INVESTIGATIONS OF THE EFFECTS OF HEAT AND OF ULTRASOUND ON TERMINALLY-DIFFERENTIATED CELLS: USING THE ERYTHROCYTE AS A MODEL SYSTEM.

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Physiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 1992

Urbana, Illinois

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Glossary of Abbreviations

2,3-DPG	- 2,3-diphosphoglycerate.
4,6-P	- 4 and 6 phosphates of inositol pentaphosphate.
CHO	- Chinese hamster ovary cells.
CW	- Continuous wave.
FID	- Free induction decay.
Hb	- Hemoglobin.
Hct	- Hematocrit.
HS	- Heat shock.
HSP	- Heat shock protein.
IP	- Intraperitoneal.
IP_5	- Inositol pentaphosphate.
RMCHC	- Relative mean corpuscular hemoglobin concentration.
MeP	- Methylphosphonate.
MOPS	- (3-[N-Mopholino] propane sulfonic acid).
NMRS	- Nuclear magnetic resonance spectroscopy.
NTP	- Nucleoside triphosphate.
O.D.	- Optical density (Absorbance).
Pi	- Inorganic phosphate.
PN	- Pyridine nucleotides.
ppm	- Parts per million.
RBC	- Red blood cell (Erythrocyte).
SPTA	- Spatial peak temporal average.
SPTP	- Spatial peak temporal peak.
TEP	- Triethyl phosphate $(CH_3CH_2O)_3PO$.
U.S.	- Ultrasound.
VT	- Variable temperature unit.
W/cm^2	- Watts per square centimeter.

Abstract

The effects of heat and of ultrasound were investigated in the terminally-differentiated erythrocyte using hemolysis as an end-point. An assay was developed in which the optical density of supernatant suspension at 410 nm was related to percent hemolysis. It was determined that the absorption coefficient was most stable at 410 nm.

Using the hemolytic assay the heat induced hemolysis of both chicken and rat RBC's was determined at various temperatures ranging from 45 to 51.5°C. Rat RBC's were found to be more heat sensitive than chicken RBC's. The threshold level for damage in rat RBC's was found to occur near 60 min of heating at 50.5°C. This threshold was also confirmed using kinetic studies of damage during incubation at 35°C. The heat response of rat RBC's differing by age was determined by separating cells according to age using ultracentrifugation. It was determined that cell density had no effect on survival except for the least dense fraction which may have been enriched with reticulocytes. The survival following split-heating of RBC's was the same as the survival following continuous heating when the interval between heating was 20 min, while survival decreased significantly when the incubation time was increased up to 50 hr. This indicates that during incubation at 22°C potentiation of heat damage occurs.

The inducibility of thermotolerance was investigated in RBC's. Induction of thermotolerance was achieved in fresh chicken RBC's by chronically heating cells at 42.4°C for 2 hr. Induction of thermotolerance in fresh rat RBC's was not achieved. This may indicate that rat RBC's cannot achieve thermotolerance. Otherwise the pre-incubation temperature below 38°C had no effect on the survival of rat RBC's following heating at 50.5°C.

Investigations, using ^{31}P NMRS to monitor changes occurring in the phosphorus metabolites during heating indicated that there was no difference in the levels of nucleoside triphosphates to differentiate heat-shocked RBC's from non-heat-shocked RBC's. The intracellular pH of RBC's significantly changed with temperature, but the pH was the same at a given temperature in both heat-shocked and non-heat-shocked cells. Differences observed between cells heated using the two protocols were that the line width of peaks in cells that were heat-shocked were significantly broadened during heat shock and 51.5°C challenge indicating that the hemoglobin in these cells were more deoxygenated than in control cells. In addition, a time-dependent left-ward shift of the peak representing the 4,6-P of IP_5 was observed during heat shock and this peak was significantly shifted 0.419 ppm to the left when cells were returned to 23°C, but returned to its original position when cells were reoxygenated. This suggests that the oxygen-hemoglobin affinity may be reduced in RBC's undergoing a heat shock.

Investigations using U.S. to induce hemolysis in RBC's showed that the extent of damage produced in the RBC could be monitored and compared to effects of heat, but that RBC's are not a sensitive enough model to use in detecting damage resulting from intensities commonly used in the clinic. The rate of damage and kinetics of damage following the two modalities were significantly different indicating that the two modalities act on different targets or affect the same targets differently. The results suggest that the mechanism of U.S. damage is different from the effects of other physical agents.

General Introduction (Abstract)

Cancer may be the leading cause of death in the United States by the year 2000. Age-adjusted cancer mortality rates are relatively stable, but mortality resulting from other diseases, especially heart disease, declined significantly over the past 40 years. Additionally, the likelihood for contracting cancer increases as more of the population attains longer life expectancies.

There are several proven strategies for treating cancer including surgery, radiation therapy, chemotherapy, or a combination of these. Innovative treatments such as antibody targeted toxins, classified under the general heading of recombinant toxins, are in development. The major limiting factor for all cancer therapies, old and new, is damage to surrounding non-malignant tissues. These tissues are often composed largely of nonproliferating terminally-differentiated cells such as nerve, muscle, and connective tissue. The oncologist designs a therapy protocol primarily dependent on minimizing damage to the healthy tissues that are exposed during treatment. For this reason, it is very important to understand the mechanisms of the responses of these healthy cells.

Interest in using heat as a treatment for cancer is increasing. Hyperthermia is primarily used as an adjunct to ionizing radiation. There are several physiological incentives for combining heat and radiation.

(1) The effects of heat and of radiation vary differently during the stages of the cell cycle. Cells in S phase are most sensitive to heat, whereas M and G_2 cells are most sensitive to radiation.

(2) Cells found in the hypoxic core of larger tumors are more resistant to radiation than oxygenated cells, but they are more sensitive to heat.

(3) Heat interferes with repair of radiation damage.

The usefulness of hyperthermia in the clinic is still being evaluated. To benefit future therapeutic techniques it is important to understand the effects of heat on terminally-differentiated tissues that unavoidably get exposed during heating.

My study is part of a project undertaken to develop a model for the effects of heat and of radiation on terminally-differentiated cells. This is an area of radiotherapeutic significance, but for which there are no satisfactory models. The erythrocyte was chosen as a model cell because there is extensive literature describing its function, and because a simple quantitative end-point is available. Additionally, the role of the nucleus in injury can be investigated by comparing the effects of agents on mammalian erythrocytes, which are enucleate, with the effects on avian erythrocytes, which are nucleate.

This study has five parts: 1. To develop an assay for determining heat damage, using hemolysis as an end-point; 2. To determine the kinetics of heat damage in chicken and rat red blood cells (RBC's) for various temperatures and at various pH values; 3. To determine whether thermotolerance can be induced in chicken or rat RBC's; 4. To investigate the deleterious effects of heat and the development of thermotolerance in chicken RBC's using ^{31}P nuclear magnetic resonance spectroscopy (NMRS) (specifically, to determine whether thermal responses could be correlated with levels of high energy phosphates, or with other spectroscopic changes); 5. To determine whether the effects of ultrasound (U.S.) radiation, including possible non-thermal effects, could be detected and compared to damage produced by hyperthermia.

A method was developed to assay for RBC hemolysis based on measuring the absorbance at 410 nm of 0.07 % hematocrit (Hct) RBC suspension supernatant. Using this assay the kinetics of damage following heating was determined by

exposing RBC's to temperatures between 45 and 51.5°C for various times, then incubating them for various durations before determining extent of hemolysis.

Results indicate that chicken RBC's can develop thermotolerance after heating at 42.4°C for two hours. After numerous heating schemes, however, I failed to detect any development of thermotolerance in fresh rat RBC's.

Studies using ^{31}P NMRS to monitor changes in the red cell intracellular milieu caused by heat, including the onset of thermotolerance, indicated that nucleoside phosphate levels could not be correlated with thermotolerance; however, a shift in the resonance frequency of a peak corresponding to an inositol phosphate in the avian RBC, along with the broadening of all the observed peaks during heating at 42.4°C, may yield a clue about why heat shocked cells are more thermotolerant during heating at 51.5°C.

Preliminary U.S. investigations on rat RBC's indicated that the maximum U.S. intensity that could be generated with the available equipment (60 W/cm^2) significantly affected survival of RBC's when assayed immediately after exposure. Exposures below 50 W/cm^2 did not cause significant immediate hemolysis or hemolysis following incubation. Survival curves were generated using cells that were sonicated or sonicated and then heated at 50.5°C for 60 minutes. Survival curves following sonication were compared to survival of cells that were continuously heated or split-heated (heated for 15 to 75 min at 50.5°C before incubating at 22°C for 20 min and heating at 50.5°C for 1 hr) to determine a heat equivalence (iso-effect) value for U.S. damage. Kinetic studies of iso-effect exposures to heat and U.S. showed that the kinetics of damage following each modality are different, suggesting that each modality acts on a different target(s) in the cell or affects the same target(s) differently.

Chapter 1

General Background

RBC Model

RBC's were chosen because they are easily acquired, provide a quantitative end-point for injury, and much is known about them. Additionally, any distinction between nucleated and enucleated cells could be examined by comparing mammalian and avian RBC's.

There are several other differences between avian and mammalian RBC's. The presence of a nucleus in the avian RBC affects the shape of the cell. While mammalian erythrocytes are biconcave disks, RBC's containing nuclei are flattened biconvex ellipsoidal disks, allowing for the nuclear volume (Ngai and Lazarides,1989).

There are at least two additional cytoskeletal structures present in the avian RBC membrane skeleton that are not found in mammalian RBC's, marginal bands and transmarginal bands. Marginal bands are an aggregation of microtubules that appear to attach to the plasma membrane. These bands may be involved in maintaining the RBC's ellipsoidal shape and provide resistance to deformations resulting from the mechanical stresses encountered in the circulation (Barrett and Dawson, 1974). Transmarginal bands are filamentous networks that span the cytoplasm and appear to enmesh the centrally located nucleus. These bands seem to be involved in nuclear anchoring (Ngai and Lazarides, 1989).

The phosphorus metabolite content of the two cell types also varies. Mammalian RBC's contain approximately 7.2 - 10.2 mmoles/(liter of packed RBC's) 2,3-diphosphoglycerate (2,3-DPG), while avian RBC's contain approximately 8.5 mmoles/(liter of packed RBC's) inositol pentaphosphate (IP_5). The RBC's of avian embryos contain 2,3-DPG without any IP_5, but it is completely replaced with IP_5

within eight days after hatching. Both 2,3-DPG and IP_5 function to decrease the affinity of hemoglobin (Hb) to oxygen by binding in the central core of the Hb molecule stabilizing deoxyhemoglobin.

The synthesis and degradation of 2,3-DPG is a detour in the glycolytic pathway between the hydrolysis of 1,3-diphosphoglycerate to 3-phosphoglycerate. Diphosphoglycerate mutase transfers a phosphoryl group from the 1-position of 1,3-DPG to the 2-position of 3-phosphoglycerate to form 2,3-DPG. 2,3-DPG is hydrolyzed to 3-phosphoglycerate by 2,3-diphosphoglycerate phosphatase. In addition to regulating oxygen transport in the RBC, 2,3-DPG serves as a cofactor in the conversion of 3-phosphoglycerate to 2 phosphoglycerate which is the next step in the glycolytic pathway (Stryer, 1981). Mammalian RBC's have a much higher concentration of 2,3-DPG than other mammalian cells (Prankerd, 1961).

Avian and reptilian RBC's contain inositol pentaphosphate (IP_5). The pathway for its formation is not known. It is thought that the initial step in its synthesis is phosphorylation of inositol (Carpenter *et al.*, 1989). The formation of IP_5 is thought to originate from inositol as shown by incorporating radiolabeled [^{14}C]inositol into IP_5 (Isaacks, *et al.*, 1982). It is not likely that IP_5 is formed from glucose since [^{14}C]glucose is not incorporated into IP_5. Membrane phospholipids are not likely precursors for IP_5, since IP_5 lacks a D-1 phosphate which is present after phosphodiesteratic cleavage of phospholipids. IP_5 is formed directly when IP_4 kinase phosphorylates IP_4. This reaction requires ATP (Stephans, *et al.*, 1988). The product of the IP_4 kinase when incubated in the presence of [γ-^{32}P] ATP is D-Ins[1-^{32}P]1,3,4,5,6)P. Therefore this enzyme catalyses the transfer of the γ-phosphate group of ATP to the L-3 (D-1) hydroxy group of L-Ins(1,4,5,6)P_4. It is not known how IP_5 is broken down or whether it interacts with components other than Hb in the avian red cell. Isaacks *et al.* (1983) found that mature RBC's can

synthesize IP_5 but cannot catabolize it and that IP_5 levels didn't increase during anemia as expected. They suggested that IP_5 regulation of Hb is not as adaptable to the changing needs of the organism as is regulation by 2,3-DPG.

Both avian and mammalian RBC's metabolize glucose via the glycolytic pathway. Although avian RBC's maintain their mitochondria, not all the enzymes required in the Krebs cycle are present. Additionally, both RBC types can metabolize glucose via the pentose phosphate pathway (Kregenow, 1977).

Mature mammalian erythrocytes cannot carry out protein synthesis, since the nucleus is expelled during formation of the reticulocyte. As the reticulocyte continues to mature, the remaining organelles are eliminated. Translation of hemoglobin only continues until a mature erythrocyte is formed. The mature mammalian erythrocyte contains no nucleus, mitochondria, lysosomes, ribosomes, membranous endoplasmic reticulum, or golgi apparatus. Consequently, they cannot synthesize nucleic acids or proteins (Smith *et al.*, 1983).

Mature avian erythrocytes contain a nucleus that occupies 10-30 percent of total cell volume. The synthetic activity of the nucleus in these RBC's is greatly reduced. Zentgraf *et al.* (1975) reported that hen RBC's were able to transcribe DNA at very reduced levels, but that this pre-mRNA was confined to the nucleus and was not translated. They suggested that transcription occurring in mature erythrocytes was residual activity from earlier stages of electropoiesis. Ninety percent of protein production in the reticulocyte is globin (Smith *et al.*, 1983).

