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Red Blood Cell Stability in Uremic Rats

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Red Blood Cell Stability in Uremic Rats

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Biochemistry in the Department of Biochemistry School of Medicine at the Medical College of Virginia

By

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Abbreviations

ATP	Adenosine Triphosphate
G-6-PD	Glucose 6-phosphate Dehydrogenase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
G 3-PD	Glyceraldehyde 3-phosphate Dehydrogenase
HK	Hexokinase
HMP	Hexose Monophosphate Shunt
HPT	Hemolysis Peroxide Test
LDH	Lactate Dehydrogenase
NPN	Non-protein Nitrogen
PFK	Phosphofructokinase
PGI	Phosphoglucose Isomerase
PGK	Phosphoglycerate Kinase
PGM	Phosphoglyceromutase
PK	Pyruvate Kinase
PTH	Parathyroid Hormone
PUFA	Polyunsaturated Fatty Acid
RBC	Red Blood Cell
SEM	Standard Error of the Mean
6-PGD	6-Phosphogluconate Dehydrogenase

Abstract

RED BLOOD CELL STABILITY IN UREMIC RATS

Cecilia Mermel MacCallum, Masters Degree

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Biochemistry in the Department of Biochemistry, School of Medicine at the Medical College of Virginia.
Virginia Commonwealth University, 1996

Advisor: Dr. Richard Brandt, Ph.D., Department of Biochemistry and Preventative Medicine

Determining the fragility of the red blood cell (RBC) is important for the diagnosis of and evaluation for treatment of several RBC diseases. In part RBC production is controlled through the hormone erythropoeitin secreted by the kidney. In a previous study from this laboratory, it was found that RBC were more stable in uremic male rats compared to controls. In this experiment, uremia was induced in four groups of female rats through a two stage nephrectomy. The nephrectomy involved the removal of two-thirds of the left kidney, followed by the removal of the entire right kidney one week later. The animals were divided into three groups; NX-(5/6 nephrectomy), SH-(sham surgery), and PF-(sham surgery, but were fed the same food weight as the NX animals). The samples obtained in Trial I and Trial II were divided into two categories; initial and final. The initial samples were collected 14 days after the sham and five- sixth nephrectomy surgeries. The final samples

were collected at the time of sacrifice. The samples obtained in Trial II consisted only of initial samples, taken fourteen days after the five-sixth nephrectomy and sham surgeries were completed. The samples in Trial IV were final samples, obtained at the time of sacrifice. Decreasing hypotonic %NaCl solutions were used to determine the hemolysis of RBC from the rats. RBC hemolysis was determined spectrophotometrically by monitoring hemoglobin absorbance at 540nm in the supernatant fluid. Analytic precision experiments using multiple assays of the same blood sample for 50% RBC hemolysis showed a coefficient of variation of only 1.1%. Analysis of the %NaCl at 50% RBC hemolysis did not differ significantly between the three groups of animals suggesting that although the NX animals were uremic, the RBC did not differ in stability to hypoosmotic shock. Future direction for this type of research will be extended to human studies where kidney failure patients (dialysis patients) can have both the age of the RBC and their fragility determined under therapy.

The erythrocyte hemolysis peroxide test (HPT) was also performed on rat blood samples from Trial IV and on five human blood samples, in order to determine hemolysis in the RBC. A 2% H_2O_2 solution was used to determine RBC stability and %Hemolysis was calculated by dividing the value for hemolysis due to H_2O_2 by the 100% hemolysis value and multipliying by 100. Analysis of the %Hemolysis by HPT for each animal sample also showed no significant difference between the three subgroups of animals. Future direction for this type of research will be extended to include all four trials of animals as well as human studies involving patient's with kidney failure (dialysis patients).

Introduction

A. Objectives

Renal failure can occur at any age and if untreated can result in the death of the patient. Kidney disease has a variety of causes, among which are bacterial infections, congenital defects, impaired renal blood flow, tumors, toxins and immunological injury (1). When the degree of acute renal failure is moderate, the major physiological effect is retention of salt and water. Edema results and eventually the patient will develop hypertension, usually with a blood pressure increase of 30 to 40 mm Hg. In severe cases of renal failure, uremic retention of waste products ensues and acidosis develops (2). The effect of renal failure on the body's physiological mechanisms are complex, widespread and can be quite deleterious.

Hemolytic anemia is a common and severe complication of renal failure. In some manner the disease state compromises the structural integrity of the red blood cell (RBC) membrane (3). The mechanism underlying the decreased red cell survival in uremia has not been completely elucidated. In considering possible mechanisms for the premature destruction of the red cell it has been suggested that the hexose monophosphate (HMP) shunt of uremic red cells might be defective (4). RBC due to their unique structure of high concentrations of polyunsaturated fatty acids, molecular oxygen and ferrous ions in the ligand state are particularly susceptible to oxidative damage, thus suggesting a second possible explanation for the increased destruction of RBC in renal failure patients (5). RBC have an elaborate antioxidant defense system, which includes enzymes such as superoxide dismutase and catalase among others, as well as nonenzymatic biological antioxidants such as ascorbic acid and tocopherols. However, in renal failure these systems seem to be overwhelmed resulting in greatly increased hemolysis in renal failure

patients and consequently the average 120 day life span of the mammalian RBC is significantly shortened (6).

Determining the fragility of RBC is important for diagnosis of disease as well as evaluation of treatments. The experiments presented here were designed to help determine what if any effect an acute renal insult may have on the stability of the RBC. The five-sixth nephrectomy model of chronic uremia exhibits biochemical changes resembling those of the naturally occurring disease and was therefore used as the model for uremia in these experiments (7). Table 1 lists some of the biochemical changes that occur in five-sixth nephrectomised animals that are consistent with changes in naturally uremic subjects. The animals studied were female Sprague-Dawley rats and were divided into three separate experimental groups: nephrectomised (NX), sham (SH), and pairfed (PF) (7). The animals in the NX group received a five-sixth nephrectomy in a two stage procedure. The second group, PF, received sham surgeries in which the renal capsule was removed from both kidneys, to prevent damage to the adrenal gland, but no tissue was removed. The animals in the PF group were then individually matched to a NX animal and were fed by weight only what the NX animal was fed. The third animal group, SH, received sham surgeries and there were no restrictions as to the quantity of normal rat chow or water they were allowed to consume (7). Table 2 is a summary of the criteria for the grouping of the experimental animals. The initial blood samples were drawn two weeks after the surgeries to determine the onset of uremia, although previous experiments have shown that rats receiving this protocol were uremic by this time (7, 34, 35, 41).

A modification of the method of Parpat et al. where an increase in the hypotonic environment to determine the stability of the RBC was used (8, 9). The experimental basis for this model originates from the finding that when a red cell reaches a certain increased volume due to a specific hypotonic NaCl environment, the hemoglobin diffuses to satisfy the equilibrium inside and outside the cell, usually without rupturing the plasma membrane (8). The red cells in any given population differ in the volume at which the hemoglobin will diffuse out, the osmotic resistance of such cells is believed to be inversely proportional to the fragility of the cell (10). In the past decreasing concentrations from isotonic solutes of sodium chloride, sodium oxalate, sodium

Table 1. Biochemical Changes That Occur in Five-Sixth Nephrectomised Animals (13,41,42)

Anorexia
Growth Retardation
Increased Serum Cholesterol
Increased Serum Urea
Increased Serum Phosphate
Increased Serum Potassium
Decreased Serum Calcium
Hyperparathyroidism

Table 2. Summary of Criteria Used for the Grouping of Experimental Animals

---

NX- Five-sixth nephrectomised animals
Animal received five-sixth nephrectomy
No dietary restrictions, animals fed *ad libitum*

SH- Sham animals
Animals received surgery, renal capsule was removed from both kidney, but no tissue was excised
No dietary restrictions, animals fed *ad libitum*

PF- Pairfed animals
Animals received surgery, renal capsule was removed from both kidneys, but no tissue was excised
PF animals were fed by weight what the NX animals consumed

sulfate, or sapotoxin have been used to create the hypotonic environment needed to measure RBC resistance. These reagents have been reported to cause hemolysis in different manners (11). For example, certain of these chemicals cause the cell membrane to become more permeable, while others damage the membrane causing release of the hemoglobin. Decreasing the concentration of NaCl in small increments allows for precise determination of the amount of RBC membrane permeability and for the calculation of NaCl concentrations at 50% hemolysis by spectrometric measurement of the increase in supernatant fluid hemoglobin.

RBC fragility was also determined in a group of five humans by the modified Parpat et al. method as well as by using a hydrogen peroxide oxidative hemolysis test (HPT) outlined by Lubin et al. for comparison purposes (12). Vitamin E serum levels were also determined in these samples for determination of correlation between antioxidant concentration and fragility. The HPT was also performed on a group of the five-sixth nephrectomised rats. The HPT has been used to determine RBC hemolysis due to the simplicity and reliability the procedure has demonstrated. In addition, the HPT has been shown to be easily replicated, thus making it an potentially excellent experimental tool.

B. Background

1. Chronic Renal Failure

Progressive kidney failure disturbs renal metabolic and excretory function which eventually has deleterious effects on many systems in the body (13). Some features of renal failure in humans include; edema, acidosis, hypertension, hyperlipidemia, osteomalacia, azotemia and anemia (15). The five-sixth nephrectomy served as a model for chronic renal failure in these experiments.

In humans, renal failure and the decrease in a large number of nephrons, results in the still functioning nephrons becoming severely overloaded. First, the blood flow through the glomerulus and the amount of filtrate formed by each functioning nephron increases 50 to 100 percent (2). Second, as

illustrated in Figure 1, while kidney shutdown increases, large quantities of excretory substances, such as urea, sulfates, uric acid, phosphates, water, sodium, and creatinine accumulate in the extracellular fluid. Bicarbonate will tend to decrease in the extracellular fluid as kidney failure progresses. These solutes greatly increase tubular loads of substances that are poorly reabsorbed, increasing as much as 1000 percent per nephron (2). Therefore, only a small fraction of the tubular solutes are reabsorbed, and the remaining solutes act as a osmotic diuretic, resulting in rapid flushing of tubular fluid through the tubules. The increased concentration of different substances in the extracellular fluid and the decrease in concominant tubular solute reabsorption, will lead to generalized edema, and acidosis (2).

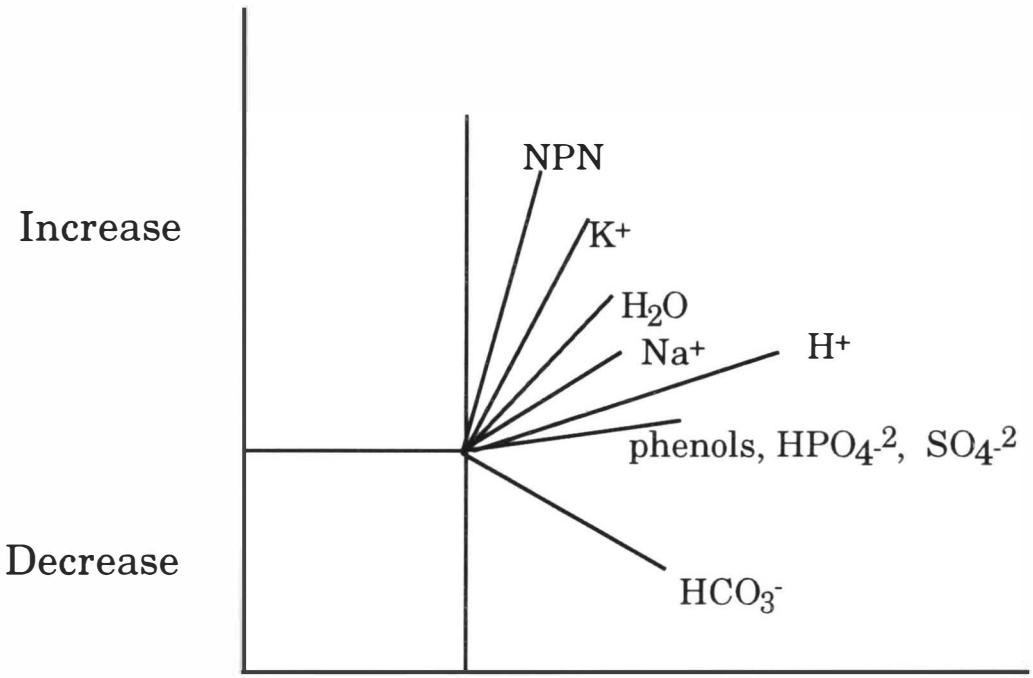
Through the normal metabolic processes there is an excess of 50 to 80 mmoles of metabolic acid produced in one day over that of metabolic alkali (2). During kidney failure, acid begins to rapidly accumulate in the body fluids. Normally, the physiological buffers can buffer a total of 500 to 1000 mmoles of acid without lethal decrease of the extracellular fluid pH. In addition, the phosphate compounds in the bones can buffer a few thousand mmoles of the acid. However, under the conditions of renal failure, the buffering capacity is eventually used up and the extracellular pH falls drastically and severe acidosis results (2).

The pathogenesis of hypertension in chronic renal failure is multi-faceted (14). Excessive renin secretion in relation to the sodium volume balance is a very important factor. There also exists some evidence that there are functional abnormalities of the sympathetic nervous system in uremic animals and humans. Stimulation of renal afferent nerves by uremic toxins such as urea evokes reflex increases in sympathetic nerve activity and blood pressure. Thus, chronic stimulation of renal afferent nerves may lead to sympathetic over activity and hypertension (15).

Cardiovascular disease accounts for up to 50% of all patients with chronic renal failure and is a major cause of morbidity among that population (16). Many factors contribute to the high prevalence of atherosclerosis in renal insufficiency including, hypertension, hyperlipidemia, vascular calcification, and endothelial injury. There is no single pathogenic mechanism for hyperlipidemia since abnormalities in lipoprotein metabolism result from

Figure 1 (2)

Change in Extracellular Constituents With Increasing Kidney Failure. As kidney failure progresses, represented on the x axis, some extracellular fluid constituents increase and some decrease as indicated by the y axis. As shown in this figure, non-protein nitrogen, potassium, water, sodium, hydrogen ions and phenols increase in the extracellular fluid. Bicarbonate decreases in the extracellular fluid as kidney failure progresses.



Kidney Shutdown

several causes (16).

Hypertriglyceridemia, resulting from decreased clearance of very low-density lipoproteins however, is the most common abnormality described. In addition to alterations in lipoprotein concentrations, abnormalities in lipid and apoprotein composition of lipoproteins are also factors. The activity of important apoproteins may be reduced and the activities of key enzymes in lipoprotein metabolism may also be decreased (16).

The disruption in metabolic and excretory functions caused by chronic renal failure will eventually lead to renal osteodystrophy (13). Osteodystrophy is described in two ways; either as osteitis fibrosa a high turnover bone disease, or as osteomalacia, a low turnover bone disease (13). Osteitis fibrosa is caused by continually increased serum levels of PTH accompanied with an elevated number and size of osteoclasts, increased osteoblast activity, and enhanced resorption of bone. Osteomalacia results from vitamin D deficiency or from hypocalcemia (13). Vitamin D (D_3. cholecalceferol) must be converted by a two stage hydroxylation, first in the liver and then in the kidney, into 1,25-dihydroxycholecalciferol before it can promote calcium absorption from the intestine. Consequently, serious damage to the kidney as in renal failure, greatly reduces the availability of calcium to the bones and osteoblast activity is markedly decreased with ensuing decrease in bone formation (2).

