A. Blake
Oligomycin and active transport reactions in cell membranes.
Nature 203: 720-724, 1964b.

Whittam, R. and M.E. Ager
The connection between active cation transport and metabolism in erythrocytes.
Biochem. J. 97: 214-227, 1965.

Widdicombe, J.H., I.T. Nathanson and E. Highland
Effects of "loop" diuretics on ion transport by dog tracheal epithelium.
Am. J. Physiol. 245: C388-C396,

Widdicombe, J.H. and A.F. Brading
A possible role of linked Na and Cl movement in active Cl uptake in smooth muscle.
Pflugers Arch. 386: 35-37, 1980.

Wieth, J.O.
Effect of some monovalent anions on chloride and sulfate permeability of human red cells.
J. Physiol. London. 207: 581-609, 1970.

Wiley, J.S. and R.A. Cooper
A furosemide-sensitive cotransport of sodium plus potassium in the human red cell.
J. Clinical Investigation. 53: 745-755, 1974.

Wolf, H.U.
Studies on a Ca^{2+}-dependent ATPase of human erythrocyte membranes. Effects of Ca^{2+} and H^{+}.
Biochem. Biophys. Acta. 266: 361-375, 1972.

Woods, K.L., D.G. Beevers and M.J. West
Racial differences in red cell cation transport and their relation to essential hypertension.
Clin. Exp. Hypertension 3: 655-662, 1981.

Woods, J.W., J.C. Parker and B.S. Watson
Perturbation of sodium-lithium countertransport in red cells.
N.E.J.M. 308: 1258-1261, 1983.

Worley, R.J., W.M. Hentschel, C. Cormier, S. Nutting, G. Pead, K. Zelenkov, J.B. Smith, K.O. Ash and R.R. Williams
Increased sodium-lithium countertransport in erythrocytes of pregnant women.
N.E.J.M. 307: 412-416, 1982.

Yu, J. and T.L. Steck
Isolation and characterization of band 3, the predominant polypeptide of the human erythrocyte membrane.
J. Biol. Chem. 250: 9170-9175, 1975.

Zidek, W., H. Vetter, K.G. Dorst, H. Zumkley and H. Losse
Intracellular Na^+ and Ca^+ activities in essential hypertension.
Clin. Sci. 63: 41s-43s, 1982.