In spite of limited gene activity, avian erythrocyte preparations were shown to synthesize heat shock proteins (HSPs) when heat stressed both *in vitro* (Morimoto and Fodor, 1984; Atkinson and Dean, 1985) and *in situ* (Dean and Atkinson, 1985). Morimoto and Fodor (1985) found that chicken blood cell preparations synthesized HSPs having molecular weights of 23K, 70K, and 89K

when heated above 42°C for 30 minutes. They attributed this HSP synthesis to the lymphocyte fraction of the cell preparation. They also determined that reticulocytes obtained from anemic chickens synthesized only a 70K HSP following heat shock (HS). This 70 K protein was determined to be present normally in both retiulocytes and mature erythrocytes at high levels.

Dean and Atkinson (1985) also found that the RBC's from both normal and anemic quails had new and/or enhanced synthesis of HSPs having molecular weights of 26K, 70K, and 90K when the core temperature of quails was raised to above 41°C for 80 minutes. They (Atkinson and Dean, 1985) suggested that synthesis of HSPs may only occur in the reticulocytes and not in mature erythrocytes. In subsequent studies of HSP synthesis in avian blood cells, they used reticulocyte enriched blood samples from both chicken (Atkinson *et al.*, 1986) and quail (Atkinson *et al.*, 1986; Atkinson *et al.*, 1990). They found that chicken reticulocytes synthesized 24K and 90K proteins in addition to the 70K protein following a 60 minute heat shock at 45°C.

While the synthesis of HSPs following heat shock in red cell preparations from normal and anemic quail was demonstrated, neither investigator examined the induction of thermotolerance in these cells. Since the induction of thermotolerance has been correlated with the synthesis of HSPs (Li *et al.*, 1982; reviewed by Subject and Shyy, 1986), it is expected that avian RBC preparations might develop thermotolerance. Mammalian erythrocytes are not expected to develop thermotolerance since they lack all nuclear components.

RBC Membrane

In order to illustrate the complexity of the RBC membrane and possibly suggest mechanisms by which heat may injure it, the major structural components of the RBC membrane will be reviewed. The RBC membrane basically consists of

a phospholipid bilayer sandwiched between outer and inner elements. The outer layer is mainly composed of adsorbed albumen interspersed between surface proteins such as glycophorin and band III (Bull and Brailsford, 1989). The inner layer is mainly composed of cytoskeletal elements such as spectrin. Glycophorin, band III, and spectrin account for more than 60 % (by weight) of the total membrane protein.

Glycophorin and band III are the two major integral transmembrane proteins which are exposed at the RBC's outer surface. Glycophorin carries the majority of the total RBC surface carbohydrate and therefore most of the negative charge. Glycophorin is a single-pass membrane protein which passes through the lipid bilayer as a single α-helix. Glycophorin is found only in the RBC and its function remains unknown.

Band III, having a molecular weight of 100,000, serves as an anion transporter. It extends across the bilayer several times possibly functioning as an anion channel for Cl^- and HCO_3^-.

The inner element of the RBC membrane is composed of the cytoskeleton which mainly consists of spectrin in the form of tetramers interconnected in a net-like array. Spectrin is a long fibrous molecule that constitutes about 30% of the membrane protein mass and is loosely associated with the cytoplasmic side of the RBC membrane. Six spectrin molecules come together to bind one molecule of actin. Spectrin along with actin and other proteins participate in the formation of the meshwork, which is involved in maintaining the biconcave shape of RBC's, while at the same time allowing the cell to deform as necessary to pass through narrow capillaries.

The major attachment site between the membrane skeleton and lipid bilayer is provided indirectly via ankyrin. Ankyrin is a globular protein with a molecular

weight of 215,000. It has separate binding sites for spectrin and the N-terminal cytoplasmic domain of band III. The ratio of spectrin dimers to ankyrin suggest that each spectrin tetramer is linked to band III by a single ankyrin.

In addition, the N-terminal cytoplasmic domain of band III, which is structurally and functionally independent of its membrane spanning region, is a site for binding other proteins such as glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, hemichromes (a denatured form of hemoglobin), and hemoglobin (Low, 1989). The binding of Hb to band III occurs rapidly, is of high affinity, and depends strongly on pH and ionic strength.

Effects of Heat on Membrane

Alterations of cell membranes during heating in numerous cell lines have been reported. These include changes in cellular surface morphology (eg., blebbing), in membrane permeability, in surface charge, in membrane fluidity, and in binding of growth factors.

The effects of heat have been studied extensively in proliferative cells. Heat can act on the cellular membrane, nucleus, or any of the other organelles. Roti Roti (1982) proposed a model for hyperthermic cell killing postulating that initial heat damage occurs at the plasma membrane, affecting membrane permeability and thus leading to changes in composition of the intracellular milieu. Such changes cause proteins to become adsorbed to chromatin, restricting the access of repair enzymes to DNA. This inhibition of DNA repair enzymes could cause radiosensitization. Portions of the DNA eventually become damaged due to excess proteins binding to chromatin, and the cell dies (reproductive death).

Because a nucleus is not present in mammalian RBC's and is repressed in mature avian red cells, it is not likely that damage to the nucleus would lead to

hemolysis. Therefore, the remainder of this discussion will concentrate on the effects of heat at the level of the membrane.

After heating, Chinese hamster ovary (CHO) cells showed a characteristic membrane deformation, termed blebbing, which was correlated with survival (Borrelli *et al.*, 1986). Borrelli investigated the induction of blebbing and changes in ion gradients across the membrane while cells received heat sufficient to decrease survival by 60 %. He found that there were no changes in ion gradients or energy levels during the onset of blebbing. Therefore, the membrane functional integrity was maintained for several hours despite the occurrence of blebbing.

Borrelli suggested that during this initial phase of blebbing the cytoskeleton might have detached from the membrane and began collapsing around the nucleus. At this stage recovery is still possible. Following severe blebbing the cytoskeleton may completely collapse around the nucleus preventing the cell from recovering.

Many investigators have studied alterations in membrane permeability during and or following heating. Major changes in the membrane permeability for ions may result in cell death. The levels of calcium have been reported to increase in a variety of cells prior to death following hyperthermia. In human erythrocytes, a temperature of 41°C was sufficient to decrease the transmembrane potential, an indication of permeability changes (Mikkelsen and Wallach, 1977).

Heating also affects the fluidity of membranes. Hyperthermia (41-45°C) induced a decrease in the fluidity of plasma membranes in pig erythrocytes (Leyko and Bartosz, 1986), possibly due to the indirect effects of changes in membrane proteins. Alterations in the conformation of bovine erythrocyte membrane proteins after hyperthermia were demonstrated using electron spin resonance with spin labels (Grzelinska *et al.*,1982). Eighty percent of bovine RBC's hemolyzed when

heated at 50°C for 15 min. During this treatment rigidification of membrane lipids and changes in the physical state of proteins were observed. Heat treatment of erythrocyte membranes labelled with 4-(N-maleimido)-2,2,6,6-tetramethylpiperidine-1-oxy(MSL), which binds to the sulphydryl groups of membrane proteins, showed an increase in the ratio of weakly-bound over strongly-bound residues, indicating conformational changes in the protein. Using a lipid-bound spin label (M12NS), a slight increase in the rigidity of the membranes with increased temperature was found. These changes did not occur in the membrane lipids of liposomes when treated under the same conditions. This may indicate that lipid disturbance in the RBC membrane are due to secondary effects mediated by proteins. It is not certain whether these changes are reversible (Grzelinska *et al.*, 1982).

Heat Shock Proteins and Thermotolerance.

The first indication that heat may alter gene expression came from Ritossa (1962) who found that heat induced distinct patterns of chromosomal puffs in *Drosophila* salivary glands. The puffing patterns indicated sites of active transcription, and their gene products were designated heat shock proteins (Tissieres *et al.*, 1974). Most all organisms form bacteria to man synthesize HSPs in response to elevated temperature (reviewed by Schlesinger *et al.*, 1982).

HSP synthesis occurs when cultured mammalian cells are either chronically heated [3 to 5 hours at sublethal temperatures] or acutely heated [short intervals at temperatures above 42.5°C followed by incubation at phsyiological temperature] (Henle and Dethlefsen, 1978). Most studies of thermotolerance in cells investigate proliferative cells. This study investigates thermotolerance in terminally-differentiated cell systems with very limited gene expression or none at all.

The mechanism by which HSPs provide thermotolerance is not known. There are four lines of evidence which support the correlation between HSP synthesis and thermotolerance (Nover *et al.*, 1984). First, the three-phase kinetics of thermotolerance induction, expression and decay exactly parallel the induction, accumulation, and degradation of HSPs (Li *et al.*, 1982; Landry *et al.*, 1982).

Secondly, several non-heat shock inducers of HSP synthesis, such as alcohol and sodium arsenite, also activate thermotolerance (Li, 1983).

Thirdly, cells which are deficient in HSP synthesis do not develop thermotolerance. Heikkila *et al.* (1985) showed that early *Xenopus laevis* embryos are not heat inducible to produce HSPs. During this time they also do not express thermotolerance; however, thermotolerance does become inducible at the same time the embryo develops the ability to express HSPs and other genes. In addition, Henle and Leeper (1982) using CHO cells showed that cycloheximide, a protein synthesis inhibitor, prevented the induction of thermotolerance.

Finally, lines of Chinese hamster fibroblasts selected for heat resistance contained an increased amount of proteins of the HSP 70 family and showed accelerated and enhanced HSP 70 synthesis after heat shock (Lazlo and Li, 1983).

Many mechanisms for induction of thermotolerance by HSPs have been proposed (reviewed by Subject and Shyy, 1986). Recent evidence suggests a role of some HSPs as accessory molecules to stabilize nascent polypeptides in order to facilitate proper folding (Beckman *et al.*, 1990). In such a role HSPs provide thermotolerance by stabilizing maturing proteins which are necessary for cell survival.

RBC Age and Heat Response

Survival curves generated following heating of many cell lines indicate that as heating time increases, a greater total percentage of cells die. This curve may

result due to the gradual accumulation of damage in individual cells or because the cell suspension contains numerous populations of cells with different sensitivities to heat. The most heat sensitive cells die during the initial heating period, while the more heat resistant cells die following longer heating periods.

A factor that may affect a RBC's heat sensitivity is age. Many changes occur during the 80 day lifetime of a rat RBC. These changes occur gradually and include increased density and fragility to osmotic lysis, decreased volume and deformability, changes in surface charge (Kay, 1990), and decreases in energetic metabolism, especially in the activity of pyruvate kinase and hexokinase (Seaman *et al.*, 1980). It is believed that many of these changes accumulate throughout the lifetime of the RBC, triggering a conformational change of a membrane protein near the 80^{th} day of circulation to form a senescent cell antigen (Noble, 1990). This newly altered membrane antigen is recognized by phagocytic cells that destroy the RBC.

Red cell preparations can be separated by age using ultracentrifugation. Borun *et al.* (1957) found a correlation between cell density and age using radiolabeled iron. Blood from human patients who had various chronic diseases was withdrawn at approximately 10 day intervals following the injected of Fe^{59}. The whole blood was centrifuged for 40 min at 2,000 x g to separate RBC's by density. Newly labeled radioactive cells would appear at the top of a centrifuged cell sample and gradually moved towards the bottom of the pellet as the time between radio-labeling and centrifuging increased (Borun *et al.*, 1957).

Murphy (1973) showed that centrifugation of cells at 30°C for 1 hour at 27,000 X g in a fixed angle rotor was adequate to separate cells according to density. At the warmer temperature the viscosity of the red cell pellet is decreased allowing the cells to circulate into density gradients within the centrifuge tube.

More recent separation techniques can separate smaller volume fractions through the addition of a high molecular weight substance to produce a linear density gradient (Vettore *et al.*, 1980). The advantage of Murphy's method is that nothing is added to cells. Any foreign substance added to the cells might affect the cell's response to stresses such as heat.

Heat in Cancer Therapy.

The initial rationale for using heat to treat cancer arose because many have claimed that malignant cells are more heat sensitive than their normal counter parts (Hahn, 1982; reviewed by Strom *et al.*, 1977). A possible explanation for this is that a tumors surrounding neovaculature is unable to supply the rapidly proliferating cells of a tumor with adequate nourishment leading to decreased tumor energy status and pH (Gerweck,1985).

As a cell populations distance from the blood supply increases, it has a greater chance of becoming necrotic. This occurs often in the central core of a tumor (Thomlinson and Gray, 1955). In the region just outside the central core, where the supply of both oxygen and glucose is reduced, a reduction in the intracellular ATP level occurs. Thermal sensitivity is increased greatly as ATP levels decrease (Gerweck *et al.*, 1984). It is also in this thermo-sensitive region of tumors where cells are most resistant to radiation, since the concentration of oxygen is lowest.

In addition tumor cells, which are chronically hypoxic, undergo anerobic glycolysis to produce large quantities of H^+. Therefore, tumors generally have an average pH more acidic than the surrounding healthy tissues, which have a pH of approximately 7.3 (Gerweck,1985). A decreased pH was found to sensitize cells to heat and was designated the "pH effect" (Gerweck, 1977).

Another consequence of a deficient blood supply is that excess heat cannot be dissipated adequately. While healthy tissues dissipate excess heat by increasing blood flow, tumor tissues cannot increase flow, therefore they reach higher temperatures than the surrounding healthy tissue.

Additional rational for using heat in conjunction with radiation for cancer therapy is that the heat sensitivity of cells in different phases of the cell cycle, complements the sensitivity of cells to radiation (Hahn, 1982). Westra and Dewey, using synchronized CHO cells, showed that the most heat-sensitive phases of the cell cycle were M and late S. Cells were most resistant to heat in early G1. Terasima and Tolmach (1963) showed that greatest sensitivity to radiation occurred in late G1 and M phases, while greatest resistance occurred in late S. Thus by using both heat and radiation together, populations of cells in all phases of the cell cycle would be inactivated.