Excessive bleeding is also an important complication of uremia. Recent animal experiments suggest that this bleeding tendency in uremia is due to an excessive formation of nitric oxide, an endogenous vasoactive molecule which also inhibits platelet function (17). Profound abnormalities in the coagulation system are characteristic of uremia, precise pathophysiology of the problem is still poorly understood (18).

A decrease in red blood cell survival seems to be a common factor amongst patients with renal failure, consequently a patient with chronic renal failure almost always develops anemia (19). The kidneys normally secrete erythropoietin which stimulates the bone marrow to produce red cells (2). The erythrocyte has no nucleus, endoplasmic reticulum, mitochondria or other organelles required for cell maintenance, and consequently has a short lifespan of only about 120 days in the human. As erythrocytes age, changes in their plasma membrane make them susceptible to recognition and ingestion by

macrophages in the spleen, marrow and liver (2). Damaged kidneys are unable to form sufficient quantities of erythropoietin, which leads to diminished RBC production and consequent anemia. However, other factors may play a role in causing the anemia such as the high plasma concentrations of urea, hydrogen ions and other waste products (2).

2. Oxidative damage in the Red Blood Cell

Oxidative damage to cell components has been established as a factor in various pathological conditions (Table 3), including renal failure. RBC are particularly susceptible to oxidative damage as a result of the high polyunsaturated fatty acid (PUFA) content of their membranes and high cellular concentrations of hemoglobin and oxygen with hemoglobin being a potentially powerful promoter of oxidative processes (4). In addition during their relatively short lives in which no protein synthesis occurs, the RBC whose principal function it is to carry oxygen to and from the tissues and organs and to return carbon dioxide to the lungs (20), comes in close contact with free radicals from various sources: (1) the normal oxidation of hemoglobin, which ultimately produces the very reactive superoxide radical; (2) oxidation-reduction of drugs or (3) xenobiotics that are transported by the blood (21). In healthy RBC significant oxidative damage is prevented by a very efficient antioxidant system (Table 4), consisting of a number of antioxidant compounds and enzymes including catalase, superoxide dismutase, glutathione with a regenerating system, glutathione peroxidase, and vitamin E, as well as effective compartmentalization of RBC components (6, 20). However, in disease states such as uremia, these antioxidant defense systems can be overwhelmed and the RBC becomes susceptible to oxidative damage.

Even though mature RBC do not have a nucleus, mitochondria or endoplasmic reticulum, they do have cytoplasmic enzymes capable of metabolizing glucose and forming small amounts of adenosine triphosphate (ATP). ATP is used by the RBC in several ways: (1) helping to maintain the pliability of the membrane, (2) maintaining membrane transport of ions, (3) keeping the iron of the cell's hemoglobin in the ferrous form and (4) preventing oxidation of the proteins in the cell (2). With time, however, these metabolic

Table 3. Pathological Conditions in Which Oxidative Damage is a Factor (31)

1. Hemolytic anemia
2. Retinopathy
3. Atherosclerosis
4. Cancer
5. Rheumatoid arthritis
6. Reoxygenation injury
7. Drug-associated toxicity
8. Viral infections

Table 4. Some Significant Red Blood Cell Antioxidant Compounds and Enzymes Commonly found in Healthy Red Blood Cells (31,32,33)

1.Vitamin E

2. Catalase

3. Glutathione Peroxidase

4. Ascorbate

5. Selenium

6. Beta Carotene

processes become less active and the cells become more fragile. Once the RBC membrane becomes too fragile, it will rupture when the cell passes through tight regions of the circulation (2). The spleen, due to the small spaces between the structural trabeculae of the red pulp, is a common site for RBC fragmentation and cell death.

a. Generation of Reactive species in the RBC

RBC are continuously exposed to reactive oxygen species from both internal and external sources. Within the cytosol of the RBC, under physiological conditions, it is estimated that as much as 3% of the total body hemoglobin ($Hb\text{-}Fe^{+2}$) is converted into methemoglobin ($Hb\text{-}Fe^{+3}$) each day, as a result, the conversion of oxyhemoglobin to methemoglobin serves as a constant source of superoxide radicals (5). Superoxide radicals will spontaneously or enzymatically dismutate yielding hydrogen peroxide. The presence of both peroxide and the superoxide radical generates hydroxyl radicals as shown in equation one (5):

$$\cdot O_2^- + H_2O_2 \text{ ----> } \cdot OH + OH^- + O_2 \qquad \text{(eqn 1)}$$

It has recently been found that in systems generating superoxide radicals and peroxide, such as shown above in the RBC, the presence of iron will act as a catalyst in the formation the hydroxyl radical as shown in the following equations (5):

$$\cdot O_2^- + Fe^{+3} \text{ ----> } Fe^{+2} + O_2 \qquad \text{(eqn 2)}$$

$$\cdot O_2^- + HO_2\cdot \text{ ----> } O_2 + H_2O_2 \qquad \text{(eqn 3)}$$

$$Fe^{+2} + H_2O_2 \text{ ----> } Fe^{+3} + \cdot OH + OH^- \qquad \text{(eqn 4)}$$

Thus, the reaction of hydrogen peroxide with ferrous iron will generate the hydroxyl radical at an increased rate (22). The hydroxyl radical (OH·) is very damaging to the RBC because it has the potential to initiate lipid

peroxidation of the membrane, it can inactivate red cell enzymes and denature the hemoglobin molecule itself (21).

$$—CH + OH\cdot \text{ ----} > —C\cdot + RH \qquad \text{(eqn 5)}$$

The newly formed carbon centered radical reacts rapidly with oxygen:

$$—C\cdot + O_2 \text{ ----} > —CO_2\cdot \qquad \text{(eqn 6)}$$

Consequently a fatty acid side chain peroxyl radical (lipid peroxyl radical) is formed which can attack adjacent fatty acid side chains and propagate lipid peroxidation (23). Thus, once initiated, the process of lipid peroxidation can be self-propagating.

The interaction of hemoglobin in the RBC with xenobiotics or redox drugs can generate free radicals and oxidized or even denature hemoglobin (22). Heme bound oxygen can oxidize some compounds, as is the case with phenylhydrazine in the following manner (22):

$$Hb^{+2}\, O_{2^-} + RH_2 \text{ ----} > Hb^{+2} \cdot H_2O_2 + R\cdot \qquad \text{(eqn 7)}$$

Compounds such as quinones can be reduced by the heme iron:

$$Hb^{+2}\, O_2 + R \text{ ----} > Hb^{+3} + O_2 + R\cdot \qquad \text{(eqn 8)}$$

Subsequently these drug radicals (R·) can undergo secondary reactions with hemoglobin, molecular oxygen, or other cell constituents giving rise to superoxide radicals, peroxyl radicals and hydrogen peroxide, which can go onto induce membrane lipid peroxidation and hemolysis (22).

b. Mechanisms of Oxidation in the RBC

Polyunsaturated fatty acids are some of the most susceptible molecules to oxidative degradation present in nature (22). Membrane susceptibility to peroxidation is largely influenced by the amount of polyunsaturated fatty acids

present and their degree of unsaturation. Erythrocytes have an extremely heterogeneous composition of lipids in their membranes (Table 5) (25). During the lifespan of the RBC they undergo many structural and dimensional changes. Younger RBC contain appreciably more lipids than older cells, which seem to lose cholesterol and phospholipid in the aging process (23). The erythrocyte, rich in polyunsaturated fatty acids is a readily available target for oxidation by free radicals.

In lipid peroxidation, a primary reactive free radical, (·OH), like those in equations 5 and 6, interacts with a PUFA in the RBC membrane to initiate a complex series of reactions that result in a variety of degredation products that can result in cell lysis (25). PUFA in the erythrocyte membrane appear to be particularly sensitive to oxidative damage due to lowered bond dissociation energy of their allylic hydrogens (22). Initiation of peroxidation usually occurs by the attack of a free radical that is capable of abstracting a hydrogen from the RBC methlyenic carbon, for example, the hydroxy radical (·OH) or the protonated form of the superoxide radical, perhydroxyl radical (HO_2 ·) (22,26) :

$$LH + \text{Initiator} \text{ ---- } > L\cdot + H_2O_2 \qquad \text{(eqn 9)}$$

c. Antioxidant defenses of the RBC

Since the mature mammalian erythrocyte is a non-nucleated cell it cannot synthesize protein, therefore it is more difficult for the cell to replace damaged components. Consequently a strong antioxidant defense system is mandatory for the RBC to survive in its aerobic environment (22. Hemoglobin is usually protected from oxidation by two processes: the reducing power of the RBC metabolism and the stable quartenary structure of the hemoglobin molecule (22).

The RBC antioxidant defense systems can be found in two separate domains of the cell; one group is found in the cytosol and the second is found in the membrane itself.

Superoxide dismutase is present in the RBC cytosol. The primary function of superoxide dismutase is to scavenge the superoxide radical

Table 5. Lipids of the Erythrocyte (27)

Lipid Type	μmole x 10^{-10} /cell
Cholesterol	3.2
Free fatty acids, glycerides, sterol esters	< 0.1
Phosphatidylcholine	1.2
Phosphatidylserine	0.6
Phosphatidylethanolamine	1.1
Phosphatidylinositol	0.03
Sphingomyelin	1.0
Lysolecithin	0.04
Phosphatidic acid	0.04
Glycosphingolipids	0.2

generated by the oxidation of hemoglobin to methemoglobin (22,22). The enzyme uses the superoxide radical as its substrate:

$$2 \cdot O_2^- + 2H \text{ ----} > H_2O_2 + O_2 \qquad \text{(eqn 10)}$$

The resulting hydrogen peroxide is eliminated by glutathione and glutathione peroxidase:

$$2\ H_2O_2 \text{ ----} > 2\ H_2O + O_2 \qquad \text{(eqn 11)}$$

A high GSH/GSSG ratio is maintained in normal cells through an enzymatic mechanism for reducing the GSSG back to GSH. This is accomplished by the glutathione reductase enzymes, which catalyses this reaction (20,21,26):

$$GSSG + NADPH + H^+ \text{ ----} > 2\ GSH + NADP^+ \qquad \text{(eqn 12)}$$

In addition to glutathione peroxidase, RBC also contain catalase, an enzyme that acts as a scavenger of hydrogen peroxide. The main reaction catalyzed by the enzyme in the following reaction:

$$2\ H_2O_2 \text{ ----} > 2\ H_2O + O_2 \qquad \text{(eqn 13)}$$

The physiological role of catalase is not clear. However, it has been demonstrated that catalase activity becomes important when the concentration of hydrogen peroxide is raised (22).

Vitamin E is the major RBC membrane-bound antioxidant defense. Vitamin E has been shown to scavenge both the oxygen radicals attacking from the outside of the RBC and suppress the oxidation of phospholipids induced by free radicals generated in the cytoplasm or lipid peroxyl radicals generated in the liposomal membrane itself (27). Vitamin E donates a hydrogen atom to peroxyl radicals converting them to hydroperoxides. The hydroperoxides are then decomposed to a non-toxic hydroxy compound by glutathione peroxidase (22). Thus, vitamin E plays a crucial role in protecting

the RBC membrane by interrupting the propagation of lipid peroxidation. A vitamin E radical is formed by the oxidation of the vitamin, however it is fairly stable because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure of the tocopherol molecule or the tocopheroxyl radical can be reduced back to vitamin E by ascorbate (29). Although tocopherol and ascorbate are sequestered and separated in their respective lipid and aqueous phases, a significant decrease in lipid peroxidation is observed when both vitamins are present (30). Vitamin C by itself has been shown to be an sufficient antioxidant in the aqueous phase, but is much less effective when radicals are generated in the membrane itself, perhaps because it cannot penetrate the lipid phase in order to interact with the peroxyl radical (30). Thus, the likely relationship between tocopherol and ascorbate that allows the combination of vitamins to be a more effective antioxidant defense, is that vitamin C reduces the tocopheroxyl radical back to alpha-tocopherol. This may be the mechanism whereby high concentrations of vitamin C can help protect against peroxidation (21). In addition, these antioxidants are renewable from dietary sources.

Compartmentalization in the RBC of various components is another important means of defense against oxidative stress (26). By keeping iron bound to transport or storage proteins cells prevent the conversion of H_2O_2 into the hydroxyl radical (26).

$$Fe^{+2} + H_2O_2 \text{ ---- } > \cdot OH + OH^- + Fe^{+3} \qquad \text{(eqn 14)}$$

Hence, a major determinant of the nature of the damage done by the excess generation of reactive species may be the availability and location of the metal ion catalysts.

3. Defect in the Hexose Monophosphate Shunt

Glucose is the most important source of energy utilized by the RBC. The RBC has no citric acid cycle and therefore is unable to further oxidize lactate or pyruvate formed during glycolysis (20). Inosine, fructose, mannose and galactose could be metabolized by the RBC for energy, but they are not

present in the plasma in sufficient concentration to be useful (20). Thus the RBC is dependent on the metabolism of glucose for most of its energy.

Figure 2 illustrates the metabolic pathways utilized by the RBC (20). Once glucose 6-phosphate is formed through the hexokinase reaction, it may traverse two different pathways; the anaerobic pathway or the direct oxidative pathway (the hexose monophosphate shunt). Under normal conditions, 90% of the glucose taken up by the RBC goes through the anaerobic pathway (20).

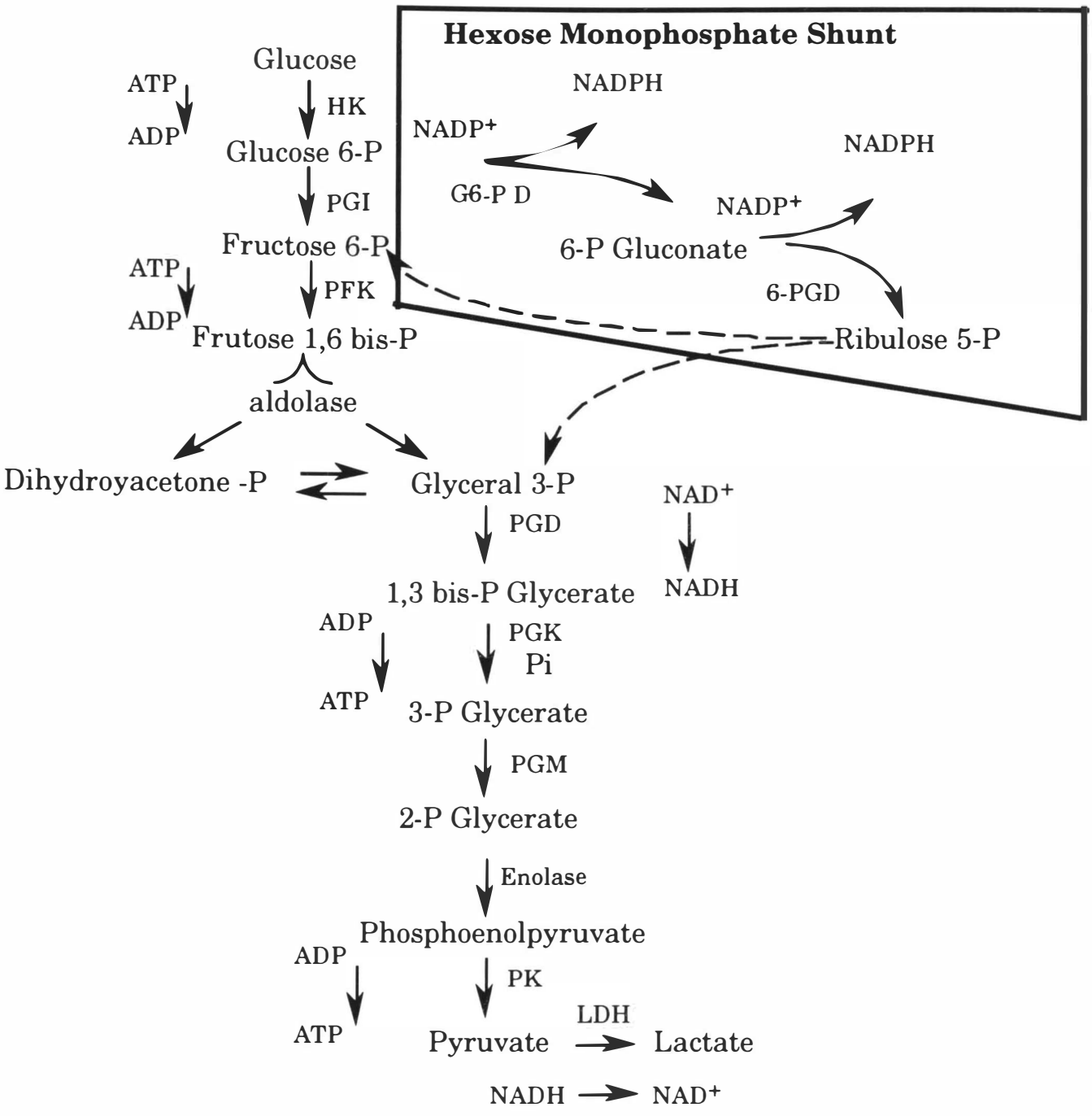
The main function of the HMP shunt in the RBC is to provide reducing equivalents for the reduction of oxidized glutathione (GSSG) to form reduced glutathione (GSH) (5, 20, 24). This is achieved through the formation of the reduced coenzyme NADPH, in the first two major steps of the pathway. GSSG is reduced by NADPH using glutathione reductase as shown in eqn 12 (5, 20, 22).

The HMP shunt through its production of NADPH functions to protect the RBC contents from oxidative denaturation through the regeneration of glutathione which is an essential component of the RBC antioxidant defense (6, 20, 24, 25). Any defect in this metabolic pathway could lead to increased RBC destruction associated with oxidation of cellular glutathione and both the oxidative denaturation of hemoglobin into Heinz bodies and the oxidation of the cell membrane (4). Yawata et al. (4) suggested that a circulating factor accumulates in uremic patients that is believed to inhibit the RBC transketolase activity. This enzyme is involved in the recycling of glucose through the HMP shunt (4, 20). Another purposed mechanism behind the failure of the HMP shunt is a blockade in the main glycolytic pathway which would result in the accumulation of intermediates which in turn prevents shunting of metabolites into the main pathway, ultimately resulting in deficient recycling of glucose through the HMP shunt (19, 30).The most likely glycolytic intermediates that have been shown to accumulate in uremic cells are fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate, the central entrance points from the HMP shunt to the glycolytic pathway (30). The likely site of blockade then appears to involve phosphoglyceromutase (30). Regardless of the site of the blockade of the HMP shunt, any defect in the shunt will severely impair the RBC capacity to defend against oxidative damage.

Figure 2 (18)

Metabolic Pathways Utilized by the Red Blood Cell.

Specifically glucose 6-phosphate can be metabolized by the anaerobic pathway (Glycolysis) or the hexose monophosphate shunt.


Hexose Monophosphate Shunt
Glucose
ATP
ADP
HK
Glucose 6-P
PGI
Fructose 6-P
ATP
ADP
PFK
Frutose 1,6 bis-P
aldolase
Dihydroyacetone -P
Glyceral 3-P
NADPH
NADP+
G6-P D
NADPH
NADP+
6-P Gluconate
6-PGD
Ribulose 5-P
NAD+
PGD
NADH
1,3 bis-P Glycerate
ADP
ATP
PGK
Pi
3-P Glycerate
PGM
2-P Glycerate
Enolase
Phosphoenolpyruvate
ADP
ATP
PK
LDH
Pyruvate
Lactate
NADH
NAD+

C. Summary

The biochemical and molecular changes associated with renal failure are widespread and can be quite severe. Edema, acidosis, hypertension, hyperlipidemia, osteomalacia and anemia are common complications of renal failure. Among these, intermediates that have been shown to accumulate in uremic cells are fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate, the central entrance points from the HMP shunt to the glycolytic pathway (30). Hemolytic anemia can be the most troublesome complication of uremia (2). The anemia associated with renal failure has been partially attributed to the shortened RBC survival. Erythrocytes are highly susceptible to peroxidation. Their membranes are rich in PUFA, they are continuously exposed to oxygen, and they contain a powerful transition metal catalyst, iron (22). Oxidation of the RBC is held in check by a powerful antioxidant defense system. However in renal failure, this system can be overwhelmed and result in greatly increased destruction of the RBC. In addition, defects in the HMP shunt commonly observed in renal failure, may shorten RBC survival (2).

Using the five-sixth nephrectomy model for uremia, we attempted to find out if indeed there was an increased destruction of the RBC in chronic uremia. The fragility of the RBC was determined by using a modification of the Parpat et al. method of increasing the hypotonic enviroment of the RBC (8, 9).

II
Materials and Methods
Red Blood Cell Stability in Uremic Rats Study

A. Animals

Female rats were purchased from Charles River Breeding Laboratories (Wilmington, MA.). Animals were housed and fed individually in order to better monitor their food intake. The animal room had a controlled temperature of 21-23 °C with a 14 hr light, 10 hr dark schedule.

The animals were separated into three experimental groups: nephrectomised (NX), sham (SH), and pairfed (PF) (11, 34, 35). The animals in the NX group received a five-sixth nephrectomy in a two stage procedure. The second group, PF, received sham surgeries in which the renal capsules were removed, to prevent damage to the adrenal gland, but no tissue was removed. The animals in the PF group were individually matched to a NX animal and were fed by weight only what the NX animal consumed. The third animal group, SH, received sham surgeries in which the renal capsule was removed, but no tissue was removed. There were no restrictions as to the quantity of chow the SH animals were allowed to consume (10, 34, 35).

Four separate experimental trials are reported here; Trial I, Trial II, Trial III and Trial IV. The samples obtained in Trial I and Trial III are divided into two categories; initial and final. The initial samples were collected 14 days after the five-sixth nephrectomy was completed, and the final samples were collected at the time of sacrifice. The samples obtained in Trial II consisted only of initial samples and those in Trial IV were only final samples. The animals in all four trials were Sprague-Dawley rats and were maintained as described above. The criteria for division of animals into three subgroups: NX, SH and PF was the same in every trial and followed the guidelines described in

the preceding paragraph.

B. Diet

The animals were maintained on standard rodent laboratory chow (Ralston-Purina Co., St. Louis, MO.) in powdered form. The NX animals were fed chow *ad libitum* and the PF group were fed by weight the same quantity of food that the NX group consumed. The SH group were fed chow *ad libitum*.

C. Age and Weight

The mean weight of the rats in Trial I on arrival in the laboratory was 182g and the range of ages were between 51-56 days. In Trial II the mean weight of the animals on arrival to the lab was 109g and the range of ages were between 36-39 days. In Trial III the mean weight of the animals on arrival to the laboratory was 175g and the range of ages were between 51-56 days. In Trial IV, the mean weight of the animals on arrival to the laboratory was 160g and the range ages were between 45-50 days.

On the day of sampling, approximately four weeks after arrival in the laboratory and approximately two weeks after surgical procedures were performed, the individual weights of each animal were taken and they are shown in Table 6 in the Results Section. The mean weight of the animals in Trial I was 268g with a SEM of +/- 5.19 for a sample size of 22, in Trial II the mean wieght was 220g with a SEM of +/- 3.28 for a sample size of 28, in Trial III the mean weight was 226g with a SEM of +/- 2.81 for a sample size of 26, and in Trial IV the mean weight was 220g with a SEM of +/- 3.83 for a sample size of 28. A significant increase in the animals weight was observed in all samples between the time of their arrival to the laboratory and the time of sampling.

D. Experiments

1. Surgical Procedure Used to Induce Uremia

The experimental model used in these trials, the five-sixth nephrectomy, has been shown to produce a state of mild to moderate uremia in Sprague Dawley rats by 14 days after the procedure (11, 35).

a. Five-sixth Nephrectomy Procedure

The five-sixth nephrectomy was performed by Drs. Krieg and Tokieda in the laboratory of Dr. Krieg, in a two stage procedure, on the NX subgroup of experimental animals. This same procedure was used in all four trials with the only exception being the type of anesthesia. In Trial I, II and IV all procedures were conducted using ether anesthesia, in Trial III, all procedures were conducted using brevital anesthesia.

In the NX group of animals, in all four trials, the left kidney was exposed through a postero-lateral incision (10, 34, 35). The renal capsule was removed to avoid injuring the adrenal gland, the upper and lower thirds of the kidney were then excised (11, 34, 35). Bleeding was controlled by applying manual pressure to the cut surfaces. The remaining third of the kidney was returned to the abdominal cavity, the wound sutured, and the animal was placed on a heating pad. One week later, the second stage of the nephrectomy was performed. The right kidney was exposed and also decapsulated to avoid damaging the adrenal gland. The entire right kidney was then removed (11, 34, 35).

The SH and PF experimental animal groups received the same surgeries as described above, however, no tissue was removed.

b. Catheterization

Four days before sampling, a polyvinyl catheter (Dural Plastics Ltd., Auburn, Australia) was inserted, under brevital anesthesia, through the right external jugular vein into the right atrium (11, 34, 35). To prevent removal of the catheter by the rat, the heparin filled catheter (50 IU/ml) was tunneled under the skin of the rat to exit dorsally (11, 34, 35). The catheter was plugged and the animals were returned to their cages.

c. Sampling

Blood samples used to determine RBC hemolysis were taken on two separate occasions. The initial sample was taken 14 days after the five-sixth nephrectomy was completed (11). The final sample was drawn as the animal was sacrificed.

On the day of blood sampling, a 50 cm polyvinyl extension was attached to the catheter by a piece of stainless steel tubing (11). The blood was withdrawn in heparin-treated syringes. The initial blood samples were replaced with saline (11).

2. Assay for Determination of RBC Hemolysis With Increasing Hypotonic NaCl Environment

The procedure used to determine RBC hemolysis was a modified form of that described by Parpart et al. (8, 9, 34, 35). The degree of resistance of RBC to a decrease in the salt content of their environment has been used experimentally as one measure of their viability (8). The RBC has the tendency to undergo increasing hemolysis as the concentrations of a buffered sodium chloride solution is decreased from 0.9% . On the basis of these findings, in this experiment the concentrations of a buffered sodium chloride solution were decreased incrementally from 0.8% to 0.1% and the 50% hemolysis point was determined by interpolation.

a. Reagents

Buffered NaCl Solutions (0.1% to 0.9% NaCl) were prepared using sodium chloride (Fisher), sodium phosphate (Fisher), sodium phosphate heptahydrate (Fisher) and deionized water. The final pH of the solution was 7.4.

b. Equipment

An IEC clinical centrifuge (International Equipment Co., Needam Hts., MA.) was used to separate the plasma from the RBC. Pasteur pipettes 15cm long (Fisher) were used to remove the plasma. The plasma was then stored in 2ml cryovials (Nalge), at -70 °C in a freezer for future HPLC vitamin E analysis. Disposable pipettes 5.0ml (Fisher) were used to add 4.0ml NaCl buffered solutions to 16 x 100mm disposable culture tubes (Fisher). Continuously adjustable digital microliter pipettes were used for the addition of the sample to the culture tubes. Incubation and shaking was accomplished through a water bath shaker (Eberbach Corporation Ann Arbor, Mi.). The absorbance was determined using a Spectronic 20D (Milton Ray Co.) and B&L cuvettes. Hematocrit, whenever possible was measured to determine the percent of RBC per volume.

c. Procedure

For the initial sample, blood was withdrawn from the existing catheter into a heparin-treated syringe and approximately 1ml was removed for RBC hemolysis determination. The removed volume was replaced with 0.9% NaCl buffered saline solution. For the final sample, blood was removed from the abdominal aorta into a heparin-treated syringe and again approximately 1ml was removed for RBC hemolysis determination.

The buffered saline was prepared using 9g NaCl, 2.7g $Na_2HPO_4 \cdot 7H_2O$,, 0.255g $NaH_2PO_4 \cdot H_2O$ diluted with H_2O to one liter (1% solution), pH 7.4. The blood sample was centrifuged for five minutes and the plasma was removed and stored for further analysis. The removed volume was replaced with 0.3ml of 0.9% buffered-NaCl, and mixed. 40µl of blood was then added to 4ml of 0.10, 0.25, 0.40, 0.45, 0.475, 0.50, 0.60, 0.80% buffered NaCl. The blood sample and buffered saline were then incubated at 30 °C for 0.5 hr with gentle shaking. The sample was centrifuged for 6 minutes and the remaining RBC pellet sedemented on the bottom of the culture tube, with the hemoglobin in solution. A sample of the supernatant fluid was transferred to a cuvette with a Pasteur pipette and the absorbancy was determined at 540nm (the wavelength for hemoglobin absorbance) against a water blank. The coefficient of variation for this procedure was found to be only 1.1%.

d. Calculations

1. Percent Hemolysis With Increasing Hypotonic NaCl Environment

In order to calculate the %Hemolysis, the optical density (OD) for the 0.8 (A) buffered %NaCl solution was subtracted from the OD of the remaining concentrations of buffered %NaCl solutions; 0.60 (B), 0.50 (C), 0.475(D), 0.45 (E), 0.40 (F), 0.25 (G) and 0.10(H). The values for A, B, C, D, E, F, G and H were then divided by the value obtained for H (which represents OD for 100% hemolysis) and multiplied by 100 to determine percent hemolysis. Graphs were drawn with the ordinate, %Hemolysis and the abscissa, %NaCl concentrations. The 50% hemolysis point was interpolated from the graphs. Mean 50% hemolysis graphs for NX, PF and SH initial samples for animals in Trial II are shown in figures 3 ,4 or 5 in the Results Section. Mean 50% hemolysis graphs for NX, PF and SH final samples for animals in Trial III are in figures 6, 7 or 8 in the Results Section.

2. Correlation Analysis

For all animals in all four trials, the correlation coefficient for the linear regression was determined from graphs with the ordinate, concentrations of buffered %NaCl and the abscissa, %Hemolysis (calculated as previously discussed in Percent Hemolysis Section) (36). As shown in the Result Section, figures 9, 10 and 11 are examples of correlation graphs for SH, PF and NX initial samples in Trial I. These graphs supplied the "r" or correlation coefficient value for each animal, the mean "r" value for each subgroup was calculated from the linear equation from the graph trial data, as well as a mean value for %NaCl concentration at 0% hemolysis and a mean value for %NaCl concentration at 50% hemolysis.

Table 8 combines data from the linear correlation graphs completed for all initial and final samples in each trial with data calculated on these same samples from the %Hemolysis versus the %NaCl concentration graphs. The first column of table 8, (%NaCl concentration for 0% hemolysis, linear

correlation graph), is the %NaCl concentration at zero percent hemolysis as calculated from the linear correlation graphs for all samples, examples figures 9, 10, 11. The second column, (%NaCl for 50% hemolysis, linear correlation graph), is the %NaCl concentration at 50% RBC hemolysis as calculated from the linear correlation graphs, example figures, 9,10, and 11. Finally, the third column, (%NaCl for 50% hemolysis, graphical interpolation), represents the concentration of %NaCl for 50% hemolysis as calculated from the sigmoidal graphs done on each animal with the abscissa of %Hemolysis and ordinate %NaCl, example figures 3-8. All analyses were performed using Statstix and Cricket Graph software on a Macintosh LC II.