A final consideration is that heat can potentiate radiation damage by inhibiting repair of sublethal radiation damage (Gerweck *et al.*, 1975). Heating during or following radiation treatment increases cell death. Mills and Meyn (1983) showed that hyperthermia increased the number of unrejoined single strand breaks and this was correlated with cell killing. Heat may directly or indirectly affect the repair enzymes, or it may cause the DNA lesion to become inaccessible to the repair enzyme.

NMR and Energetics

The NMR studies were begun to determine whether changes in the high energy phosphate levels found in chicken RBC's could be correlated with the induction of thermotolerance. It appeared possible that changes in the levels of ATP during heat shock in chicken RBC's may be responsible for the observed thermotolerance. The RBC was the first living system investigated with high

resolution ^{31}P NMRS (Moon and Richards, 1973). Thus phosphorus NMR was first shown to be useful as a sensitive, non-invasive indicator of intracellular pH.

In red blood cells ATP is essential for the maintenance of cation balance driven by the sodium potassium pump. When mammalian RBC's are suspended *in vitro* in their own serum without added substrate, the following changes resulting from ATP depletion occur: The shape of the cell transformations from a disc to a sphere (10-24 hrs). The cell loses potassium and accumulates sodium (10 hrs). The RBC swells (10-24 hrs), and then shrinks (24 hrs +). The cell loses membrane lipid and surface area (24 hrs+). The deformability of the membrane is decreased (4 hrs). There is a decrease in cell filterability (5 hrs), and an accumulation of calcium (10 hrs) (Weed and Lacelle, 1969).

^{31}P NMR allows one to monitor all the major unbound phosphate compounds present in a system. Only the nuclei found in mobile molecules give rise to sharp NMR signals. Nuclei of the same chemical species have different resonant frequencies, termed chemical shift, depending on their magnetic environments. The relationship between resonant frequency and magnetic field is described by the Larmour equation:

$$\omega_o = \gamma \beta_o$$

where γ is the gyromagnetic ratio of the nucleus, and β_o is the magnetic field acting on the nuclei. Since the gyromagnetic ratio is constant for a given nucleus, it is the magnetic field acting on the nuclei that determines the resonant frequency for the molecule. The spectrometers strong magnetic field is affected at the molecular level by the shielding of electrons and other nuclei in the molecule. Since the charge and conformation of a molecule are dependent on its environment, information about the chemical environment can be obtained as well.

The mobile phosphates in 28-month old chicken RBC's consist of the following compounds: 1.2 µmol/ml RBC of inorganic phosphate; 7.5 µmol/ml RBC of ATP, and 8.5 µmol/ml RBC of IP_5 (Isaacks, *et al.*, 1987).

Ultrasound and Therapy

In 1944, Horvath was the first to clinically utilize ultrasound (U.S.) to treat skin carcinomas and sarcomas and observe reduced tumor growth (Marchal, 1992). Today, U.S. is the method of choice by many clinicians for the hyperthermic treatment of deep tumors. As the U.S. pressure wave passes through the inelastic media of tissue, energy in the form of heat is transferred to the tissue. U.S. has several advantages to other methods of heating. The major advantage is that U.S. penetrates muscle tissue well without preferentially heating fat. Since its wavelength at 1 MHz is 0.15 cm, accurate focussing to a depth of 15 cm is possible (Hahn, 1982). In addition, U.S. is reflected at a tissue-air interface so the energy is confined to the patient and the equipment operator remains safe from stray sonication. This tissue-air reflectivity can make it difficult to treat tumors near the lungs or other air passages. U.S. is also reflected by bone, making it difficult to treat brain tumors non-invasively. Clinicians can utilize the reflected beam advantageously to sonicate regions directly adjacent to bone or air pockets more efficiently. In addition, U.S. imaging used in conjunction with U.S. heating allows for accurate placement of the focussed beam.

The greatest disadvantage in using U.S. to heat tumors is the same as with all other heating modalities; it is difficult to monitor accurately the temperature distribution within the treated tissues. Presently the most accurate thermometry method relies on thermocouples that are pierced through the tissues which are heated. The number of thermocouples used is dependent on the size of the area being treated. This technique has limited resolution so that hot or cold spots may

occur between thermocouples. An accurate non-invasive thermometry system will be required to utilize the full benefits of ultrasound hyperthermia.

Diagnostic ultrasonic imaging has recently become a routine procedure for monitoring the health of developing human fetuses. Much attention is now devoted to determine how U.S. interacts with the delicate proliferating tissues of the fetus. Clinically it appears to be safe, although long term studies have not been completed. Ultrasound is also routinely used for therapy and diagnostics in muscle tissues.

It is important to learn the effects of U.S. on tissues since the modality used to heat cells may affect survival of cells and thermotolerance initiation. Anderson *et al.* (1984) showed that heating mouse fibroblasts during exposure to ultrasound sonication, below the cavitational threshold, decreased both the clonogenic survival and rate of attachment to a greater extent than when using heat alone. This suggests that ultrasound has a non-thermal component that can damage tissues.

Physical Properties of Ultrasound.

Ultrasound consists of all frequencies of sound greater than 20 kHz, which is the approximate upper limit of human audibility. Ultrasonic waves can propagate transversely (shear waves) and longitudinally in solid media. Biological tissues behave as fluid-like media, since they cannot support transverse acoustic waves.

There are three major biological effects of ultrasound; thermal phenomena, cavitation, and radiation forces. Thermal effects are those which occur as a result of increased temperatures occurring in the irradiated tissue. The same biological effects would occur if the temperature elevation occurred without U.S. As a plane wave of ultrasound travels through a homogenous medium its intensity, I,

decreases, since its energy is absorbed and converted to heat. The intensity (I) for a plane wave traveling along x at any position can be determined by:

$$I = I_0 e^{-2\alpha x}$$

where α is the absorption coefficient and I_0 is the intensity at x=0. Due to absorption $2\alpha I$ units of heat are generated per unit volume per unit time in any region where the intensity is I. The temperature in a region where heat is not being transported away by conduction will increase at the rate:

$$dT/dt = 2\alpha I/\rho c_m$$

where ρ is the density and c_m is the specific heat per unit mass.

Due to a low absorption coefficient (< 0.034 Nepers/cm) for whole blood irradiated at 1 MHz (Goss *et al.*, 1978), thermal changes are insignificant. A neper is a dimensionless unit equal to 8.68 decibels.

The intensity or force exerted by the beam is represented by the amplitude of the waveform and is generally expressed in Watts per unit area. Ultrasound can be generated either as a continuous wave (CW) or as a series of distinct pulses. The parameters of a given CW exposure include frequency, intensity, and exposure time. In quantitatively describing a pulsed beam exposure, additional factors are considered, such as the duration of the pulse, the interval between pulses, or the duty cycle, which is the ratio of pulse on time to the total time a single pulse is on and off. Expressions for ultrasonic intensity for the pulsed beam can be defined in two ways. 1) Temporal peak - peak intensity of the ultrasound pulse: 2) Temporal average - peak intensity of the ultrasound pulse averaged over the pulse period plus the wait period or peak intensity times duty cycle.

For therapeutic purposes ultrasound is generally delivered in a continuous wave mode at frequencies near 1 MHz, and intensities of 1-5 W/cm^2. Ultrasound employed for most diagnostic purposes is generally delivered in a pulsed mode

(duty cycle of approximately 0.001) at frequencies of 1-10 MHz. Temporal average acoustic intensity of such beams generally lies in the range of a few mW/cm^2 to approximately 30 mW/cm^2 while temporal peak intensities may be as great as 100 W/cm^2 (Waldman, 1982).

Chapter 2

Heat Responses

It was first necessary to establish conditions and characterize the hemolytic effect of heat on both chicken and rat RBC's. This chapter describes the hemolytic assay and the heat response of chicken and rat RBC's, including the induction of thermotolerance, to various temperatures and heating protocols.

Materials and Methods

Acquisition of Cells.

Red blood cells were acquired from White Leghorn chickens (*Gallus domesticus)* and Sprague Dawley rats (Rattus rattus). A volume of 7 ml of chicken blood was withdrawn from the brachial vein using a 24 gauge 1/2 inch needle and heparinized syringe. Rat blood was collected by heart puncture using a 3/4 or 1 inch 23 gauge needle and heparinized syringe. Rats were first anesthesized (IP) with pentobarbitol (0.8 ml/kg) or by using ether as an inhalant. Chicken blood was obtained with the kind assistance of Dr. Janice Bahr at the Animal Genetics Lab. Rat blood was generously supplied by Dr. Zehr's group and Dr. Meisami's group.

Solutions.

The red blood cells were washed three times in an isotonic MOPS (3-[N-Mopholino] propane sulfonic acid)-buffered glucose-saline solution to remove the blood plasma and buffy coat. For each wash the samples were centrifuged at 900 x g for 5 minutes. Washed cells were resuspended at 0.7% Hct. The solution used for rat RBC's was 300 milliosmolar and contained 131 mM NaCl, 7 mM KCl, 10 mM glucose, 1.5 mM $MgCl_2$, and 10 mM MOPS. The solution used to suspend chicken RBC's was 325 milliosmole and contained 140 mM NaCl, 10 mM KCl, 10 mM glucose, 1.5 mM $MgCl_2$, and 10 mM MOPS. In both cases the solutions were buffered to pH 7.4 by adding NaOH unless otherwise specified.

MOPS buffer (pKa=7.2, -0.006 pKa/°C) was used since it has a pKa near physiologic pH and the pKa value is more resistant to changes with temperature than the other zwitterionic buffers.

Hemolysis Assay

This method, based on measuring the absorbance of free hemoglobin in RBC suspension supernatant, was developed to score RBC damage since methods based on colony formation were inappropriate. Free hemoglobin was separated from the exposed RBC suspension by centrifuging the suspension at 900 x g for five minutes. Reference samples, which were cell suspensions maintained at room temperature (23°C) for the duration of heat exposure of the sample cells, were centrifuged along with the exposed samples to account for any additional hemolysis that may occur during cetrifugation.

Changes in absorbance measurements during lengthy incubation periods could result from changes in either the concentration of hemoglobin or the absorption coefficient of hemoglobin or both. Accurate measurement of changes in Hb concentrations using the hemolytic assay requires that the absorption coefficient of Hb at a fixed wavelength remains constant. Figure 1 shows the absorption spectra for a 0.7% Hct rat RBC supernatant after various durations of incubation at 35°C ±1°C following complete hypotonic lysis. This solution was prepared by hypotonically lysing a 0.07% Hct red cell solution using distilled water. The figure indicates that the peak of maximum absorbance varied considerably between 19 and 71 hours. This may result from the hemoglobin becoming deoxygenated or denatured during the incubation period.

A wavelength of 410 nm was chosen for the hemolytic assay because least variability of the absorption coefficient, for up to 48 hours near the maximum absorption peak, occurs at a wavelength of 410 nm. Also, by choosing a

wavelength near the maximum peak increased resolution is obtained. Choosing a wavelength where the absorption coefficient was constant even while the absorption peak varied, insured that changes in absorption values were due to changes in Hb concentration resulting from hemolysis, rather than to conformational changes of the Hb molecule. Therefore, the amount of hemoglobin released from the cells was then determined by measuring the absorbance [optical density (O.D.)] of sample supernatant at 410 nm using a Beckman DB (split beam) spectrophotometer. The supernatant from reference samples served as reference in the spectrophotometer.

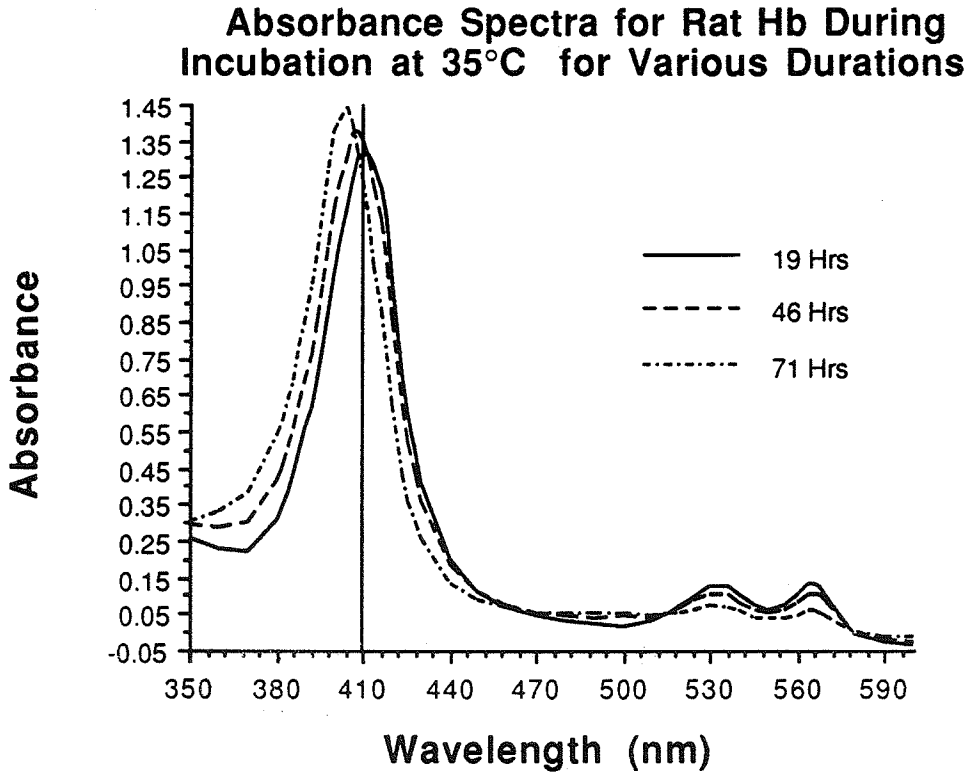

Figure 1. Absorbance Spectra for Rat Hb During Incubation at 35°C for Various Durations. Rat RBC supernatant is shown at 19, 46, and 71 hours of incubation at 35°C ±1°C. The figure indicates that the absorption coefficient of rat Hb varies over time at 35°C. The absorbance at 410 nm is indicated since least variability in the absorbance coefficient for up to 46 hrs occurs at this wavelength.