3. Red Blood Cell Hemolysis Peroxide Test

The erythrocyte hemolysis peroxide test (HPT) due to its reliability and ease with which it can be repeated has proven to be another excellent method by which to determine hemolysis in the RBC (36). The procedure used was preformed by a modification of the method of Lubin et al. (36).

a. Experimental Subjects

The experimental subjects for the HPT consisted of two separate groups. Group one contained five adult female human subjects and Group two used female Sprague-Dawley rats. The rats were separated into three experimental groups; NX, SH and PF. All rat blood samples were from Trial IV of the RBC Stability in Uremic Rats Study and consisted only of final samples i.e. collected at time of sacrifice.

b. Reagents

Buffered NaCl solution of 0.9% was prepared using sodium chloride (Fisher), sodium phosphate heptahydrate (Fisher) sodium phosphate (Fisher), and deionized water. A 2% H_2O_2 solution was prepared by diluting 3ml of 30% H_2O_2 with 0.9% buffered NaCl solution to a total volume of 50ml.

c. Equipment

The samples were placed in 16 X 100mm disposable culture tubes (Fisher) and an IEC clinical centrifuge (International Equipment Co., Needam Hts., MA.) was used to separate the plasma from the RBC. Pasteur pipettes 15cm long (Fisher) were used to remove the plasma from the samples and the plasma was then stored in 2ml cryovials (Nalge), at -70°C in a freezer for further analysis. Five volumes of 0.9% buffered NaCl solution were added using a 5.0 x 1/10ml disposable pipettes (Fisher). The samples were centrifuged and supernatant fluid was removed by Pasteur pipette. The RBC were washed three times in this manner.

A continuously (Ranin) adjustable microliter pipette was used to add the suspended RBC to 0.9% phosphate buffered saline, this was done in triplicate for each sample. In two 16 x 100mm disposable culture tubes (Fisher) 0.25ml of the RBC suspension was then added to 2% H_2O_2 solution using an adjustable pipette. The RBC suspension was added to 0.9% phosphate buffered saline to determine any initial RBC hemolysis. The samples were incubated and gently shaken in a water bath shaker (Eberbach Corporation Ann Arbor, Mi.).

Distilled water was added to the solution in one 100mm disposable culture tube and 0.9% phosphate buffered saline was added to the other two culture tubes. Samples were mixed using a Vortex, and were centrifuged with an IEC clinical centrifuge (International Equipment Co., Needam Hts., MA.). Absorbance was determined using a Spectronic 20D (Milton Ray Co.) and B&L cuvettes. Hematocrit was measured to determine the percent of RBC per volume.

d. Procedure

Blood samples from both experimental groups were collected in heparinized tubes, were centrifuged and plasma was removed. The RBC were then washed three times with 5 volumes of 0.9% saline solution. Following the third wash, 0.4ml of packed RBC was added to 9.6ml 0.9% phosphate buffered saline at pH 7.4, creating a 4% suspension of RBC. In two separate culture

tubes, tube #1 and tube #2, 0.25ml of the 4% RBC suspension was added to 0.25ml of 2% H_2O_2. In a third tube, tube #3, 0.25ml of the 4% RBC suspension was added to 0.25ml distilled water.

The samples were incubated for 3hr at 37°C with gentle shaking. After incubation, 4.5ml of 0.9% phosphate buffered saline was added to tube #1 to determine hemolysis resulting from H_2O_2. In tube #2, 4.5ml distilled water was added to determine 100% hemolysis. Finally, in tube #3, 4.5ml 0.9% phosphate buffered saline was added to determine how much hemolysis existed before the addition of H_2O_2.

All samples were mixed and centrifuged for six minutes. The RBC pellet accumulated at the bottom of the culture tube with the hemoglobin in solution. A sample of the supernatant fluid was transferred to a cuvette with a Pasteur pipette and the absorbance was determined at 540nm.

e. Calculations

1. Percent Hemolysis for HPT

The 100% hemolysis value was obtained from the absorbance of tube #2, the tube with H_2O_2 added before incubation and distilled water added after incubation. A value for the hemolysis due to the H_2O_2 was determined by the absorbance of tube #1, which had H_2O_2 added before incubation and 0.9% phosphate buffered NaCl added after incubation. To calculate the percent hemolysis of the RBC in the HPT, the value for hemolysis due to H_2O_2 (tube #1) was divided by the 100 % hemolysis value (tube #2) and then multiplied by 100. A mean % Hemolysis was determined for all rat and human samples for the HPT and are shown in Table 10.

2. Percent Hemolysis With Increasing Hypotonic NaCl Environment

Percent hemolysis was determined for both the human and rat samples using the assay for determination of RBC hemolysis with decreasing hypotonic NaCl environment and the appropriate calculations as described above. A

graph was then drawn with the ordinate % Hemolysis and the abscissa %NaCl. The 50% hemolysis value was interpolated from the graph. Figures 12 and 13 represent examples of 50% hemolysis graphs for a human sample and a rat sample. Table 9 presents mean %NaCl concentrations for 50% RBC hemolysis for all samples in the HPT. All calculations were performed using Cricket Graph software.

3. Correlation Analysis

The %NaCl for 50% hemolysis versus %Hemolysis as calculated in the HPT for each human sample was correlated on a linear correlation graph, shown in figure 14, with the ordinate 50% hemolysis and abscissa $\%H_2O_2$ (37). The %NaCl for 50% hemolysis versus %Hemolysis as calculated in the HPT for each animal, NX, PF, and SH, was graphed on a linear correlation graph, as shown in figure 15, with the ordinate 50% hemolysis and the abscissa $\%H_2O_2$. All analyses were performed using Cricket Graph and Statstix software.

4. Assay for Vitamin E Quantitation- HPLC method

Plasma samples were obtained from Sprague-Dawley experimental rats and from the five human subject samples studied in the HPT. The blood samples were centrifuged for 5 min at 1000 x g and the plasma removed and placed in 2ml cryostat tubes. Plasma samples were stored at -70°C freezer until used for analysis.

a. Extraction Procedure (38)

The samples were thawed for analysis and a 250µl aliquot was added to a 17 x 125mm screw cap tube. 750µl of 1% sodium ascorbate and 2.0ml absolute ethanol was added to the sample. The samples were mixed for 30 sec and allowed to remain shielded from the light for 5 min. To extract the vitamin E, 5ml n-hexane was added. As an internal standard, 20µl of ethyl-ß-apo-8-carotenoid was added. The tubes were then covered with foil, capped, mixed for 1.5 min and centrifuged for 10 min at 1000 x g. A 4.0ml aliquot of the hexane

layer was removed from the samples and placed in conical bottom 17 x 118mm tubes. In a 40°C water-bath using a stream of nitrogen, the hexane layer was evaporated. The oily residue was dissolved in 1.0ml of the mobile phase and 200µl was injected onto a high performance liquid chromatography (HPLC) column (38).

b. HPLC Procedure

HPLC was performed at room temperature with a Waters model 510 pump and Waters model 490 programmable multiwavelength detector at 292 nm at a flow rate of 1.5 ml/min. Data was collected using a Shimadzu CR 501 integrator. Separation was performed on a Supercosil LC-18 25 cm column with a Waters Guard-Pak u-Bondapak precolumn module. The standard mobile phase was 80:20 methanol:toluene, which had been filtered and degassed. All solvents were HPLC grade and were from Fisher Scientific. Vitamin E standards were obtained from Sigma Chemical Co., St. Louis, MO. All of the standards were initially dissolved in 50:50 methanol:toluene and were diluted to appropriate concentrations with the 80:20 mobile phase. Concentrations of the standards were determined spectrophotometrically using an extinction coefficient ($E_{1\%}$) of 2494 at 292 nm for AT. Variable amounts of the standards (4-30 ng) were injected into the column and integration was performed on the resultant peak areas. Standard curves (area versus amount injected) were prepared for AT and the concentration of the sample components was calculated from these standard curves in µg/dL . The spectra scans for the samples showed that the AT spectra were virtually identical between the standards and the plasma samples. The retention times of the sample peak agreed with those of the standards. An AT standard was also determined daily to adjust the retention time within the programmed analysis ($\pm$ 5 seconds).

III
Results

A. Red Blood Cell stability in Uremic Rats Study

The female Sprague-Dawley rats used in this experiment were divided into three separate experimental groups. As shown in Table 2, animals that received a five-sixth nephrectomy and had no dietary restrictions were placed in the NX group. Animals that received surgery, but no tissue was removed and had no dietary restrictions were placed in the SH group. Finally, animals that received surgery, but no tissue was removed and had dietary restrictions were placed in the PF group. Samples were collected in four trials. Initial samples, taken from the animal two weeks following surgery and final samples, taken as the animal was sacrificed, were obtained in Trial I and Trial III. Only initial samples were used in Trial II and in Trial IV, only final samples were analyzed. The animals were weighed at the time of sampling and individual weights of each animal and the mean wieght of the animals in each trial are given in Table 6, along with the mean values for each group.

1. Determination of RBC Hemolysis With Increasing Hypotonic NaCl Enviroment

For each sample in every trial, the percent hemolysis was calculated and graphed. In order to calculate the %Hemolysis, the optical density (OD) for the 0.8 (A) buffered NaCl solution was subtracted from the OD of the remaining concentrations of buffered %NaCl solutions; 0.60 (B), 0.50 (C), 0.475(D), 0.45 (E), 0.40 (F), 0.25 (G) and 0.10(H). The values for A, B, C, D, E, F, G and H were then divided by the value obtained for H (which represents OD for 100% hemolysis) and multiplied by 100 to determine percent hemolysis.

Table 6. Body Weight of Animals at Time of Sampling [a]

Trial I	**Trial II**	**Trial III**	**Trial IV**
235	207	226	208
258	202	230	238
218	221	240	203
268	212	223	204
244	199	218	222
268	215	214	180
274	201	197	216
280	207	231	231
256	186	234	224
289	207	243	233
245	227	214	206
231	205	228	215
270	217	232	221
254	250	242	238
278	230	203	204
271	213	231	259
280	212	212	222
296	234	239	227
295	259	222	208
286	234	202	182
283	221	217	235
323	216	237	199
	237	232	257
	240	218	237
	251	255	213
	208	246	197
	228		256
	228		217
268 (5.19, 22)[b]	220 (3.28, 28)[c]	226 (2.81, 26)[d]	220 (3.83, 28)[e]

a Values for body weight of experimental animals is give in grams

b Mean (+/-SEM, 22)

c Mean (+/-SEM, 28)

d Mean (+/-SEM, 26)

e Mean (+/- SEM, 28)

For all samples, graphs were drawn with the ordinate, %Hemolysis as calculated above and the abscissa %NaCl concentrations. The 50% hemolysis point was interpolated from the graph. Figure 3 is an example of a NX initial sample from Trial III, and the %NaCl concentration at 50% hemolysis of the RBC was determined to be 0.420. Figure 4 is an example of the graph of %Hemolysis versus %NaCl concentration for a PF initial sample from Trial II, %NaCl concentration at 50% hemolysis of the RBC was found to be 0.410. Figure 5 is an example of the graph obtained for a SH initial sample from Trial II, the %NaCl concentration at 50% hemolysis of the RBC is 0.420. Similar graphs were drawn with the ordinate, %Hemolysis as calculated above and the abscissa, %NaCl concentrations for the final samples of all animals in every trial. The 50% hemolysis point was interpolated from the graph. Figure 6 is an example of a graph for NX final sample from Trial III, the %NaCl concentration at 50% hemolysis of the RBC is 0.430. Figure 7 is a graph for a PF final sample from Trial III, the %NaCl concentration at %50 hemolysis of the RBC is 0.450. Figure 8 is a graph of a SH final sample from Trial III, the %NaCl concentration at 50% hemolysis for the RBC is 0.430.

The 50% hemolysis points obtained from the %NaCl concentration versus %Hemolysis graphs were averaged using Statstix software within each animal group and Trial. These results were then grouped as to initial or final sample and Table 7 shows the mean %NaCl concentration from these graphs at 50% RBC hemolysis for all four trials.

Linear correlation graphs were constructed for every sample in each Trial. Buffered %NaCl concentrations versus %Hemolysis of the RBC were correlated. The linear regression equation obtained from the correlation graphs provides the linear coefficient "r" and an equation that allows for the calculation of %NaCl concentration at 0% hemolysis. Figure 9 is an example of the linear correlation graph obtained for SH initial samples in Trial I, the correlation coefficient was found to be 0.931, which is significant at the $p<0.05$. Figure 10 is a linear correlation graph of PF initial samples in Trial I and the correlation coefficient was found to be 0.931, which is significant at $p<0.05$. Figure 11 is an example of a linear correlation graph for NX initial samples in Trial I and the correlation coefficient was found to be 0.730, which is significant at $p<0.05$. Linear correlation graphs such as these were done for every sample.

Figure 3

%Hemolysis versus the %NaCl concentration. Mean data for NX initial samples Trial II.
%NaCl concentration at 50% hemolysis is extrapolated from the graph and is 0.420.
The error bars are the SEM.

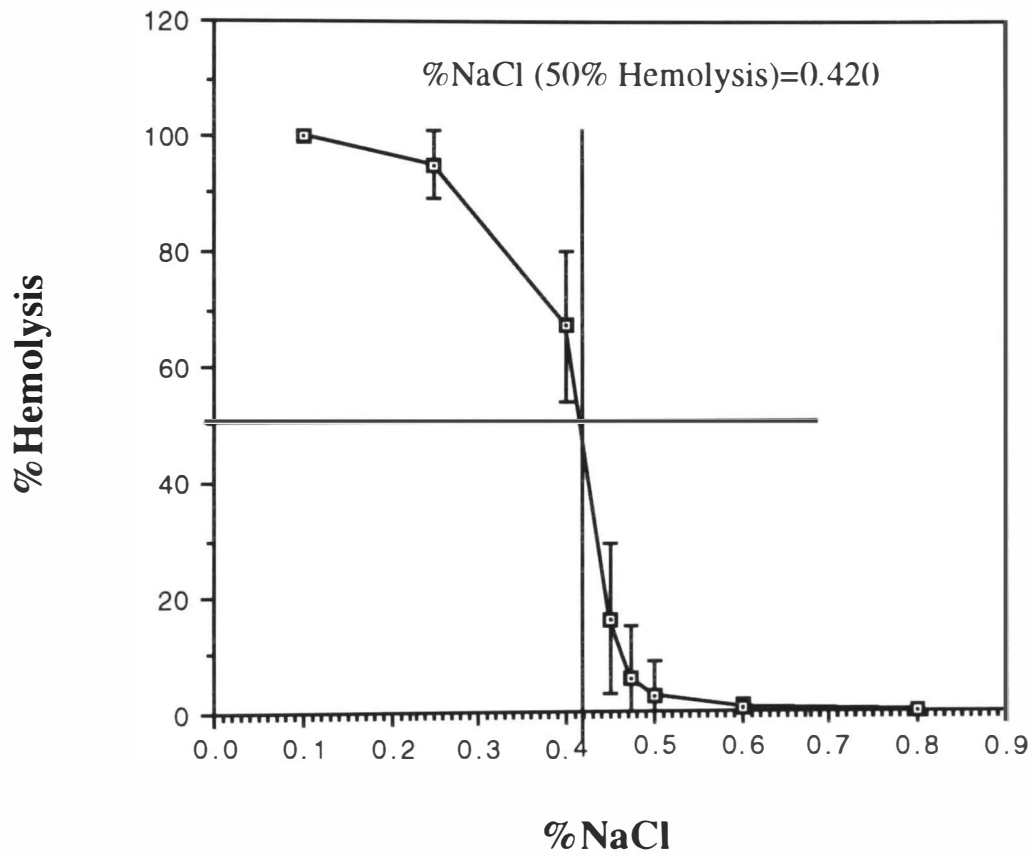

%NaCl (50% Hemolysis)=0.420
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
% Hemolysis
%NaCl

Figure 4

Percent hemolysis versus the %NaCl concentration. Mean data for PF initial samples Trial II.
%NaCl concentration at 50% hemolysis is extrapolated from the graph and is 0.410.
The error bars are the SEM.

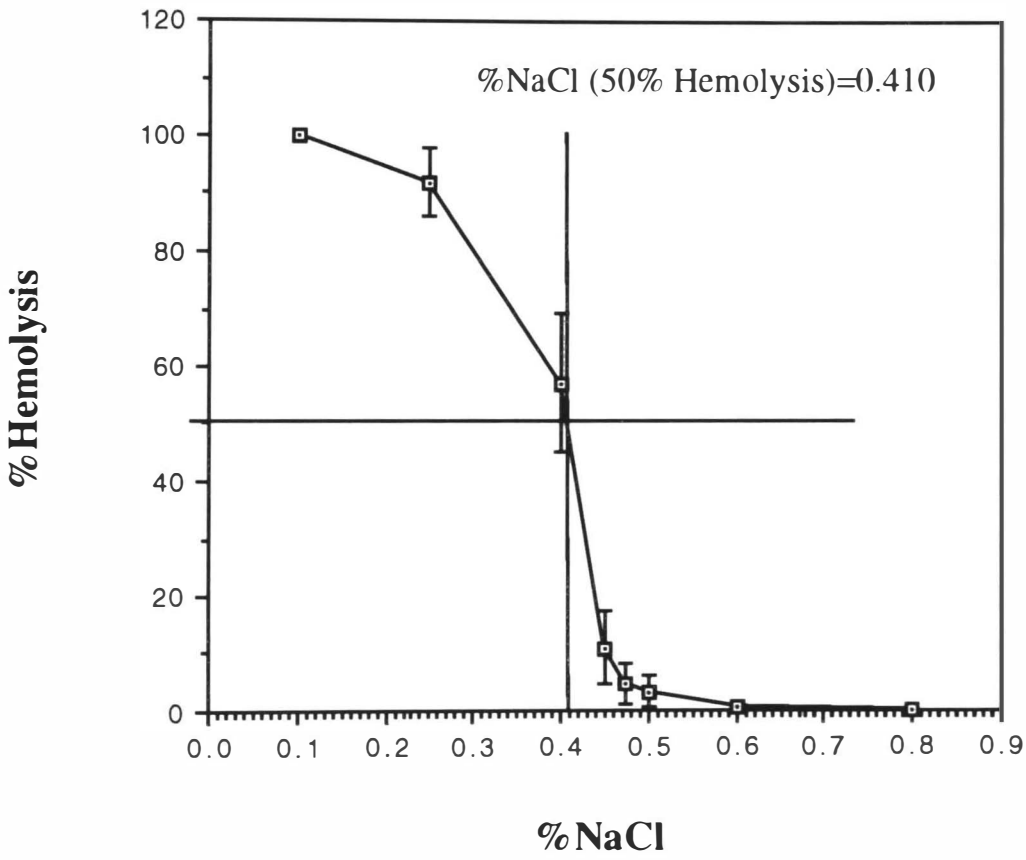

%NaCl (50% Hemolysis)=0.410
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
%Hemolysis
%NaCl

Figure 5

Percent hemolysis versus the %NaCl concentration. Mean data for SH initial samples Trial II.
%NaCl concentration at 50% hemolysis is extrapolated from the graph and is 0.420.
The error bars are the SEM.

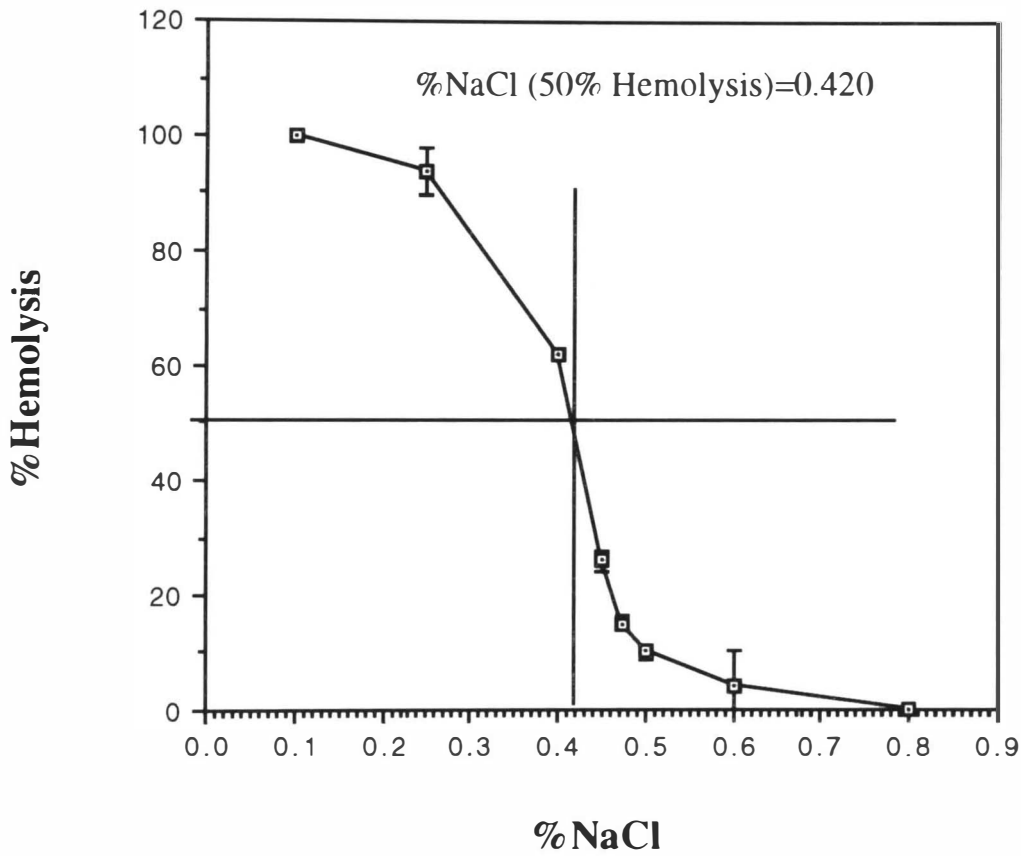

%NaCl (50% Hemolysis)=0.420
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
%Hemolysis
%NaCl

Figure 6

Percent hemolysis versus the %NaCl concentration. Mean data for NX final samples Trial III. %NaCl concentration at 50% hemolysis is extrapolated from the graph and is equal to 0.430. The error bars are the SEM.

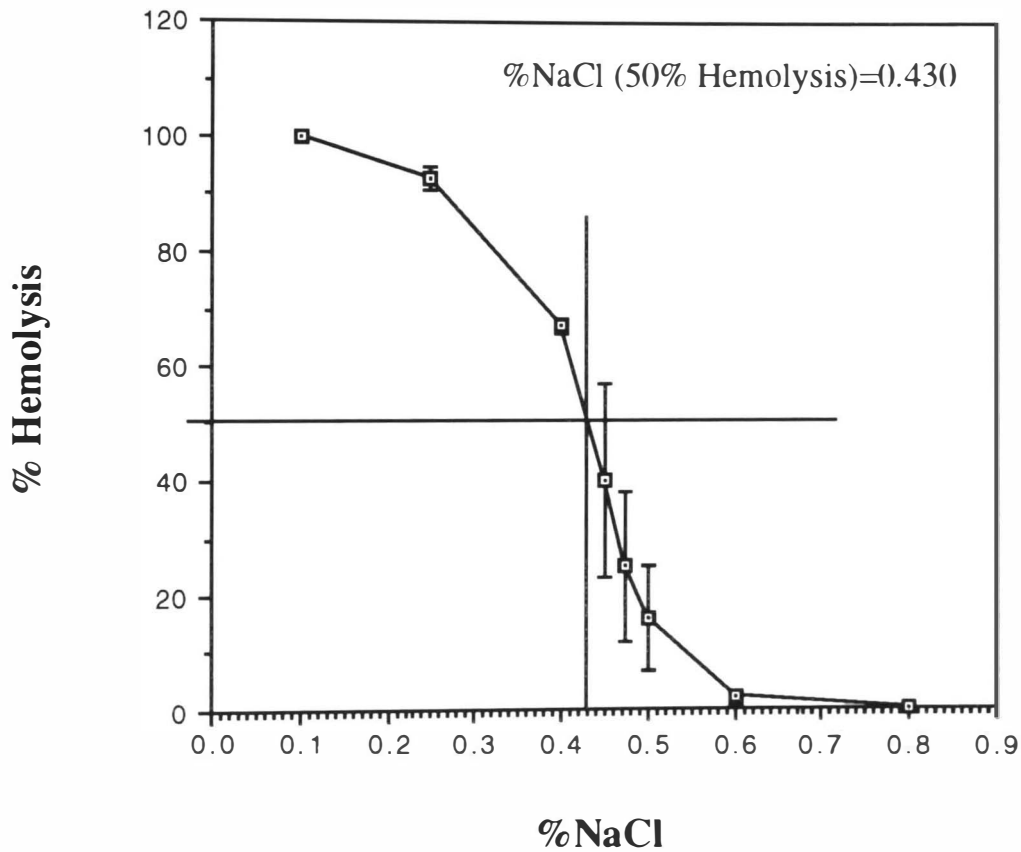

%NaCl (50% Hemolysis)=0.430
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
% Hemolysis
%NaCl

Figure 7

Percent hemolysis versus the %NaCl concentration. Mean data for PF final samples Trial III.
%NaCl concentration at 50% hemolysis is extrapolated from the graph and is 0.450.
The error bars are the SEM.

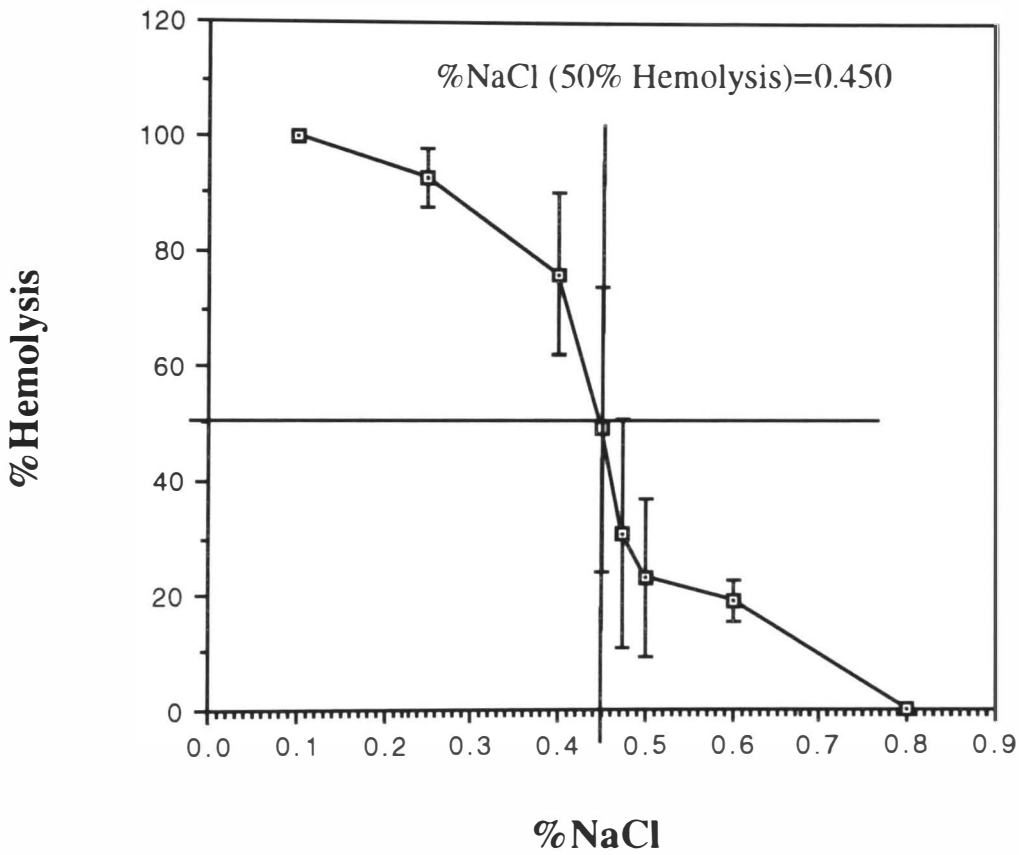

%NaCl (50% Hemolysis)=0.450
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
%Hemolysis
%NaCl

Figure 8

Percent hemolysis versus the %NaCl concentration. Mean data for SH final samples Trial III.
%NaCl concentration at 50% hemolysis is extrapolated from the graph and is found to be equal to 0.430.
The error bars are the SEM.

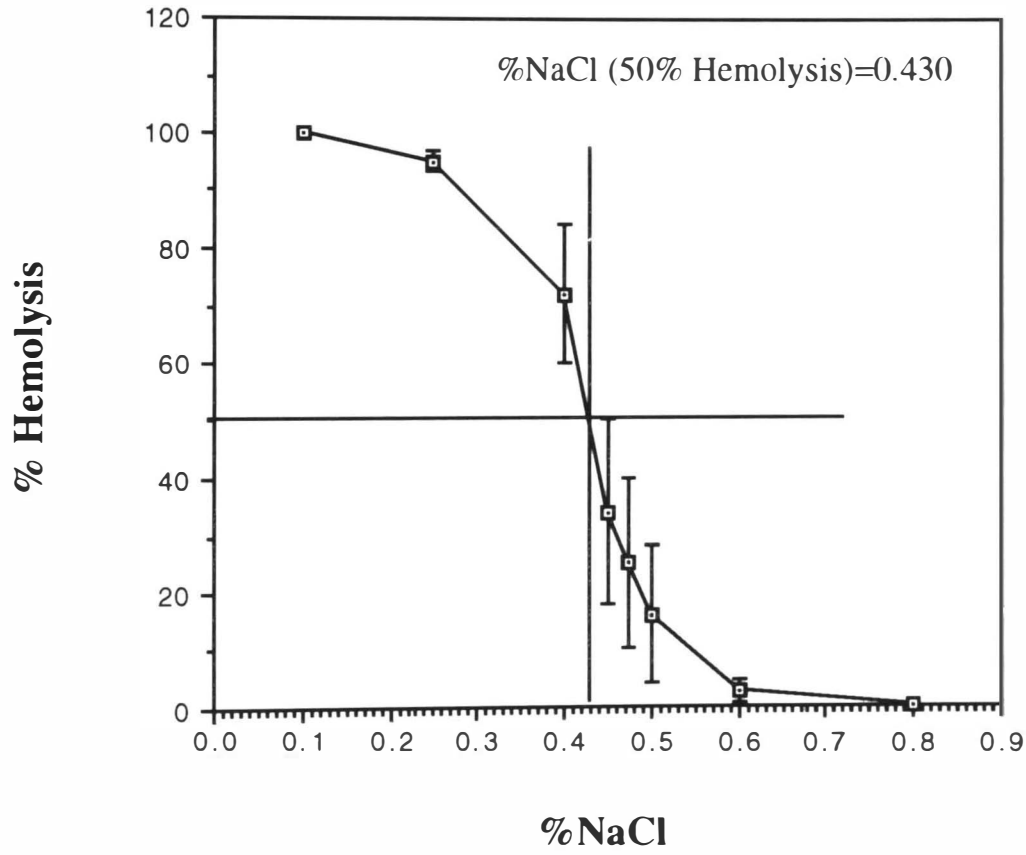

%NaCl (50% Hemolysis)=0.430
% Hemolysis
%NaCl
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Table 7. Mean %NaCl Concentration for 50% Red Blood Cell Hemolysis for Trials I, II, III and IV as Determined by Interpolation of the %NaCl Concentration Versus %Hemolysis Graphs

Protocol	Initial	Final[a]
NX	0.414 (.001, 16)	0.417 (.005, 25)
PF	0.412 (.004, 28)	0.422 (.006, 35)
SH	0.416 (.005, 19)	0.420 (.004, 29)

[a] Mean (+/- SEM, N), not significantly significant from each other at $p>0.05$.