The O.D. values of samples were converted to percent hemolysis by dividing them by the optical density values of samples which were completely hemolyzed in

deionized water within each experiment:

$$\% \text{ Hemolysis} = \frac{\text{O.D. of sample}}{\text{O.D. of completely hemolyzed sample}} \times 100\ \%.$$

By dividing by the optical density value of a completely hemolyzed sample from each blood sample, errors resulting from differences in whole blood hematocrits were avoided. The percentage of intact cells remaining, "Survival", was calculated by:

$$\% \text{ intact cells (Survival)} = 100\% - \% \text{ hemolysis.}$$

Heating Procedure.

Cells were exposed to heat, 42.0°C to 51.5°C (± 0.1°C), using a Neslab exacel water bath. Temperature measurements were performed using a self-calibrating thermometer and three separate thermistor probes. The temperature of the bath was also checked using a calibrated glass thermometer set against a thermometer traceable to a National Bureau of Standards calibrated thermometer. Using the three thermocouples positioned in the bath around the sample tubes, it was determined that the temperature of the bath was constant within the area that sample tubes were placed. The temperature of the bath did fluctuate ± 0.1°C during the exposure time.

Polystyrene centrifuge tubes containing 9.0 ml of isotonic, buffered, glucose-saline solution were placed in the bath at least 15 minutes prior to the addition of 1.0 ml of RBC suspension (0.7% Hct. for both chicken and rat). This allowed the solution to equilibrate to the temperature of the bath, ensuring that when cells were added they would be exposed for the specified length of time. It was determined by monitoring the temperature with a thermocouple in three separate sample tubes containing glucose-saline, that 13 minutes was required to raise the temperature of the saline from 21°C to 51.5°C.

Immediately after heating the tubes were placed in 15°C water for 5 minutes to cool the cells. It was determined by monitoring the temperature with a thermocouple in theree separate sample tubes containing glucose-saline, that 5 minutes at 15°C was sufficient to cool cells from 51.5 to 23°C. Some samples were scored immediately while other samples were incubated at 34 to 37°C for up to 72 hours before scoring.

Heat Shock.

Heat shock refers to the mild heat exposure that might induce thermotolerance. Challenge refers to a more severe heat exposure which tests for the induction of thermotolerance. Reference samples were used as references in the split beam spectrophotometer and were usually maintained at room temperature.

Acute Heat Shock. Acute heat shock is a short term exposure (less than 15 minutes) to temperatures which are at least 5°C greater than the normal physiological temperature of extracellular fluid, followed by incubation at 34 - 37°C for several hours. In this investigation, sample tubes containing chicken or rat RBC's were briefly exposed to various temperatures ranging from 43.5 to 50.6°C for 1 to 20 minutes. These tubes, together with controls, tubes which did not receive heat shock prior to challenge, and reference tubes were placed in an incubator at 35°C ± 1°C for a maximum duration of 5 hours before exposure to the heat challenge. RBC's were then challenged by exposing them to between 48.6 and 51.5°C. When exposing cells to a challenge, sample and control tubes were placed in the water bath directly from the incubator. The sample and control tubes were allowed to equilibrate to the bath temperature (7 minutes) before timing for the challenge was begun. It was determined by monitoring the temperature with a thermocouple in three separate sample tubes containing glucose-saline, that 8.5

minutes was required to raise the temperature of the saline from 37°C to 50.5°C. Seven minutes was required to raise the temperature to 50.3°C. During 50.5°C challenge the reference tubes were returned to 23°C.

Chronic heat shock. Chronic heat shock is a long term exposure (greater than 1 hour) to temperatures which are less than 5°C greater than the normal physiological temperature of extracellular fluid. In this investigation sample tubes containing chicken or rat RBC's were exposed to temperatures ranging from 40.6 to 42.7°C for 1 to 4 hours. These temperatures were chosen because a variety of other cells lines develop thermotolerance following a chronic heat shock at temperatures 1 to 2°C greater than the normal physiological temperature of the cells. The mean core temperature in rats is 39°C while in chickens is 41.0°C.

During heat shock, control and reference tubes remained at 23°C. Sample, control, and reference tubes were then placed into an incubator at 35°C ± 1°C for about 15 minutes (the time required to raise the temperature of the water bath to that of the challenge temperature). Sample and control tubes were allowed to equilibrate for 7 minutes in the bath before timing for the challenge was begun. Reference tubes were returned to 23°C during challenge.

Density Separation by Ultracentrifugation

In these preliminary experiments to determine whether RBC's of various age respond differently to heating, cells were separated by density using an ultracentrifuge. Five to eight ml of whole rat blood was collected by heart puncture as described previously. After centrifugation at 900 X g for 5 min, the sample supernatant, containing plasma, was removed and saved. The white buffy coat was removed and discarded using a pasteur pippette. Cells were then washed once in glucose-saline and resuspended in plasma to 80% Hct. Approximately 0.5 ml of RBC suspension was removed to serve as a non-ultracentrifuge control.

Cells were centrifuged at 27,000 X g for 1 hr at 37°C using a Beckman J2-21 centrifuge with a fixed-angle JA20.1 rotor.

Following centrifugation the supernatant plasma was removed from the resulting 3 to 5 ml pellet by aspirating with a pasteur pippette. Separation of three fractions of different densities located in the top, middle, and bottom regions of the pellet was performed as follows. First, approximately 0.5 ml from the top of the pellet was removed by aspirating with a pasteur pippette and labeled fraction I. When aspiring cells, the tip of the pasteur pipette was carefully controlled so that it remained at the surface of the pellet and passed over the entire surface area of the pellet. Since the cells in this first fraction were at the top of the pellet, they were the least dense cells.

Next, approximately 0.5 - 1.5 ml of RBC's were aspired from the pellet and saved as part of a pooled ultracentrifuged control containing RBC samples from different regions of the pellet. Then 0.5 ml of RBC's were carefully aspired from the pellet and labeled fraction II. Again 0.5 - 1.5 ml of RBC's was aspired and pooled with the ultracentrifuged control sample. Finally, 0.5 ml of the bottom fraction was aspired and labeled fraction III. This population of cells found at the bottom of the centrifuged RBC sample were the most dense population of cells. Any remaining cells were pooled with the ultracentrifuge control sample.

Using the above method, three RBC populations of different densities and two controls were separated from whole blood. The first control was a population of RBC's that was not ultracentrifuged. The second control consisted of cells from different regions of the pellet following centrifugation at 27,000 X g for 1 hr. By comparing the heat response of the two controls, it was possible to determine whether ultracentrifugation affects heat induced hemolysis of red cells.

All fractions and controls were washed twice and resuspended in MOPS buffered glucose-saline to approximately 30% Hct. To determine whether each fraction contained RBC's of different density, the mean relative hemoglobin concentration per cell of each fraction was calculated.

Hemoglobin Concentration/Cell Determination

The relative Hb density of each fraction was determined only to verify that the ultracentrifugation succesfully separated the RBC's by density. The relative mean corpuscular hemoglobin concentration (RMCHC) for each fraction was determined by dividing the Hb concentration (in an aliquot diluted 200 fold in saline) by the RBC Hct. Since the absolute quantity of Hb occurring in a mature RBC is fixed, the Hb concentration of a RBC varies directly with the density of the cell.

Hematocrit measurements were done in duplicate by removing approximately 50 µL of RBC suspension from each fraction into Microhematocrit capillary tubes. These tubes were centrifuged in an International micro-capillary centrifuge at 500 X g for 3 minutes. Using a Lancer Spiracrit micro-hematocrit capillary tube reader that reads the percentage of packed cell volume relative to the entire sample size, the hematocrit for each fraction was recorded.

The concentration of Hb in each fraction was determined indirectly by measuring the optical density at 540 nm of aliqots that were diluted 200 fold and completely hemolyzed. The optical density of a Hb solution is directly related to the concentration of Hb in the solution by Beer's law. Dilution and lysis of aliquots was done by adding 50 µL of RBC fraction to 10 ml of deionized water. References for these measurements consist of 50 µL of RBC fraction added to 10 ml of isotonic MOPS buffered glucose-saline centrifuged along with samples. The density of

each RBC fraction in terms of the RMCHC was calculated. The MRHCH was defined to be:

$$RMCHC = \frac{O.D. * 200}{\% \text{ Hct} \div 100\ \%}$$

Since it has been established that density of cells is correlated with age, different density values of separated fractions indicated a difference in age.

If ultracentrifugation separation was successful, then each fraction sample along with controls was washed one time and diluted to 0.7% hct.. Samples and ultracentrifuged controls were heated at 50.5°C (± 0.1°C) using a Neslab exacel water bath as previously described. Survival was scored immediately following heating using the hemolysis assay previously described.

Results

Hemolysis Assay.

The hemolysis assay determines extent of hemolysis by monitoring the release of hemoglobin from the RBC. This is accomplished by recording the absorbance of the RBC suspension supernatant after the various heating protocols. It is common practice to determine Hb concentrations by measuring absorbance at 540 nm following addition of a cyanide-based Drabkin's solution. Nothing was added to our samples to stabilize the Hb molecule. During oxygen binding Hb changes conformation affecting its absorption coefficient between 540-550 nm. It was therefore necessary to determine the absorption spectrum for Hb at various times after heating, so that a wavelength could be chosen where the absorption coefficient would remain constant during lengthy incubations.

Figure 1 (page 23) shows the absorption spectrum of 0.07% Hct Hb solutions at various times during incubation at 35 ± 1°C. Figure 1 shows that the absorption spectrum for hemoglobin does vary with time during incubation at 35°C. Absorbance decreases between 530-560 nm over time. Additionally, peak

absorbance near 410 nm shifts to shorter wavelengths while increasing slightly in magnitude. After 3 days the color of the Hb solution turned dark red indicating an increase in the concentration of deoxyhemoglobin. The least variability in the absorption coefficient during the first 46 hrs of incubation occurred near 410 nm. Therefore, 410 nm was chosen to assay for hemolysis.

Survival After Heating.

Figures 2 and 3 show heat dose response curves for chicken and rat RBC's, respectively, at various temperatures when hemolysis was scored immediately after heating. The chicken RBC's are more tolerant to heat than the rat RBC's. This might be the result of better osmoregulation mechanisms present in avian RBC's (Siebens and Kregenow, 1985). Also, since the mean physiological temperature for a chicken is 41.0°C while that for a rat is 39°C, the chicken RBC's, which exist in a higher temperature environment, may experience less stress than rat RBC's during 50.5°C hyperthermia.

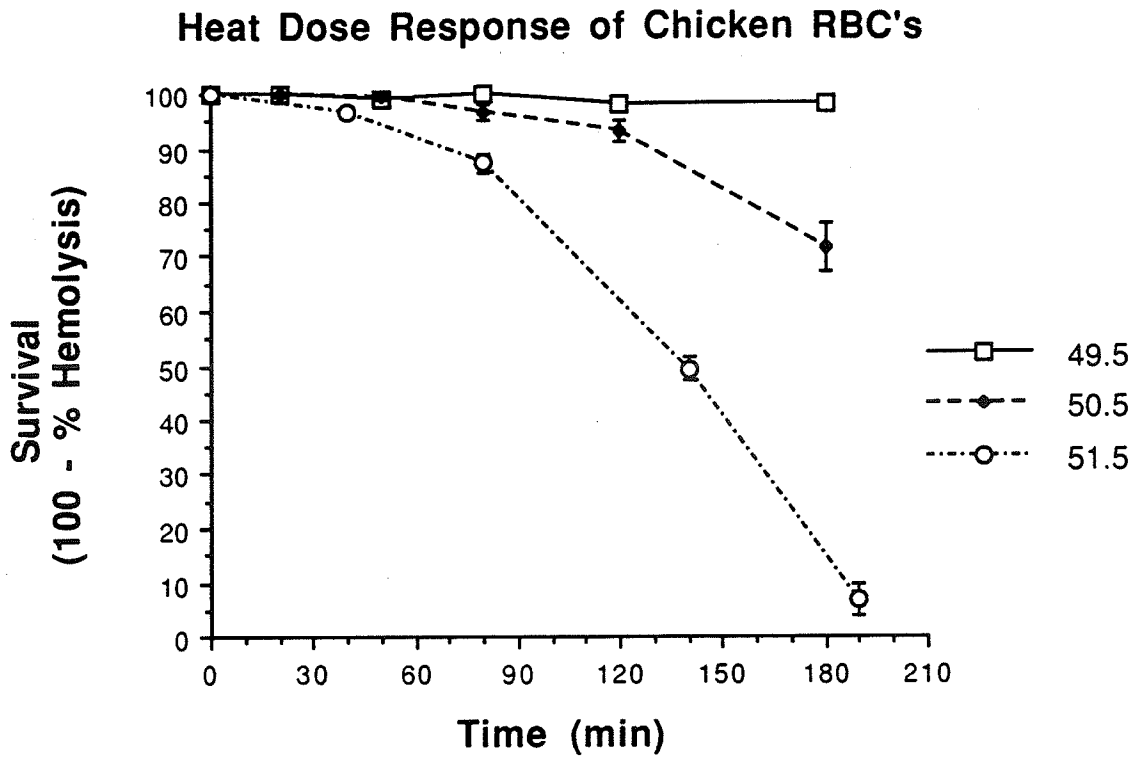

Figure 2. Heat Dose Response of Chicken RBC's.
The heat dose response of chicken RBC's exposed to 49.5°C, 50.5°C, and 51.5°C is shown. Cells were scored for survival immediately after exposures. Plotted as mean ± S.E.M. (n=5)