Figure 9

%NaCl concentration versus %Hemolysis of RBC for SH initial animals in Trial I.

The linear regression equation is:

$$y = 0.181 \times 10^{-3}X + 0.582 \quad R^2 = 0.867$$

The R value for the plot is significant for an N=8 showing a linear relationship for these parameters.

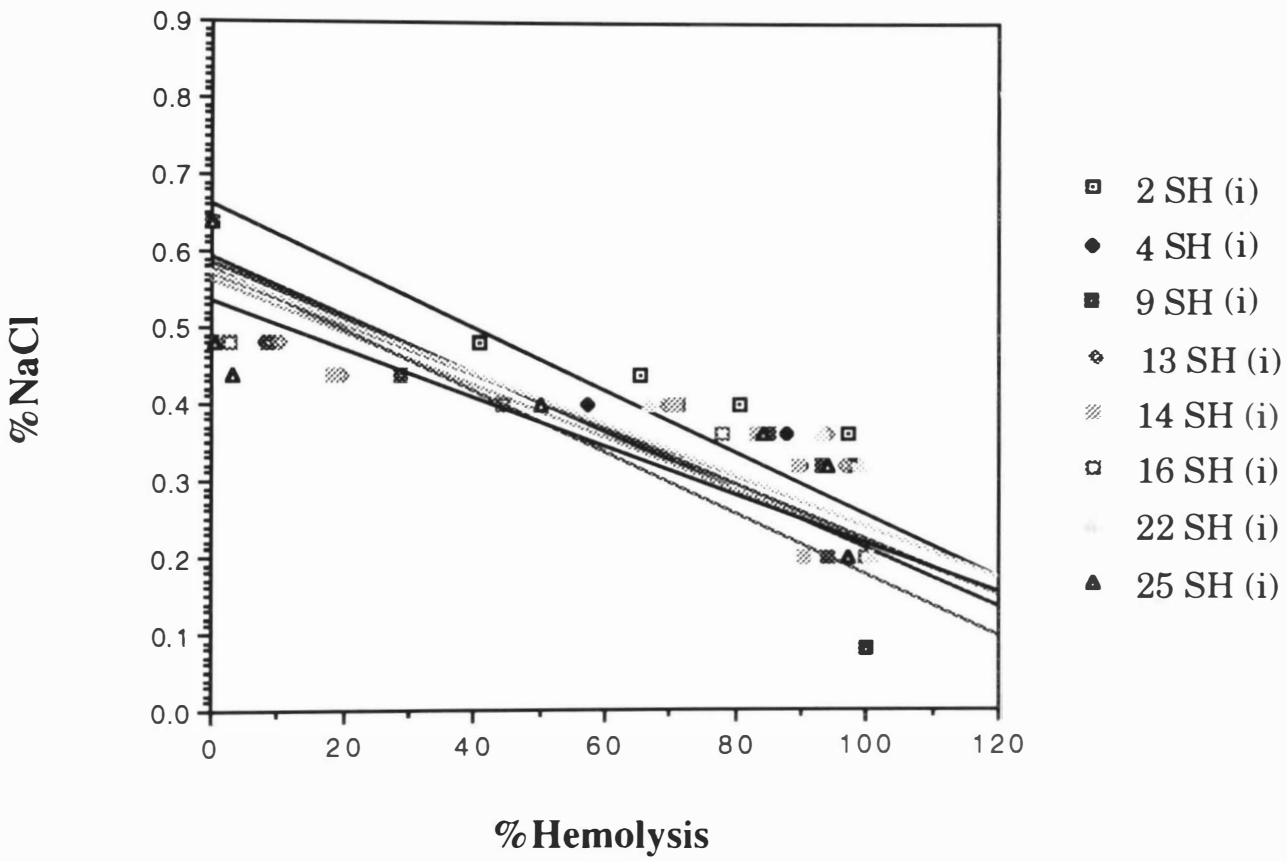

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
%NaCl
0
20
40
60
80
100
120
%Hemolysis
2 SH (i)
4 SH (i)
9 SH (i)
13 SH (i)
14 SH (i)
16 SH (i)
22 SH (i)
25 SH (i)

Figure 10

%NaCl concentration versus %Hemolysis of RBC for PF initial animals in Trial I.

The linear regression equation is:

$$y = 0.391 \times 10^{-3} X + 0.568 \quad R^2 = 0.867$$

The R value for the plot is significant for an N=7 showing a linear relationship for these parameters.

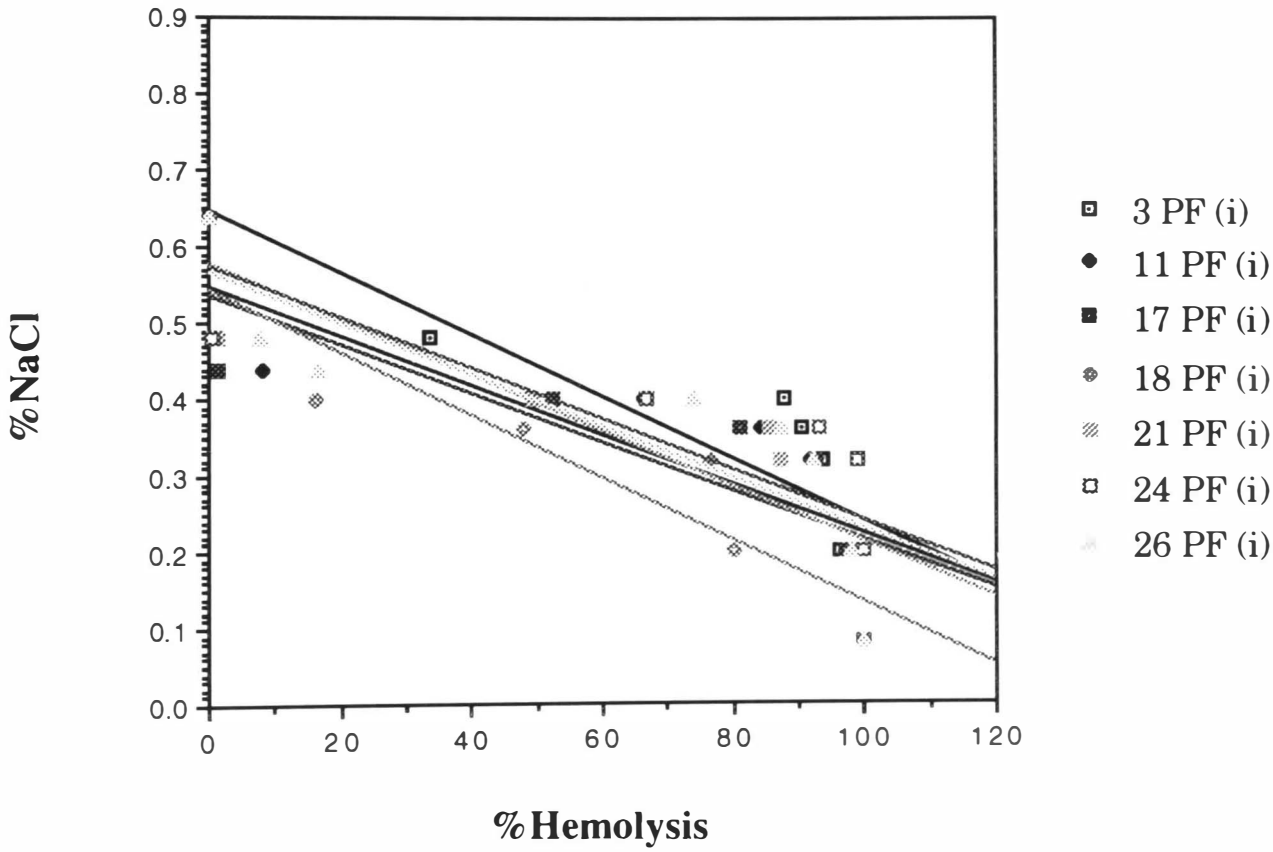

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
%NaCl
0
20
40
60
80
100
120
%Hemolysis
3 PF (i)
11 PF (i)
17 PF (i)
18 PF (i)
21 PF (i)
24 PF (i)
26 PF (i)

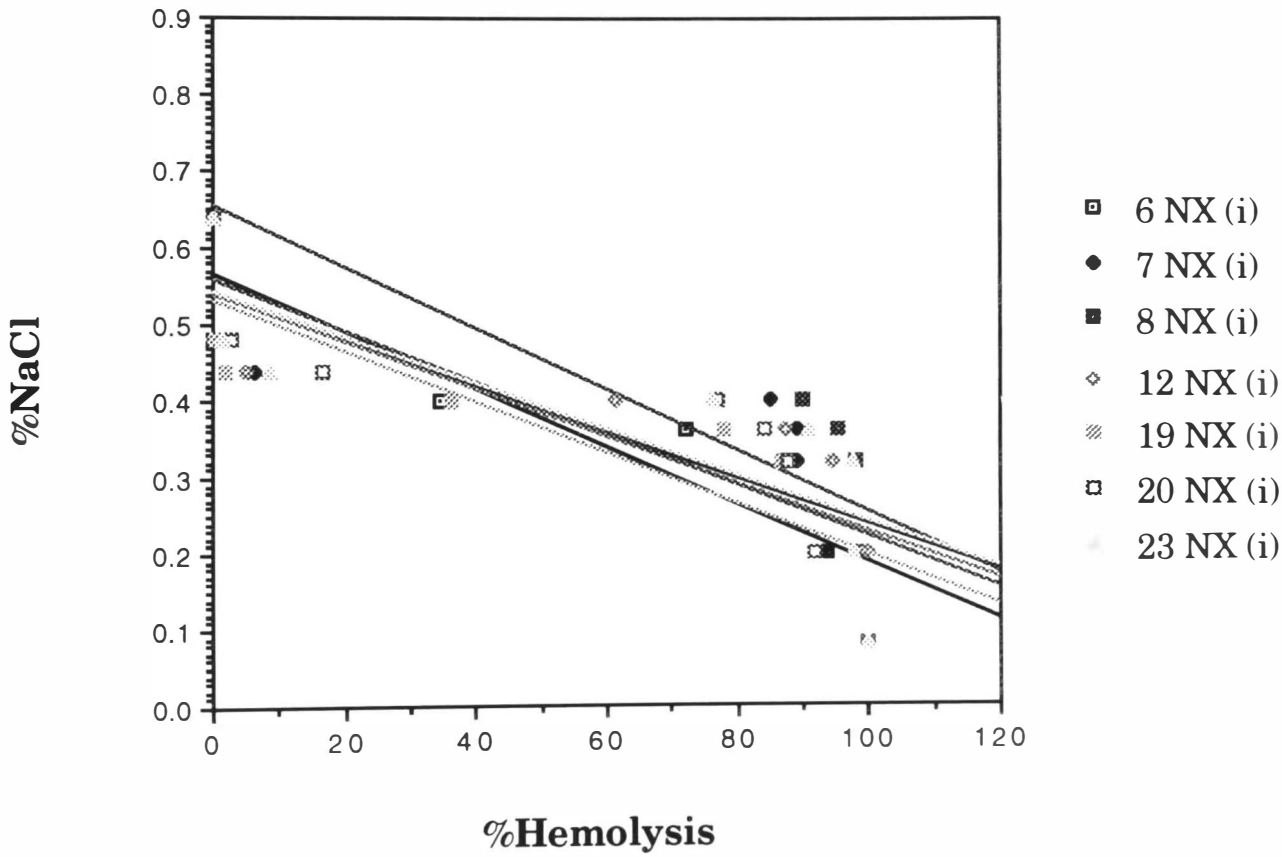
Figure 11

%NaCl concentration versus %Hemolysis of RBC for NX initial animals in Trial I.

The linear regression equation is:

$$y = 0.370 \times 10^{-3} X + 0.533 \quad R^2 = 0.533$$

The R value for the plot is significant for an N=7 showing a linear relationship for these parameters.


%NaCl
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
120
%Hemolysis
6 NX (i)
7 NX (i)
8 NX (i)
12 NX (i)
19 NX (i)
20 NX (i)
23 NX (i)

To calculate the %NaCl concentration for 0% and 50% hemolysis, the equation $y= b + m^{e-3x}$ was used. Column one of Table 8 represents the concentraion of buffered %NaCl when the x axis is equal to zero, i.e. 0% hemolysis of RBC for all animal samples. Column two of Table 8 lists the concentrations of buffered %NaCl when the x axis is equal to fifty, i.e. 50% hemolysis of RBC for all animal samples. Using the equation $y= b + m^{e-3x}$, the x intercept was placed at zero or 50 as described above, and b and m were obtained from the linear correlation graphs (example figures 9-11). Column three of Table 8 lists the concentration of buffered %NaCl for 50% hemolysis as calculated from the %Hemolysis versus %NaCl concentration graphs for all animal samples (example figures 3-8).

The %NaCl concentration for 0% hemolysis as determined from the linear correlation graph indicates a significant difference between; the NX (i) and NX (f) samples in Trial I, the NX (i) and SH (i) samples in Trial II and the NX (i) and NX (f) and SH (i) and SH (f) samples in Trial III. The %NaCl for 50% hemolysis as obtained from the linear correlation graphs indicated a significant difference between; the NX (i) and NX (f) samples in Trial I and the NX (i) and NX (f) samples in Trial III. The %NaCl concentraion for 50% hemolysis as determined from the %Hemolysis versus %NaCl graphs indicated a significant difference between; the NX (i) and NX (f) samples in Trial III and the SH (i) and SH (f) in Trial III.