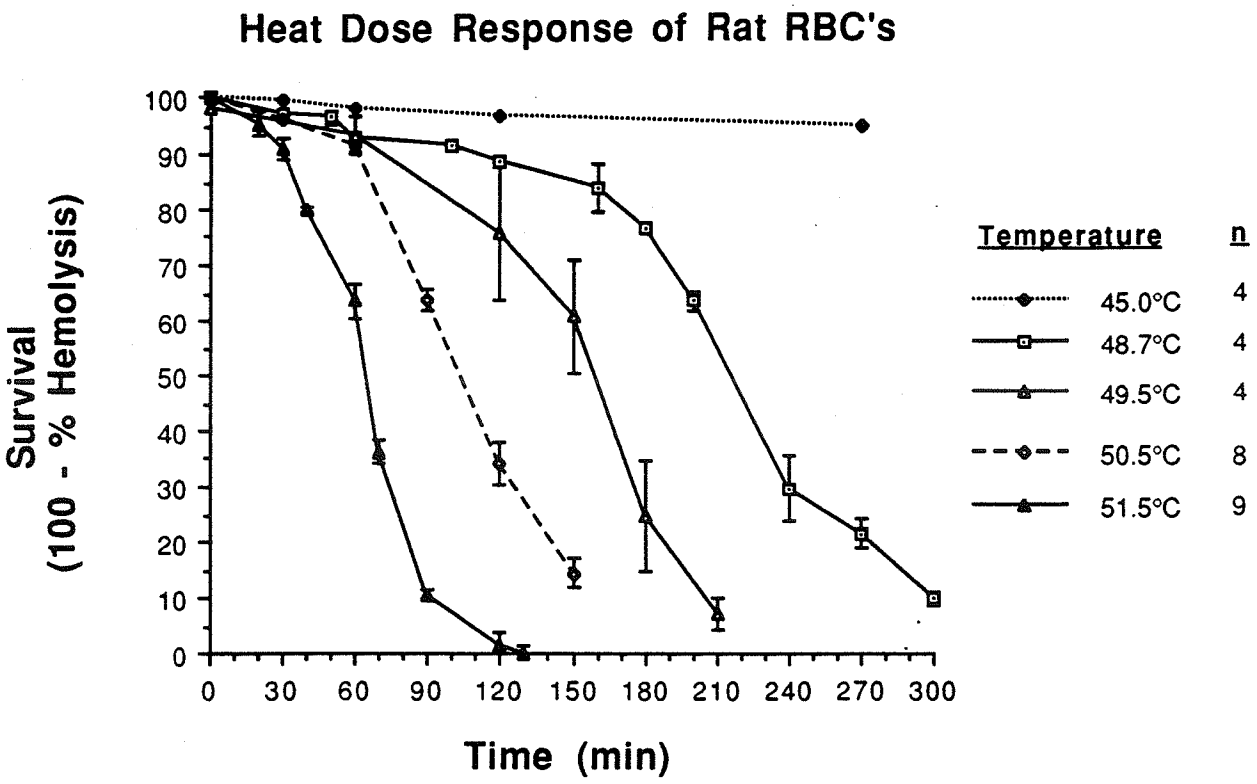

Figure 3. Heat Dose Response of Rat RBC's.
The heat dose response of rat RBC's exposed at various temperatures: 45°C, 48.7°C, 49.5°C, 50.5°C, and 51.5°C is shown. Cells were scored immediately after exposures. Plotted as mean ± S.E.M.

Figure 4 shows the survival of rat RBC's heated at 50.5°C for various times and the equations that fit the two phases of the curve. A shoulder exists for the first 60 minutes of heating. For exposure times greater than 60 minutes the cells hemolyze to a much greater extent.

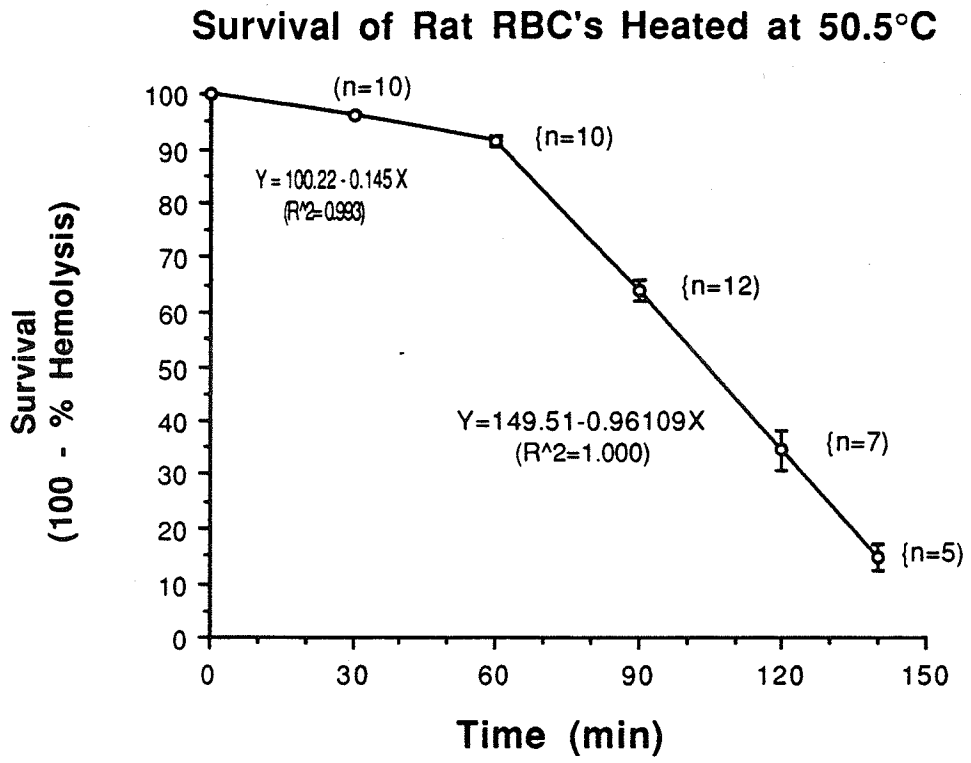

Figure 4. Survival of Rat RBC's Heated at 50.5°C.
Figure shows the survival of rat RBC's heated at 50.5°C as well as the equations fitting the two phases of the curve. Cells were scored for survival immediately after exposures. Plotted as mean ± S.E.M. The n values are as indicated.

Figure 5 shows the survival kinetics of rat RBC's heated at 50.5°C for 30 or 60 minutes followed by incubation at 35 ± 1°C for up to 54 hours. Cells heated for 60 minutes at 50.5°C hemolyze to 25 % survival after 20 hours of incubation at 35°C while cells heated for 30 minutes hemolyse only to 80 % survival during the same incubation period. This indicates that significantly more damage occurs to the RBC's during 60 minutes of exposure to 50.5°C than after 30 minutes of exposure. A component of the RBC which maintains membrane integrity is damaged between 30 and 60 minutes of heating at 50.5°C.

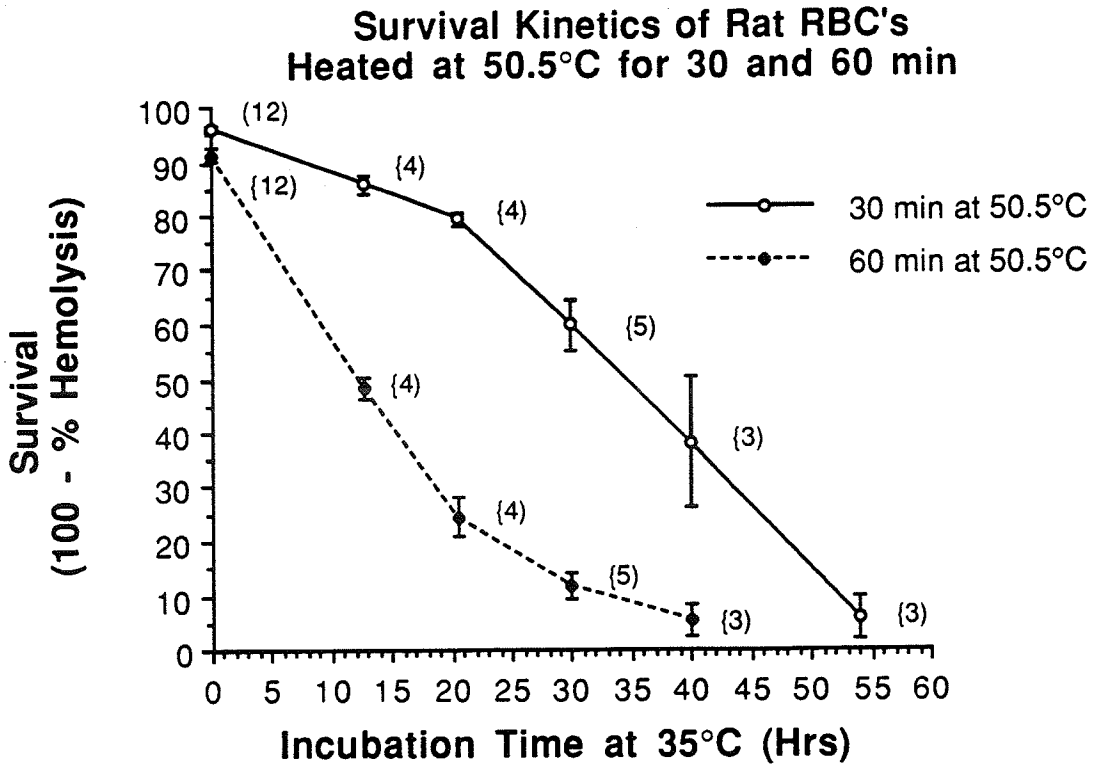

Figure 5. Survival Kinetics of Rat RBC's Heated at 50.5°C for 30 and 60 min. RBC's were heated at 50.5°C for 30 min or 60 min followed by incubation at 35±1°C. The n values are shown in parenthesis.

Effects of pH.

Preliminary experiments with chicken RBC's exposed to 50.5°C at various pH's, between 6.6 and 8.2, indicated that cells were more sensitive to heat in more acidic environments below pH = 7.4. This agrees with the observation that cells are more sensitive to heat when exposed in more acidic environments between pH 7.6 - 6.7 (Gerweck,1977). At more alkaline pH, sensitivity again increased. In a similar experiment chicken erythrocytes were exposed to 50.5°C within the pH range 7.1 to 7.5. Within the pH range 7.2 to 7.4, heat sensitivity of the cells was stable. Because resistance to heat was relatively independent of pH within this range, the red blood cell solutions of subsequent experiments were buffered to a pH between 7.2 and 7.4.

Experiments performed on rat RBC's confirmed that alkaline environments increased RBC sensitivity to heat (figure 6). RBC's in a pH 8.0 solution were more sensitive to heat than cells in pH 7.4 or 6.8.

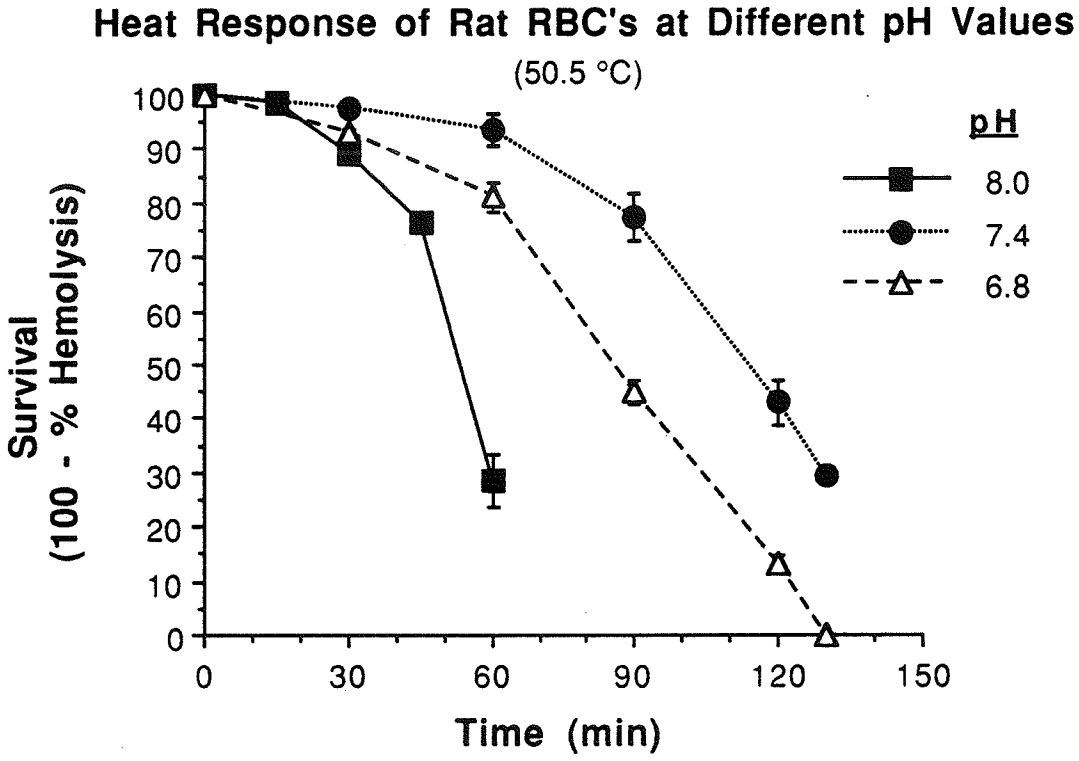

Figure 6. Heat Response of Rat RBC's at Different pH Values.
Cells were suspended in isotonic glucose-saline solutions at various pH (8.0, 7.4, and 6.8) and heated at 50.5°C for up to 130 minutes. Cells were scored immediately after heating. Results confirm that cells are more sensitive to heat in acidic environments.

Heat shock.

I was able to induce thermotolerance in chicken RBC, but was unable to induce thermotolerance in fresh rat RBC's. Many heating protocols using chicken RBC's were tested before a protocol that induced thermotolerance was found. Table 1 shows the temperatures and exposure times along with incubation periods at 37°C for the different acute heat shock protocols tested in chicken RBC's. Although some protocols did result in slight levels of thermotolerance, these were slight and not always reproducible.

Table of Acute Heat Shock Protocols Using Chicken RBC's

Temperature (°C)	HS ExposureTime (min)	Inubation Time at 37°C (hr)	Result (Tolerance)
43.5	10	3	~ 5%
44.0	10	3	None
44.5	10	3	~ 7%
46.0	5	5	None
46.0	10	5	None
46.0	15	5	None
46.2	10	2	None
47.0	5	3	~ 3 %
47.0	10	3	~ 3 %
50.0	5	2	None
50.0	10	2	None
50.5	5	3	None
50.5	10	3	None
50.6	10	2	None

Table 1. Acute Heat Shock Protocols Using Chicken RBC's. Following incubation, cells were challenged at 50.5°C or 51.5° for up to 130 min. Survival was scored immediately after challenge.