B. Red Blood Cell Hemolysis Peroxide Test

The HPT was performed on two separate groups of samples. The experimental animals from Trial IV of the RBC Stability in Uremic Rats Study were used, and therefore consisted only of samples taken at the time of sacrifice. The second group of blood samples were from five adult female humans; A, B, F, G, H. The modified Parpart procedure (8, 9, 34, 35) as described in Methods was also performed on these same samples. Figure 12 is an example of the %NaCl concentration versus %Hemolysis for a human sample in the HPT. The %NaCl concentration at 50% hemolysis for human sample H was calculated from the graph and is 0.410. Figure 13 is an example of the %NaCl concentration versus %Hemolysis for a SH rat sample in the

Table 8.%NaCl for 0% Hemolysis and 50% Hemolysis as Described by Linear Correlation Graphs and %NaCl for 50% Hemolysis as Decribed by NaCl Graphs

Protocol	% NaCl for 0%[a,b] Hemolysis Linear Correlation	%NaCl for 50% Hemolysis Linear Correlation[a,c]	%NaCl for 50% Hemolysis Graphical Interpolation[a,d]
Trial I			
NX (i)	0.533 (0.016, 7)	0.370 (0.014, 7)	0.414 (0.011, 7)
NX (f)	0.616 (0.015, 7)	0.407 (0.006, 7)	0.423 (0.005, 7)
SH (i)	0.582 (0.013, 8)	0.410 (0.009, 8)	0.417 (0.007, 8)
SH (f)	0.603 (0.023, 8)	0.395 (0.015, 8)	0.430 (0.007, 8)
PF (i)	0.568 (0.014, 7)	0.391 (0.012, 7)	0.408 (0.010, 7)
PF (f)	0.561 (0.019, 7)	0.386 (0.007, 7)	0.405 (0.002, 7)
Trial II			
NX (i)	0.593 (0.009, 9)	0.387 (0.007, 9)	0.416 (0.002, 9)
SH (i)	0.631 (0.021, 8)	0.399 (0.009, 8)	0.422 (0.008, 8)
PF (i)	0.611 (0.022, 9)	0.397 (0.019, 9)	0.411 (0.006, 9)
Trial III			
NX (i)	0.586 (0.007, 3)	0.377 (0.002, 3)	0.411 (0.001, 3)
NX (f)	0.674 (0.040, 5)	0.439 (0.031, 5)	0.430 (0.002, 3)
SH (i)	0.610 (0.026, 3)	0.381 (0.018, 3)	0.397 (0.009, 4)
SH (f)	0.638 (0.038, 4)	0.432 (0.044, 4)	0.439 (0.002, 4)
PF (i)	0.640 (0.018, 12)	0.410 (0.011, 12)	0.436 (0.008, 12)
PF (f)	0.665 (0.015, 15)	0.433 (0.011, 15)	0.448 (0.008, 15)
Trial IV			
NX (f)	0.583 (0.005, 9)	0.379 (0.005, 9)	0.408 (0.007, 9)
SH (f)	0.586 (0.004, 9)	0.376 (0.004, 9)	0.397 (0.006,10)
PF (f)	0.584 (0.005, 10)	0.377 (0.006, 10)	0.407 (0.005, 9)

a Mean (+/- SEM, N)

b The concentration of buffered %NaCl when the x axis is equal to zero, i.e. 0% hemolysis of RBC, calculated from linear correlation graphs for all animal samples, example figures include, figures 9-11.

c The %NaCl concentration when the x axis is equal to 50, i.e. 50% hemolysis of RBC, calculated from linear correlation graphs for all animal samples, example figures include, 9-11.

d The %NaCl concentration at 50% hemolysis calculated from %Hemolysis versus %NaCl concentration graphs for all animal samples, example figures include 3-8.

Figure 12

%Hemolysis versus the %NaCl concentration
data for Human sample H in the HPT.
%NaCl concentration at 50% hemolysis is calculated
from the graph and is 0.410.

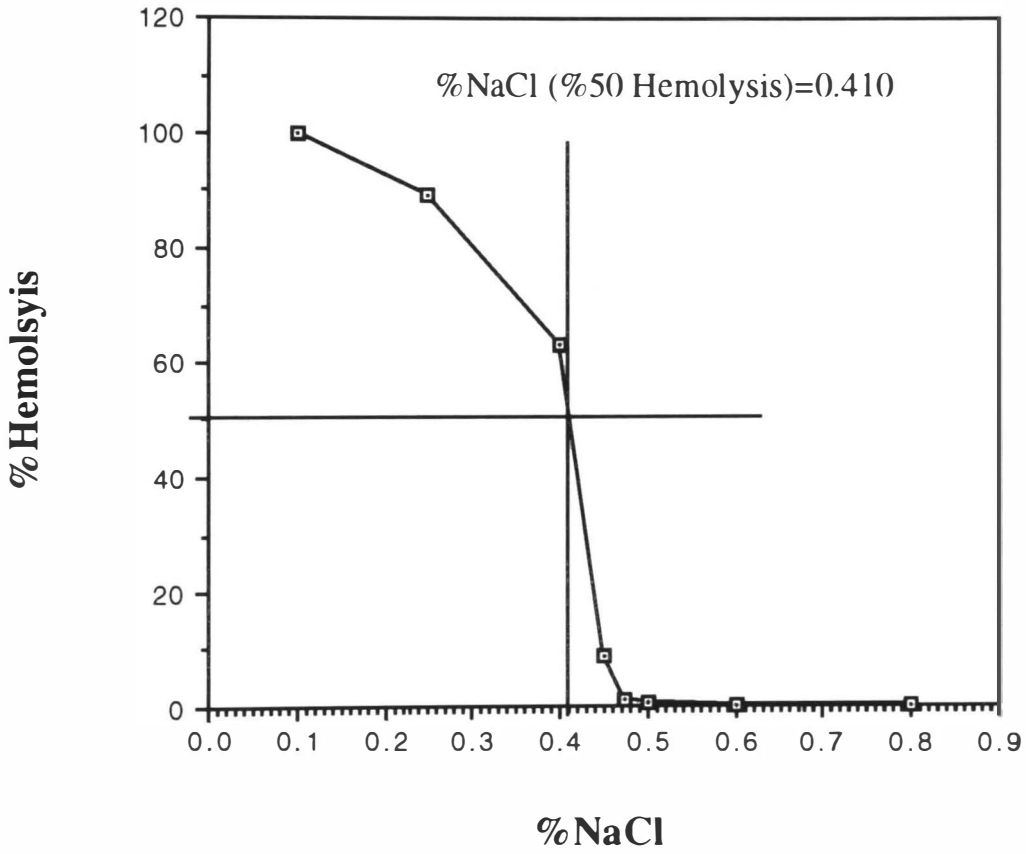
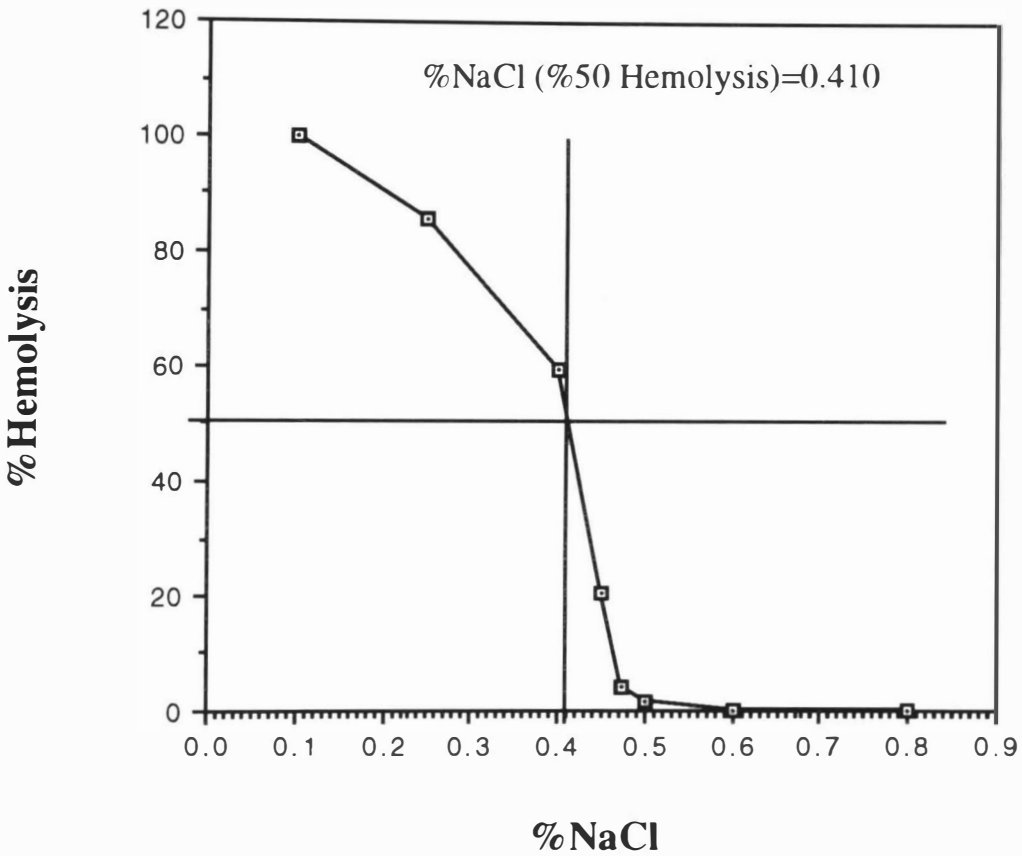

%NaCl (%50 Hemolysis)=0.410
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
%Hemolsyis
%NaCl

Figure 13

%Hemolysis versus %NaCl concentration
data for SH rat sample in the HPT.
%NaCl concentration at 50% hemolysis is calculated
from the graph and is 0.410.


%NaCl (%50 Hemolysis)=0.410
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
%Hemolysis
%NaCl

HPT. The %NaCl concentration at 50% hemolysis for the SH animal was calculated from the graph and is 0.410. Similar graphs were constructed for PF and NX animals.

The mean %NaCl concentration at 50% hemolysis for the PF animals was calculated from the graphs and is 0.397, the mean %NaCl concentration at 50% hemolysis for NX animals was calculated from the graphs and is 0.408 and the mean %NaCl concentration at 50% hemolysis for SH animals was calculated from the graphs and is 0.407. Table 9 lists the mean %NaCl concentrations for 50% hemolysis for all samples used in the HPT experiment. There is a significant difference between the PF samples and the NX samples as well as between the PF samples and the SH samples.

To calculate the percent hemolysis of the RBC in the HPT, the 100% hemolysis value was divided into the value for hemolysis due to the HPT and multiplied by 100. The 100% hemolysis value was obtained from the absorbance of tube #2, the tube with H_2O_2 added before and after incubation. A value for the hemolysis due to the HPT was determined by the absorbance of tube #1, which had H_2O_2 added before incubation and phosphate buffered 0.9% NaCl added after incubation. Table 10 gives the mean %Hemolysis for the NX, PF, SH and human samples used in this experiment. No significant difference was found between the rat samples.

Linear correlation graphs were drawn for both the rat and human samples. Figure 14 is a linear correlation graph of the %NaCl for 50% hemolysis versus the %Hemolysis as calculated from the HPT for all human samples. A linear regression equation, $y=3.529 \times 10^{-4}X + 0.406$, was obtained from the linear correlation graph and the correlation coefficient was determined to be 0.607, which is not significant at $p<0.05$ for a sample size of five. Figure 15 is a linear correlation graph of %NaCl for 50% hemolysis versus the %Hemolysis as calculated from the HPT for all rat samples. A linear regression equation, $y=0.441 \times 10^{-3}X + 0.479$, was obtained from the linear correlation graph and the correlation coefficient was determined to be 0.412, which is significant at $p<0.05$ for a sample size of 28.

Table 9. %NaCl for 50% Red Blood Cell Hemolyis for all Rat Samples used in the HPT Experiment

Protocol	Final [a]
NX	0.408 (0.007, 9)
PF	0.397 (0.001, 10)
SH	0.407 (0.003,9)
Human	0.399 (0.002, 5)

[a] Mean (+/- SEM, N), values obtained by procedure varying NaCl concentration.

Table 10. %Hemolysis for HPT Experiment

Protocol	% Hemolysis[a, b, c]
NX	11.0 (1.87, 9)
PF	10.1 (1.60, 10)
SH	11.6 (0.83, 9)
Human	37.6 (7.71, 5)

a Mean (+/- SEM, N)

b %Hemolysis=(Hemolysis of sample with H2O2 X 100)/100% Hemolysis sample

c No significant difference was found between the rat experimental groups

Figure 14

%NaCl for 50% hemolysis versus the %Hemolysis
as calculated from the HPT for all human samples.
The linear regression equaton is:

$$y = 3.529 \times 10^{-4}X + 0.406 \quad R^2 = 0.368$$

The R value for the plot was not significant for an N=5 showing no linear relationship for these parameters.

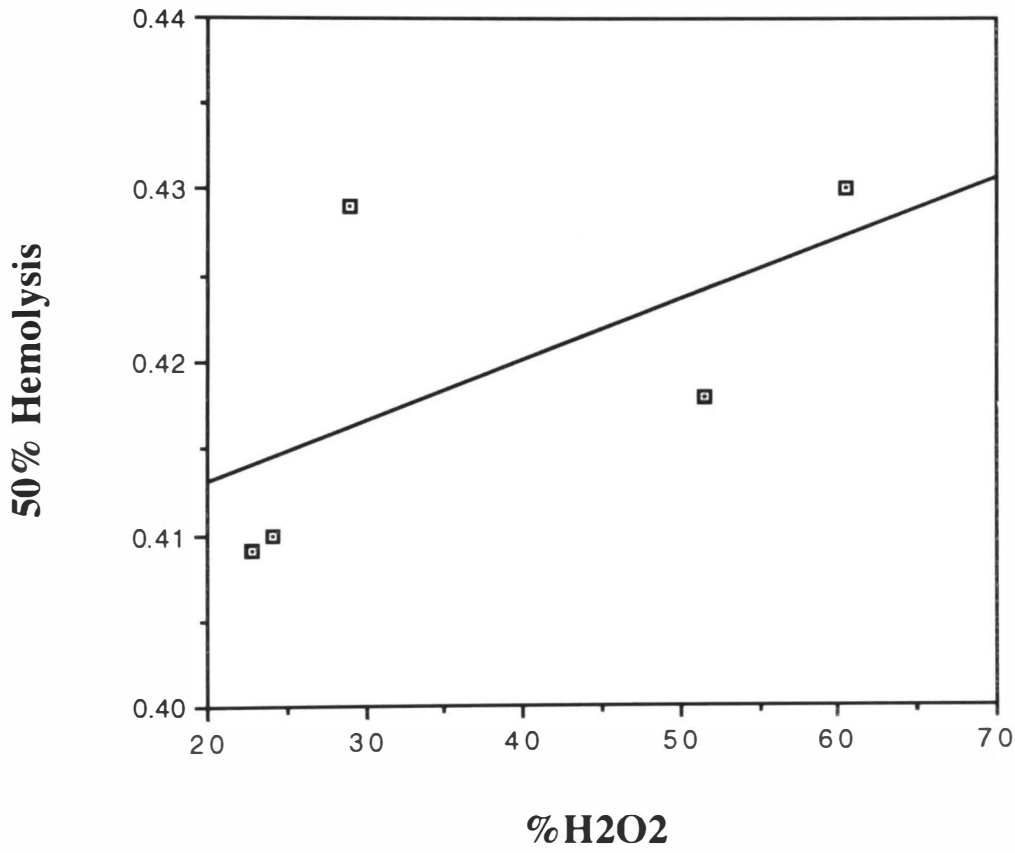
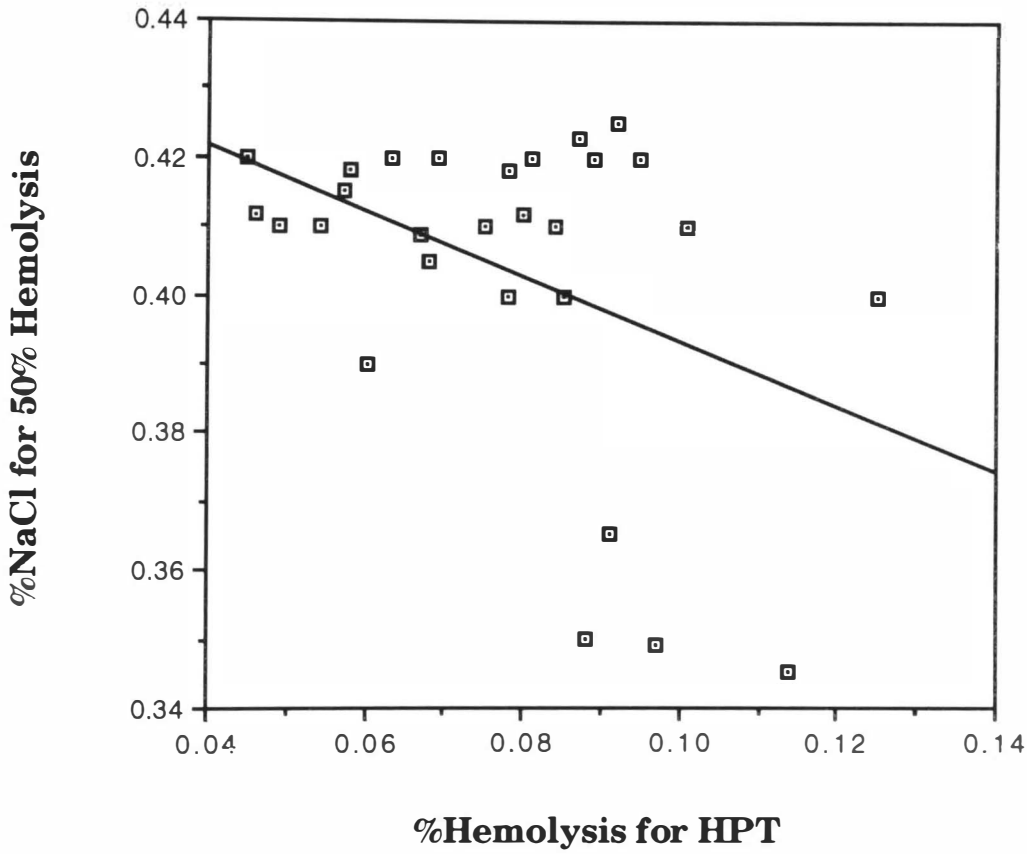