Consistant induction of thermotolerance in chicken red cells was achieved following chronic heat shock of cells at 42.4°C. The maximum amount of heat protection was generated following 2 hours at 42.4°C (Figure 7). Induction of thermotolerance was tested by exposing cells to 51.5°C for 2 hr.

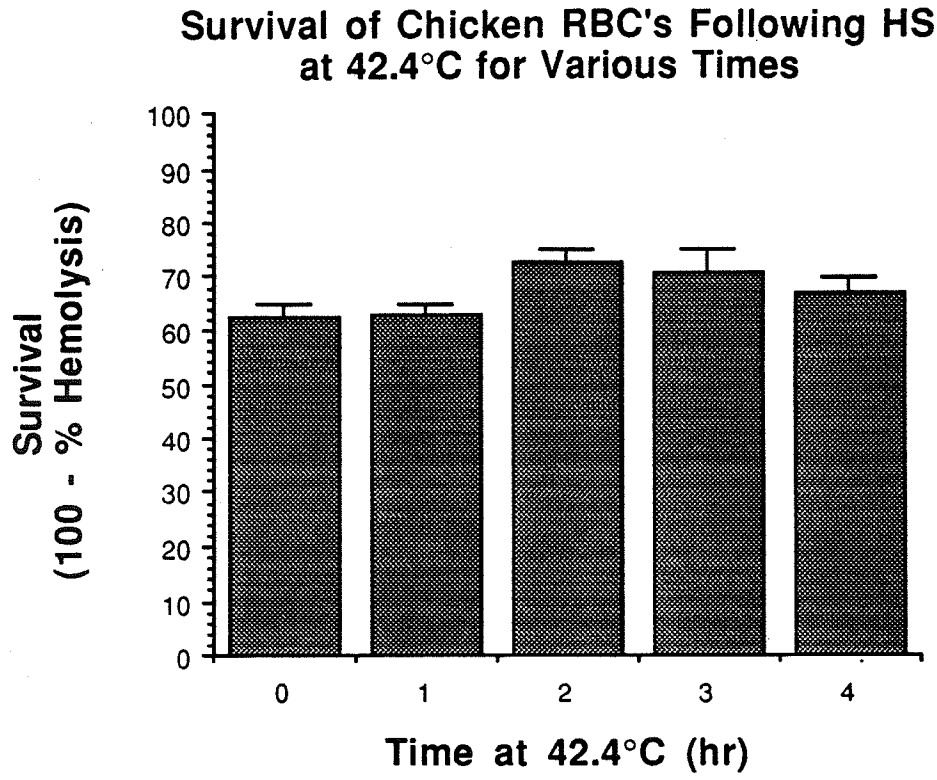

Figure 7. Survival of Chicken RBC's Following HS at 42.4°C for Various Times. Cells were heat-shocked at 42.4°C for 0 to 4 hours followed by heating at 51.5°C for 120 minutes. Cells were scored for hemolysis immediately following 51.5°C heating. Best survival occurred following 2 hours at 42.4°C. Plotted as mean ± SEM.

The level of thermotolerance following exposure to 51.5°C is shown in figure 8. Survival of cells that have been heat-shocked is compared to cells that were maintained at 23°C for two hours prior to heating at 51.5°C. It is not certain whether 42.4°C is the best temperature to induce thermotolerance in chicken RBC's. Four hour chronic exposures at temperatures of 42.2 and 42.7°C were tried with less than a 4% increase in the survival of chicken RBC's assayed immediately after heating at 51.5°C for 2 hr. Greater levels of thermotolerance was evident in these populations of cells when hemolysis was assayed after 24 hr of incubation at 37°C (Also see Lee, 1991).

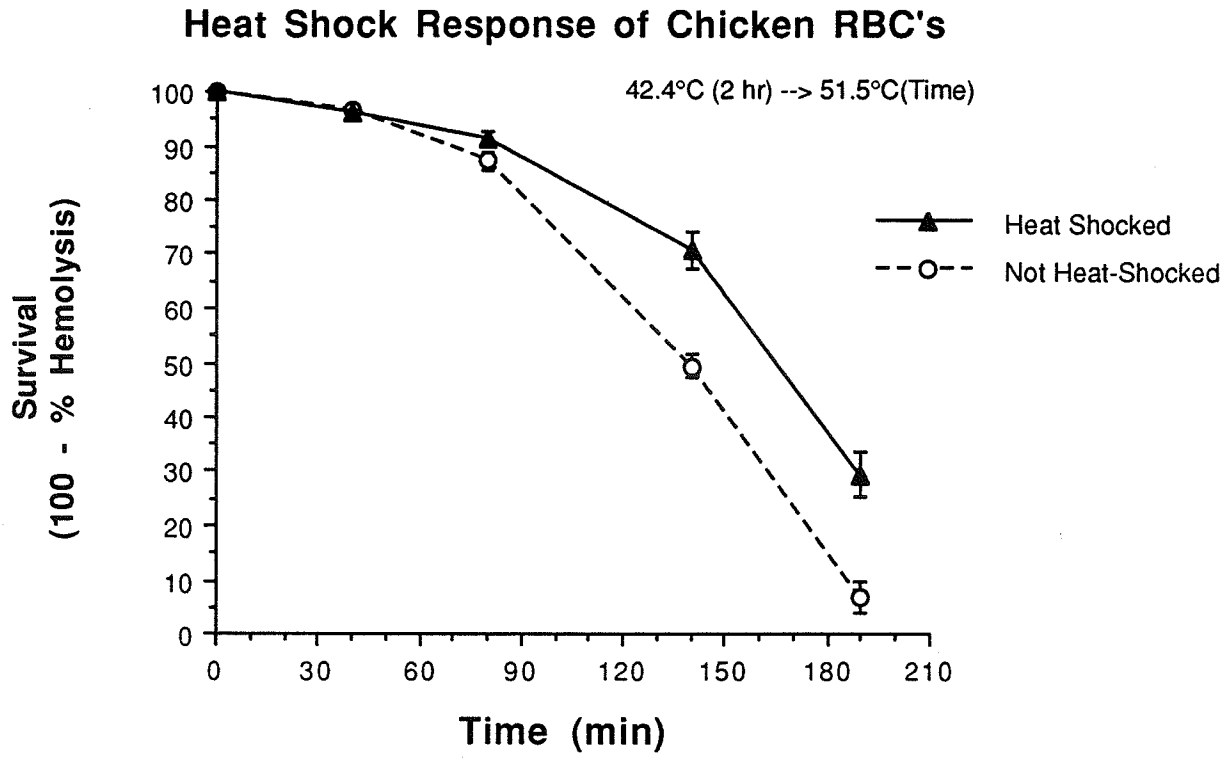

Figure 8. Heat Shock Response of Chicken RBC's.
The survival of chicken red cells exposed to 51.5°C from 0 to 190 minutes with and without a prior heat shock is shown. Heat-shocked cells were exposed to 42.4°C for 2 hr prior to 51.5°C challenge. Non-heat-shocked samples were maintained at 23°C for 2 hr prior to 51.5°C challenge. Cells were scored for extent of hemolysis immediately after heating. Results indicate significantly increased survival of heat-shocked cells. Plotted as mean ± S.E.M. (n=5).

Rat. Several chronic and acute heat shock exposures were tried on rat RBC's. Greatest thermotolerance was obtained at 48.6°C after 6 minutes exposure followed by 1 to 3 hours at 37.0°C (Table 2). In the positive trials, the induction of thermotolerance was not always repeatable. It was determined that induction of thermotolerance only occurred in those cells which were refrigerated overnight at 3°C. Although several heating protocols were tested, not all possible temperatures and heating durations were tried. Table 2 is not meant as proof that induction of thermotolerance absolutely does not occur in rat RBC. Therefore, it is possible that induction of thermotolarnce occurs in rat RBC's following a heating protocol not indicated in table 2.

Heat Shock Protocol Trials Using Rat RBC's

Chronic Exposures

Temperature (°C)	ExposureTime (hr)	Result (Tolerance)
40.6	2	None
40.6	3	None
42.5	2	None
42.5	3	None

Acute Exposures

Temperature (°C)	ExposureTime (min)	Incubation Time at 37°C (hr)	Result (Tolerance)
45.0	7	6	None
45.0	15	6	None
46.7	7	4	None
46.7	15	4	None
46.0	15	5	None
46.2	10	2	None
48.0	4	3	~ 6 %
48.0	6	3	0 to ~ 11%
48.0	7	3	0 to~ 7 %
48.0	8	3	~ 11%
48.0	10	3	0 to ~ 9%
48.3	7	3	None
48.3	15	3	None
48.6	3	3	~ 2%
48.6	6	1	~ 12%
48.6	6	2	~ 9%
48.6	6	2.5	~ 7%
48.6	6	3	0 to 14%
48.6	6	4	None
48.6	7	3	0 to ~ 5%
48.6	10	3	~ 4%
48.6	15	3	None
48.6	20	3	None
48.6	30	3	None
49.0	7	4	None
49.0	15	4	None
50.9	3	2	None
50.9	7	2	None

Table 2. Heat Shock Protocol Trials Using Rat RBC's.
Following incubation, cells were challenged at 48.7°C or 50.5° for up to 130 min. Survival was scored immediately after challenge. It was determined that the observed thermotolerance only occurred in RBC's which were refrigerated overnight.

Figure 9 compares the dose response curves of rat RBC's exposed to 50.5°C with and without a prior heat shock at 48.6°C for 6 minutes. There appears to be slight tolerance lasting up to 2 hours during exposure at 50.5°C when the cells were refrigerated over night. When fresh cells were tested no significant difference was found between heat-shocked and control cells. Therefore, the induction of thermotolerance occurring in refrigerated rat RBC's may be an artifact resulting from refrigeration.

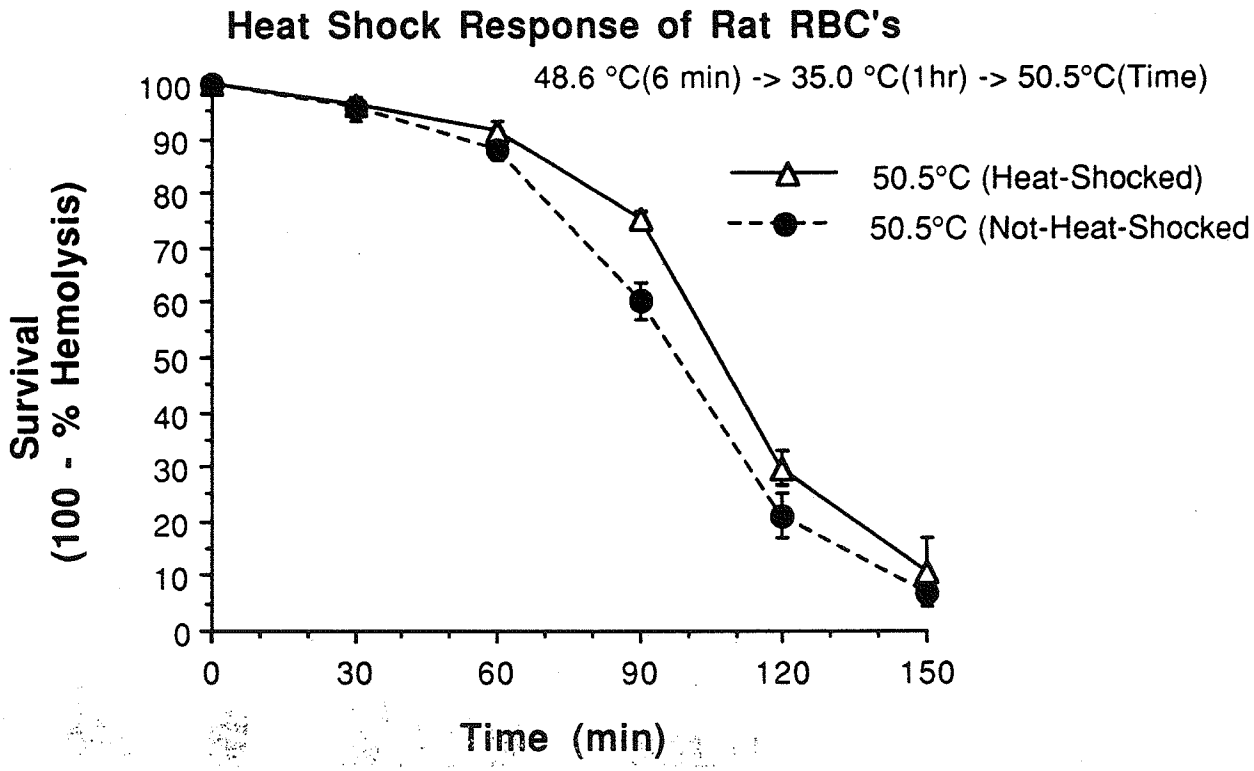

Figure 9. Heat Shock Response of Rat RBC's.
The heat response of rat RBC's to 50.5°C with and without a prior heat shock is shown in RBC's which were refrigerated overnight at 3°C. Heat shock consisted of heating cells at 48.6°C for 6 minutes then incubating cells at 35±1°C for one hour before exposure to 50.5°C. While samples were being heat shocked, controls remained at 23°C. Both heat-shocked and control samples were heated from 0 to 120 minutes at 50.5°C. Fresh RBC's did not show thermotolerance when heated and maintained greater survival when heated at 50.5°C. Plotted as mean ± S.E.M. (n=5)

There was concern that exposure to 42.4°C is not considered a heat shock, since it is only slightly above normal physiological temperature. Perhaps this

temperature maintained cells better than 22°C, the temperature of controls. To check this, rat RBC's were incubated at 0, 22, and 38°C for 2.5 hours before heating at 50.5°C. The results are shown in figure 10. The normal physiological temperature for rats is approximately 39°C. The survival after heating cells at 50.5°C following pre-incubation at three temperatures is essential the same for all temperatures. In addition, Lee (1991) showed that chicken RBC's pre-incubated for 1 hr at 5, 22, 35, and 39.5°C had the same heat dose response even when survival was scored following incubation at 37°C for three days after heat exposure.