0.44
0.43
0.42
0.41
0.40
20
30
40
50
60
70
50% Hemolysis
%H2O2

Figure 15

%NaCl for 50% hemolysis versus the %Hemolysis
as calculated from the HPT for all rat samples.
The linear regression equation is:

$$y = 0.441 \times 10^{-3}X + 0.479 \quad R^2 = 0.170$$

The R value for the plot is significant for an
N=28 showing a linear relationship for these parameters.


0.44
0.42
0.40
0.38
0.36
0.34
0.04
0.06
0.08
0.10
0.12
0.14
%NaCl for 50% Hemolysis
%Hemolysis for HPT

C. Vitamin E Concentration

Plasma was collected from the five human and from experimental animal samples. The plasma collected from the human samples was analyzed for vitamin E concentration by HPLC. Table 11 lists the vitamin E concentration for each human serum sample. A linear correlation graph of serum vitamin E concentration versus %NaCl concentration for 50% hemolysis for the human samples was constructed and is shown in figure 16. The linear correlation coefficient was calculated to be 0.283 and is not significant at $p<0.05$ for a sample size of five. All of the vitamin E values are within normal range with the mean value for the five samples of 686μg/dL +/- 122 SEM.

Table 11. Plasma Vitamin E Concentrations and %NaCl for 50% Hemolysis points for Human Samples

Protocol	PlasmaVitamin E [a,b]	%NaCl for 50% Hemolysis [c]
A	1061	0.418
B	859	0.430
F	377	0.409
G	616	0.329
H	519	0.410

a Serum Vitamin E concentrations expressed in μg/dL

b Mean +/- SEM, 5

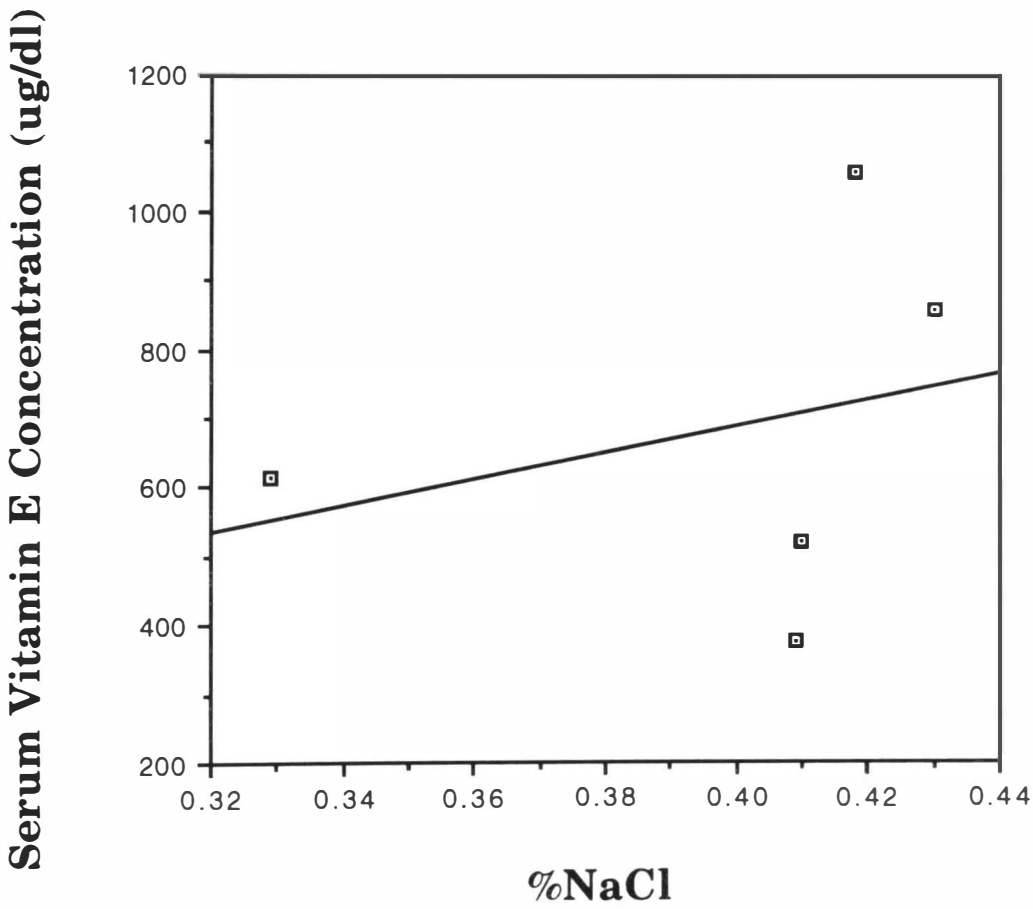
c Mean +/- SEM, 5

Figure 16

Serum vitamin E concentrations versus %NaCl for
50% hemolysis for human samples A, B, F, G, H in the HPT.
The linear regression equation is:

$$y=-1929X + 83.9 \quad R^2=0.080$$

The R value for the plot is not significant for an
N=5 showing no linear relationship for these parameters.


Serum Vitamin E Concentration (ug/dl)
1200
1000
800
600
400
200
0.32
0.34
0.36
0.38
0.40
0.42
0.44
%NaCl

IV
Discussion

A. Red Blood Cell Stability in Uremic Rats Study

1. Weight and Gender Considerations of the Research Animals

The animals used in this study were female Sprague-Dawley rats. The individual weights of each animal and the mean weights of the animals in each trial at the time of sampling are shown in table 6 of the Results Section. The mean weight of the animals in Trial I at the time of sampling was 268g with a SEM of 5.19. The mean weight of the animals in Trial II at the time of sampling was 220g with a SEM of 3.28 The mean weight of the animals in Trial III at the time of sampling was 226g with a SEM of 2.81. The mean weight of the animals in Trial IV at the time of sampling was 220g with a SEM of 3.85. The mean weight of the animals in Trial I was significantly higher than that of the animals in Trials II, III, or IV. The mean weights for the animals in Trial II, III and IV were not significantly different than each other. Whether or not any discrepancies in weight or the fact that all the animals were female played a role in RBC stability is unclear. Future studies will include samples from male rats as well as a study utilizing both female and male rat blood samples with no significant weight differences among the animal groups.

When the animals arrived at the laboratory, the mean weight for the animals in Trial I was 182g, the mean weight for the animals in trial II was 109g, the mean weight for the animals in Trial III was 175g and the mean weight for the animals in Trial IV was 160g. These weights are significantly lower than the mean weights of the animal groups at the time of sampling.

Sampling occured approximately four weeks after the animals arrived at the laboratory and approximately two weeks after surgical procedures were performed. Further research into the metabolic effects of stress on animals would be of interest.

2. Determination of RBC Hemolysis With Increasing Hypotonic NaCl Environment

The mean buffered %NaCl concentration at 50% hemolysis was determined for the NX, PF and SH samples from all four Trials for both the initial and final samples, these results are shown in Table 7 in the Results Section. No significant difference in the mean %NaCl concentration at 50% RBC hemolysis was found between the initial and final samples within any of the three groups. No significant difference in the mean %NaCl concentration at 50% RBC hemolysis was found for the initial samples between groups and no significant difference was found for the mean %NaCl concentration at 50% RBC hemolysis for the final samples between groups. Table 12 outlines the T-statistics calculated from the mean %NaCl concentration at 50% hemolysis for the initial and final samples from Table 7 and compares them in three different ways. First, mean %NaCl concentration for the initial samples were statistically compared between animal group, i.e., the mean %NaCl concentration for the initial samples of the PF animals was compared to the %NaCl concentration for the initial samples of the SH animals. Secondly, mean %NaCl concentration for the final samples were statistically compared between animal group and finally, the %NaCl concentrations for the initial and final samples were statistically compared. From these data there was found to be no significant difference between or among these sample groups.

This leads to the conclusion that, under the circumstances outlined in this study, RBC of uremic animals (NX) are niether more or less stable to hypotonic shock than are RBC of the animals who underwent the five-sixth neprectomy procedure, but did not have any tissue removed, regardless of any dietary constrictions (SH and PF). Decreased stability of the RBC in renal failure as discussed inthe Objectives Section has not been shown to be evident in this group of animals.

Table 12. T- Statistics for Mean %NaCl at 50% Hemolysis for all Four Trials

Comparison Group	t-Stats	Degree Freedom	Significance
Initial vs. initial between samples			
PF (i) vs. SH (i)	-0.54	45	0.591
PF (i) vs. NX (i)	-0.20	42	0.846
NX (i) vs. SH (i)	-0.45	33	0.654
Final vs. Final between samples			
PF (f) vs. SH (f)	0.29	62	0.775
PF (f) vs. NX (f)	0.55	58	0.587
NX (f) vs. SH (f)	-0.40	52	0.689
Initial vs. Final same sample			
PF (i) vs. PF (f)	-1.21	61	0.231
NX (i) vs. NX (f)	-0.59	39	0.557
SH (i) vs. SH (f)	-0.62	46	0.537

Analytic precision experiments using multiple assays of the same blood sample for 50% RBC hemolysis showed a coefficient of variation of 1.1% for the procedure used to determine RBC stability to hypotonic shock as described in the Methods Section.

Linear correlation graphs were constructed using initial and final samples from all four trials. The %NaCl at 50% hemolysis was determined from the %Hemolysis versus %NaCl concentration graphs for all samples. The equation y = b + m e-3x was used to calculate the %NaCl concentration at 0% hemolysis and at 50% hemolysis. Table 8 in the Results section summarizes these findings.

The %NaCl concentration for 0% hemolysis was significantly higher for the NX (f) sample as compared to the NX (i) sample in Trial I. The %NaCl concentration for 50% hemolysis as obtained from th linear correlation graph was also signiicantly higher for NX (f) as compared to NX (i) in Trial I. One purposed explanation for the increased stablilty of RBC of the initial samples as compared to the RBC in the final samples is that as uremia progresses, the RBC may become increasingly unstable.

A significant difference in %NaCl concentration for 0% hemolysis was also found between the NX (i) and SH (i) samples in Trial II. The SH (i) sample appears more stable, the high SEM of this sample (0.021), makes this data point less reliable.

Finally, in Trial III the NX (i) and NX (f) %NaCl concentrations were significantly different in every catagory, The decreased sample size in for the NX animals in Trial II may explain these data, in the future a larger sample size will be used to analysis this data. Also in Trial III, the SH (i) and SH (f) %NaCl concentrations were significantly different for the %NaCl for 0% hemolysis as well as the %NaCl for 50% hemolysis as determined from %Hemolysis versus %NaCl concentraion graphs. Again the small sample size may explain this difference.

B. Red Blood Cell Hemolysis Peroxide Test

The HPT was performed on animal samples from Trial IV of the RBC Stability in Uremic Rats Study and on five human samples in order to demonstrate an alternate method by which to determine RBC hemolysis. For each sample absorbance at 100% hemolysis was determined as well as hemolysis due to the 2% solution of H_2O_2. The %Hemolysis of the RBC was determined by dividing the hemolysis due to the H_2O_2 by the 100% hemolysis value and multiplying by 100.

Table 10 in the Results Section presents the mean %Hemolysis values for the NX, PF, SH and Human samples used in the HPT. The mean hemolysis points for the HPT for all animal samples were statistically analysed and compared to each other in a standard student's t-test as shown in Table 13. There was no significant difference between the animal samples. This is consistent with the findings in the RBC Stability in Uremic Rats Study, no significant difference was found between the animals for the %NaCl concentration at 50% hemolysis (as discussed in the Determination of RBC Hemolysis With Increasing Hypotonic NaCl Environment Section).

In addition to the HPT, the modified Papart procedure as described in the Methods Section, was also performed on these samples. Table 9 lists the mean %NaCl concentrations for 50% RBC hemolysis for all the samples in the HPT. Although the PF mean %NaCl concentration for 50% hemolysis is significantly different form both the SH and NX samples, when analysed with data from all four trials, no significant difference between the PF and SH or NX animals is found.

Linear correlation graphs were constructed in order to compare the correlation between the HPT method of determining RBC hemolysis and the determination of RBC hemolysis with an increasing hypotonic NaCl environment. Figure 14 in the Results Section is a comparison of the %NaCl concentraion at 50% hemolysis versus the %Hemolysis as determined by the HPT for the human blood samples. The correlation coefficient was 0.607 which is not significant at a $p<0.05$ for a sample size of five. Future research should include more human samples in order for such a comparison to be significant. Figure 15 in the Results Section is a comparison of the %NaCl concentraion at

Table 13. T- Statistics for All H_2O_2 Trials

Comparison Group	t-Stats	Degree of Freedom	Significance
Between samples			
SH (f) vs. PF (f)	0.787	17	0.442
NX (f) vs. PF (f)	0.364	17	0.720
SH (f) vs. NX (f)	0.284	16	0.780

50% hemolysis versus the %Hemolysis as determined by the HPT for the animal blood samples. The correlation coefficient was 0.412 which is significant at a $p<0.05$ for a sample size of 28. Consequently, for the larger sample size, the findings for the HPT method for determining RBC stability and the findings for the determination of RBC hemolysis with an increasing hypotonic NaCl environment are comparitively similar. Future research will expand the HPT method to all samples.

C. Vitamin E Analysis

Five human serum samples were analysed using the HPLC method. Figure XIV in the Results Section outlines the vitamin serum E concentration in each sample and the 50% hemolysis point for each sample. The linear correlation graph of serum vitamin E concentration versus %Hemolysis, (figure XVI in Results section), with a correlation coefficient of 0.283, shows no significant relationship between serum vitamin E concentration and %Hemolysis in these samples.

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