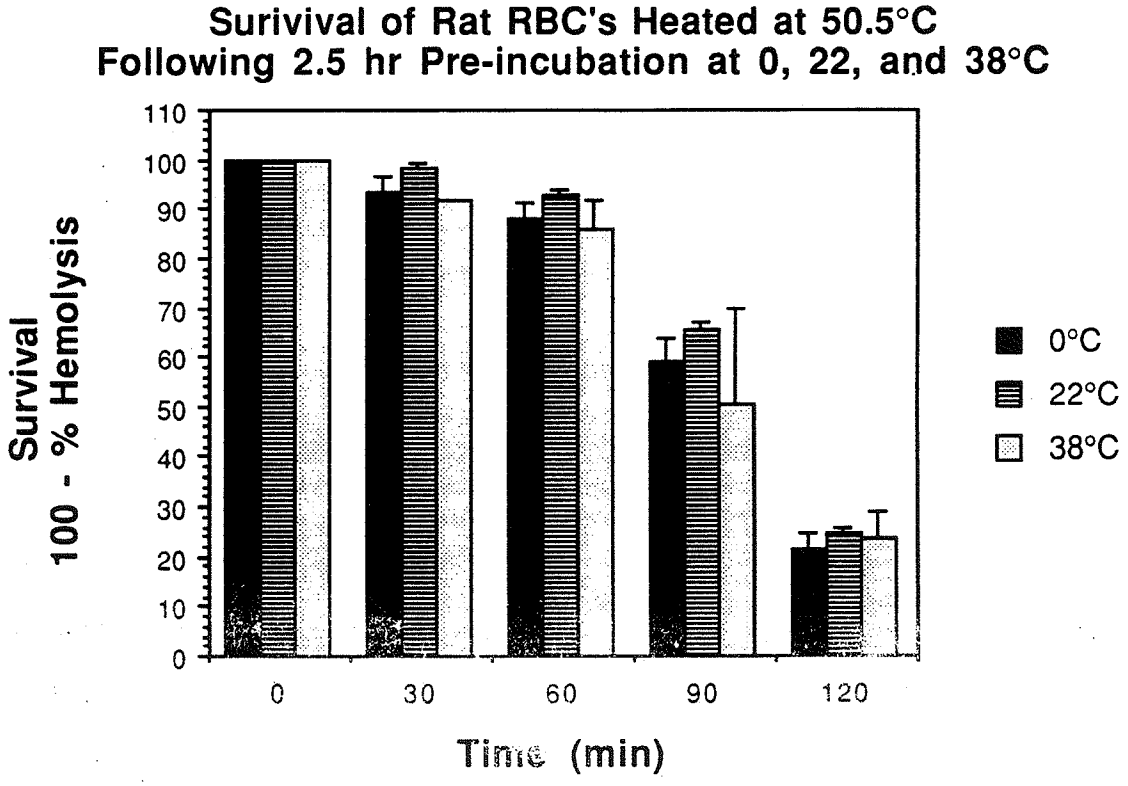

Figure 10. Survival of Rat RBC's Heated at 50.5°C Following 2.5 hrs Pre-incubation at 0, 22, and 38°C.

The survival of rat RBC's at 50.5°C following 2.5 hr. incubation at 0, 22, and 38°C. Cells were incubated at 0, 22, and 38°C for 2.5 hours before heating at 50.5°C. Results indicate that there was no significant difference in survival following either of the pre-incubation temperatures. Plotted as mean ±S.E.M. (n=3)

Age and Heat Response

Three fractions containing cell populations were separated from rat whole blood and assayed for heat response. Table 3 illustrates characteristic data for calculation of RMCHC.

Fraction	Avg. Hct.	OD_{540}	RMCHC
I	21.75	0.235	216
II	26.50	0.305	230
III	21.75	0.260	240
No Spin	22.15	0.234	212
Control	22.50	0.250	222

Table 3. Fractions and Controls Separated by Ultracentrifugation. RMCHC values calculated for each fraction indicate that separation was successful.

Fraction I is the top fraction and is calculated to be the least dense, while the bottom fraction, III, is found to be the most dense. The density of the control is approximately the average of the three fractions. The density of the control that was not centrifuged is less than any of the centrifuged fractions.

If following ultracentrifugation, cell separation by density was determined not to have occurred, based on RMCHC calculations, only two fractions of cells having the most different RMCHC values were heated at 50.5°C.

Figure 11 shows the survival curves following heating at 50.5°C of four fractions having different RMCHC values from several experiments. Survival was similar for each fraction, including non-centrifuged controls, except for the fraction that was the least dense having a RMCHC of 202. It has been shown by staining reticulocytes with new methylene blue that the least dense fraction is high in reticulocyte count (Isaacks *et al.*, 1982). If the least dense fraction in these experiments, which shows the most heat tolerance, is reticulocyte enriched, then this would indicate that the reticulocyte is more resistant to heat than the erythrocyte.

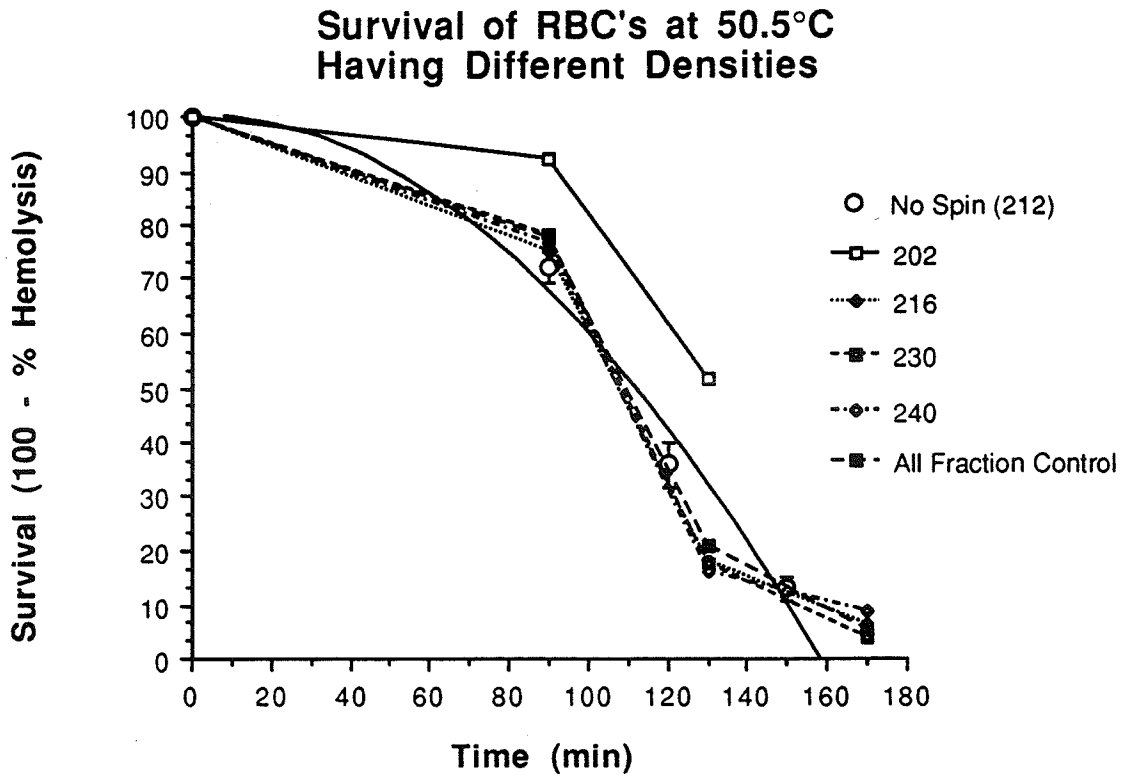

Figure 11. Survival of RBC at 50.5°C Having Different Densities. Cells were separated by centrifugation at 27,000 X g at 37°C for 1 hr. Only the least dense cell fractions response to heating is different from controls. This fraction may be reticulocyte enriched.

The preliminary data shown in figure 11 suggest that the sensitivity of mature erythrocytes to heat induced hemolysis is not age dependent.

Discussion

Effects of Heat.

Each of the heat survival curves exhibits a shoulder. This shoulder may indicate that the cellular inactivation of these RBC's is a two step process (Jung, 1986). First a non-lethal lesion is produced. If further damage does not occur the cells can recover. In the second step the non-lethal lesion is converted to a lethal lesion and the cells die. According to Jung's model, larger shoulders signify slower conversion of nonlethal lesions to lethal lesions. Therefore, the wider shoulders associated with lower temperature exposures of RBC's result because the

conversion process from a non-lethal lesion to a lesion that causes hemolysis is slower.

Another explanation for the shoulder is that multiple lesions have to occur before the cell is inactivated, similar to the multi-target theory of radiation. In this theory the cells exhibiting shoulders are able to survive sublethal damage. The larger shoulders result because a greater number of inactivating events must occur before cells are killed. According to the multi-target theory, the wider shoulders associated with the lower temperature exposures of RBC's would indicate that lower temperatures require the inactivation of more targets for hemolysis to occur. The validity of the multi-target theory in the case of RBC's would require that the mechanism of hemolysis is different at different temperatures.

We must next consider the target(s) for cellular injury in the heated RBC. Studies of the lethal effects of heat on proliferative cells usually utilize temperatures below 49.0°C and reproductive death is scored several days after heating. In this investigation, a method was developed to score damage in terminally-differentiated cells using hemolysis as the end-point for death. In these experiments cells were scored immediately after heating. A cell is killed by any stressor when its most sensitive life-limiting component succumbs to the stress. This investigation is observing a different life-limiting injury than in work with proliferative cells.

As suggested in the introduction, hyperthermia most likely acts first on RBC membrane proteins (Grzelinska *et al.*, 1982; Leyko and Bartosz, 1986). Grzelinska *et al.* (1982) showed that lipid disturbances following 50.0°C heating of RBC membranes were due to secondary effects mediated by alterations in membrane proteins. Additional work implicating membrane proteins as the target of heat damage comes from work with proliferative cells where survival is scored several days after heating by reproductive death. Several cellular proteins are implicated

by Dewey (1989) as the critical target of heat . Bowler (1981) postulated that impairment of the regulation of permeability or inactivation of membrane enzymes could be a primary lesion in heat injury causing a cascade of secondary events leading to death. Glass *et al.* (1985), showed a correlation between 45.0°C hyperthermia and the disappearances of actin stress fibers in CHO cells. The loss of fibers caused the cell shape to change. The stress fibers reappeared in 90 percent of the cells within four hours of incubation at 37°C which paralleled the recovery of protein synthesis including HSPs. In addition, the protein synthesis inhibitors cycloheximide and puromycin prevented the reappearance of stress fibers suggesting that newly synthesized proteins are required. Lepock (1982) also suggests that proteins are involved in membrane damage by heat, but that alterations in lipid fluidity greatly influence membrane protein heat sensitivity.

The RBC membrane protein that may act as the critical target at the temperatures used in this investigation is spectrin Using scanning calorimetry and circular dichroism on human RBC's, four distinct thermal transition occurred within the temperature range of 45.0 - 80.0°C (Brandts *et al.*, 1977). These transitions indicate a loss of secondary structure. The reversible A transition was centered at 49.5°C and was due to spectrin as shown by extraction experiments. The other three transitions involved other membrane proteins, but occurred above 58°C which is well above the temperatures used in this study. A change in the structure of spectrin may affect the integrity of the RBC membrane. Since only one thermal transition was observed by Brandts *et al.* (1977) within the temperature range of our investigations, it is unlikely that different mechanisms are acting to cause hemolysis at the various temperatures used in this investigation.

Cellular inactivation by heat seems to follow Jung's model. An initial lesion is produced that is converted to a lethal lesion as heating continues. This is

supported by figures 4 and 5. Figure 4 (pg. 33) shows a shoulder that lasts for approximately 60 minutes during heating at 50.5°C. The shoulder terminates between 30 and 60 minutes. Applying Jung's model, the threshold for the lethal lesion that causes prompt hemolysis is between 30 and 60 minutes of heating at 50.5°C to account for the termination of the shoulder on the survival curve. Figure 5 (pg. 34) shows the survival kinetics of RBC's following heating at 30 or 60 minutes at 50.5°C. Those cells heated for 60 min hemolyze within a shorter time than the cells heated for 30 min, since the lethal lesion hasn't yet formed in the sample heated for 30 min. It requires between 15 and 20 hr at 35°C to convert the sub-lethal lesion formed by heating the RBC's for 30 min at 50.5°C to a lethal lesion. This is evident by the change in slope of the survival curve to nearly match the slope of the curve heated for 60 min.

When erythrocytes are heated, several of the elaborately interacting membrane proteins may be affected. The denaturation of a minimal component may affect the stability of more abundant proteins affecting the integrity of the RBC membrane. The unfolding of spectrin described by Brandts, *et al.* (1977), may result from the denaturation of its stabilizing proteins.

pH Effects.

The pH of the suspending media affected survival as judged by heat-induced hemolysis. Changes in the pH of the extracellular fluid affect the proton gradient across the RBC membrane. This influences the intracellular pH in the same direction, but the magnitude of internal changes is less. Changes in pH affect the charge and conformation of proteins. In the erythrocyte, a decrease in pH causes Hb and inorganic phosphate to become less negative. Increased pH has the opposite effect. The decreased negative charge of Hb increases the number of anions inside the cell, increasing cell water and causing the cell to swell. This

changes the shape of the cell. As pH is lowered the RBC becomes slightly crenated (echinocyte I) and then spherical (echinocyte II). Increasing the pH produces the opposite effect, returning the cells to the discocyte stage and eventually forming cups (stomatocyte) (Bull *et al.*, 1989). As the RBC diverges from its biconcave shape it may become less stable and more sensitive to heat.

Changes in pH may also affect the stability of membrane proteins. This would affect the interactions between proteins such as band 3, spectrin, and protein 4.1. Such changes may affect the shape and membrane integrity of the cell. During heating the pH of the suspending solution becomes more acidic due to the nature of the buffer. This is investigated further in chapter 3.

Thermotolerance in RBC's.

This investigation found that thermotolerance can be induced in chicken RBC's but not in fresh rat RBC's. This might indicate that the induction of thermotolerance is dependent on a component in the chicken RBC that isn't present in the rat RBC such as the nucleus or IP_5. Although several heat shock protocols were tried in rat RBC's, not every possible combination could be tested, therefore it is not absolutely certain that rat RBC's are incapable of acquiring thermotolerance. The temperature for induction of thermotolerance in chicken RBC's, 42.4°C, was chosen based on searching through the literature for studies of various different cell lines in which induction of thermotolerance was achieved. I found that chronic induction of thermotolerance usually occurred by heat-shocking cells at a temperature approximately 1.5°C above normal physiological temperature for that cell line. Since the body temperature for chickens is approximately 41°C temperatures ranging from 42 to 43°C were tried to induce thermotolerance. In addition, Morimoto and Fodor (1985) found that chicken RBC preparations synthesized HSPs when heat shocked above 42°C. A two hour HS at

42.4°C produced the greatest amount of protection from heating at 51.5°C. Rat RBC's were heat-shocked at 40.6°C for the same reason, the average body temperature for a rat is 39°C; however, induction of thermotolerance did not occur.

In proliferating cell lines thermotolerance is usually correlated with the synthesis of heat shock proteins. No direct correlation between the kinetics of protein synthesis and development of thermotolerance, however, has been shown in red cells. Since the level of thermotolerance is relatively small, approximately 15 percent, it is possible that the thermotolerance observed in the chicken RBC preparations occurs only in the reticulocytes. Whole chicken blood contains between 10 and 20 percent reticulocytes (Lucas and Jamroz, 1961), and chicken reticulocytes were shown to synthesize HSPs when heated above 42°C for 30 minutes (Morimoto and Fodor, 1985).

A possible mechanism for the observed thermotolerance is that the induction of thermotolerance may occur as a result of the activation of HSPs that are already present in the erythrocyte. Gromov and Celis (1991) found two molecular chaperons of the HSP 70 family (HSX70, inducible and HSC 70 constitutive) and HSP 90 present in human RBC's. It is thought that these chaperon proteins help in protein folding and stabilize protein structure stressed by environmental changes. Subject *et al.* (1985) found a 68K and 89K protein associated with sheep erythrocte ghosts that co-electrophoresed with HSPs from CHO cells. These constituitive HSPs may normally be in an inactive state until the cell is stressed. Once activated these proteins could help stabilize membrane proteins to maintain membrane integrity. If this is the mechanism for induction of thermotolerance in chicken RBC's, then thermotolerance would also be expected to occur in rat RBC's.

Another mechanism to explain the observed thermotolerance in chicken RBC's is that following a stress, HSPs are translated from the mobilization of a

cytroplasmic pool of free, inactive mRNAs on to polyribosomes. Ferritin, an iron sequestering HSP, was shown to be translationally regulated in the avian reticulocyte (Atkinson *et al.*, 1990). By protecting the erythrocyte from free iron during heating, it may invoke thermotolerance. Since large quantities of this mRNA is present in the reticulocyte, it is also likely that the un-translated mRNA for ferritin would remain present in the mature erythrocyte. Induction of thermotolerace would require that this residual mRNA be translated into protein. Zentgraf *et al.* (1975) found that mature hen erythrocytes maintained low levels of transcription, but translation of newly transcribed RNA did not occur, because the RNA was not transported out of the nucleus. They did not investigate whether translation of mRNA already present in the cytoplasm could occur.

The above propositions could be tested by separating out the reticulocytes from the blood preparation and by using protein synthesis inhibitors such as cycloheximide and actinomycin D. Reticulocytes from chickens made anemic by daily bleeding could be separated from mature RBC's by ultracentrifugation (Isaacks *et al.*, 1983). The induction of thermotolerance could then be tested in this population of reticulocyte. If the experimentally observed thermotolerance only occurs in reticulocytes, then it is expected that greater levels of thermotolerance would be observed in a population of reticulocytes. If the level of protection does not increase significantly, then it would appear that thermotolerance also occurs in both reticulocytes and mature RBC's.

Thermotolerance occurring in mature erythrocytes could result from *de novo* synthesis of HSPs or the activation of pre-existing proteins. Both cycloheximide and actinomycin D prevent protein synthesis. Actinomycin D blocks transcription, whlie cycloheximide blocks translation. If following the addition of actinomycin D, the experimentally observed induction of thermotolerance in chicken RBC's is

prevented, then it is likely that induction of thermotolerance in RBC's is due to protein synthesis from newly transcribed mRNA. If cycloheximide prevents induction of thermotolerance, while actinomycin D does not, then thermotolerance is caused by the translation of pre-existing mRNA. If neither agent blocks induction of thermotolerance and reticulocytes don't exhibit elevated levels of tolerance, then it is possible that pre-existing dormant HSPs are activated following a stessor to help stabilize the RBC, or another mechanism not involving proteins is responsible for the observed thermotolerance. Since rat RBC's contain no nucleus and organelles, they would not be expected to develop thermotolerance from *de novo* protein synthesis.

Changes in heat sensitivity at the level of the membrane have been investigated in several different cell lines. Cress and Gerner (1980) found a correlation between the amount of cholesterol in the plasma membrane and sensitivity to heat in five different cell lines. Cells with greater cholesterol content had less membrane fluidity and increased heat resistance.

Anderson *et al.* (1981) showed that homeoviscous adaptation induced by growing CHO cells at higher temperatures caused a change in the cholesterol phospholipid ratio that correlated well with changes in membrane fluidity. As growth temperature increased, the cholesterol content increased and fluidity decreased.

Changes in the membrane propeties of thermotolernant cells were investigated by Konings and Ruifrok (1985). They found no correlation between heat sensitivity and either cholesterol or phospholipid content in HeLa S3 cells, Fibroblasts and Ehrlich ascites. They also found no difference in the fraction of cholesterol and phospholipids in cells that were made thermotolerant by heat shock and non-thermotolerant cells. Also the phospholipid composition of

thermotolerant fibroblasts did not change. Further no differences were observed in the membrane fluidity of normal and thermotolerant cells using spin labels as probes.

Konings and Ruifrok (1985) suggested that the mechanism of thermal adaptation is different from the mechanism of thermotolerance since thermal adaptation takes place over a longer period, days or weeks, while thermotolerance develops within hours. It is therefore unlikely that the thermotolerance observed in these investigations is due to changes in phopholipid composition since this takes several days.

Konings and Ruifrok (1985) along with leyko and Bartosz (1986) suggest that thermotolerance requires stabilization of membrane protein structures. Stable proteins are more resistant to heat and they may also decrease membrane fluidity. Decreased fluidity appears to be associated with increased heat resistance. Thermotolerance in RBC's, therefore, might be a result of stabilization of membrane proteins by alterations in the interactions of membrane proteins. Chronic heat shock may affect the binding of membrane proteins to one another, decreasing the fluidity of membranes either directly or indirectly via changes in ion concentrations. Interactions of this kind may require ATP. This type of mechanism may have evolved in terminally-differentiated cells which cannot manufacture stabilizing proteins or enzymes to alter the non-protein components of membrane.
If the induction of thermotolerance in chicken RBC's results from increased interactions between membrane proteins causing rigidification of the membrane, then changes in pH, which affect membrane protein interactions, may cause a decreased level of thermotolerance in RBC's. This has not been shown in my investigation. The following chapter discusses the use of NMR spectroscopy to

determine whether energetic changes might be the cause for the induction of thermotolerance in chicken RBC's.

Cell Age and Heat Response

Different density fractions, which represent cells of different age, were assayed for their heat response. As RBC's age, they develop greater osmotic fragility, decreased volume, and decreased metabolism (Kay,1990). Although the mechanism that causes hemolysis of RBC's following heating is not known, it might be possible that the changes that occur as a RBC ages would affect the sensitivity of the RBC to heat induced hemolysis. Therefore, it was expected that the older, denser cell populations would hemolyze earlier than younger cell populations.

In these preliminary experiments it was determined that cell populations of different age, hemolyze to the same extent following equivalent heat doses. This suggests that none of the physiological changes that occur as the red cell ages are involved in the mechanism for heat induced hemolysis.

In several trials, the cells that were ultracentrifuged were found to be more dense than the cells that were not ultracentrifuged. This might have resulted from the RBC's undergoing changes during ultracentrifugation. It has been suggested by Murphy (1973) that there is potassium ion leakage during centrifugation at 27,000 X g. Leakage of ion during centrifugation would affect the cell volume and density, but it appears to not affect the heat induced hemolysis response. This is true since fractions that were not centrifuged had the same response to heating as samples that were centrifuged (Figure 11). In addition, Lee (1991) found no correlation between heat induced hemolysis and potassium leakage in avian RBC's following heating.

It was also determined that the least dense fraction was more heat resistant than the other fractions. It has been shown by staining reticulocytes with new

methylene blue that the least dense fraction is high in reticulocyte count (Isaacks *et al.*, 1982). If this fraction is reticulocyte enriched, then this would suggest that the reticulocyte is more resistant to heat than the erythrocyte. As described previously, this method of ultracentrifugation could be used to separate out reticulocyte enriched populations of RBC's without the use of any foreign agents. This would allow one to determine whether the reticulocyte population is in fact more resistant to heat induced hemolysis, and whether the thermotolerance observed in figures 7 and 8 is occuring in only reticulocytes.

Chapter 3

NMR Investigations

This work was initially undertaken in an attempt to monitor the levels of ATP in the RBC during various heating protocols. Published work had indicated that decreased ATP levels are associated with increased sensitivity to heat. Nagle *et al.* (1982) showed that Chinese hamster V79 fibroblast cells were significantly more sensitive to heating at 42°C when ATP levels were depleted by inhibiting glycolysis or depriving the cells of glucose. Additionally, Gerweck *et al.* (1984) showed that decreasing ATP levels by depriving CHO cells of both glucose and oxygen made these cells more sensitive to heating at 44°C. Depriving the cells of oxygen or glucose alone, which did not affect ATP levels, did not change heat sensitivity.

Other studies had the objective of determining whether ATP levels could be correlated with cell survival by monitoring ATP concentrations during heating. Lilly *et al.* (1984) investigated murine osteosarcoma cells heated to 40°C *in vivo* using ^{31}P NMR spectroscopy. They found a dose-related loss of high energy phosphates, along with an increase in inorganic phosphate (Pi), and fall in intracellular pH. These result s could stem from a decreased nutrient supply arising from an impaired vasculature; however, Henle *et al.* (1984) investigated whether a correlation existed between ATP levels and heat sensitivity in asynchronous CHO cells *in vitro*. They found no correlation between the concentration of ATP and cell survival during 45°C heating in either control or thermotolerant cells. ATP levels monitored using the chemiluminescence luciferin-luciferase reaction remained constant even when cell survival decreased 300-fold.

Since cellular ATP levels may in certain instances correlate with heat sensitivity, some groups have investigated the ATP levels during heat shock to

determine if ATP levels change during the induction of thermotolerance. Lunec and Cresswell (1983) were able to correlate the appearance and decay of thermotolerance with the ability to maintain ATP levels measured using the luciferase method in murine lymphoma (L5178YS) cells. They found no difference in the ATP levels of thermotolerant Ehrlich ascites cells which maintained ATP levels even when survival was poor.

Knop *et al.* (1985) monitored cell survival, HSP synthesis, and dynamic intracellular ATP levels in Chinese Hamster V79 cells *in vitro,* using ^{31}P NMRS. Thermotolerance was induced by exposing cells to 43°C for one hour followed by 5-hour incubation at 37°C. Cells were then exposed to 43°C for one or two hours. Cells receiving prior HS were found to be thermotolerant to the second exposure of 43°C. ATP levels in this population of cells showed no change from steady state levels during the HS. ATP levels remained constant even after the second exposure to 43°C. Depleting cellular glutathione inhibited thermotolerance induction, but did not affect ATP levels. The ATP levels in the control cells which did not receive a HS were not monitored during the 43°C challenge to establish whether the ATP levels decreased. My investigation compares the ^{31}P NMR spectra from chicken RBC's which acquired thermotolerance by heat shock with non-thermotolerant RBC's during a second heat challenge. Since the activity and interactions of several RBC membrane proteins are ATP dependent, it is likely that changes in levels of ATP may affect RBC heat sensitivity.

Materials and Methods

GN300 system.

^{31}P NMR spectra were recorded at 121.48 MHz on a GN300, 7.05 Tesla wide-bore spectrometer using a 12-mm $^{1}H/^{31}P$ probe. Data acquisition parameters were set to maximize signal to noise ratio in order to obtain precise

peak positions (pulse duration = 16 μs, 22° flip angle at 0.5 sec intervals, sweep width = ± 4000 Hz, data points = 8192, number of free induction decays (FIDs)=3500).

Prior to November 1991 a GE/Nicolet computer system running GEM software controlled the spectrometer. Free Induction Decays were Fourier transformed and phased using the GE/Nicolet system. The peak positions of selected spectra were determined by transferring transformed spectra to the Chemical Sciences Vax computer where the overlapping peak areas corresponding to inositol pentaphosphate were deconvoluted using the program NMR1 (New Methods Research, Syracuse).

After November 1991, the spectrometer was interfaced with a Macintosh IIci, using hardware provided by Tecmag. MacNMR software (Tecmag) controlled the spectrometer and also transformed spectra. The peak positions of overlapping peaks were determined by first deconvoluting peaks using a Gaussian multiplication subroutine of the software.

The areas under the peaks were determined by using the integration subroutines of the various software packages. The area under a peak can be, in certain instances, used to calculate the concentration of the compound the peak represents. The absolute concentrations of the observed phosphate metabolites could not be calculated in this study, however, due to the rapid pulse technique used. Instead, relative concentrations were determined by dividing the peak areas of interest by the total area of all the peaks corresponding to the naturally occurring phosphate compounds found in the RBC. Relative ATP concentrations were determined by calculating the area only under the peak corresponding to β-ATP, since the peaks corresponding to γ and α-ATP contain signal from ADP.

12-mm Probe and Variable Temperature Unit Calibration

Spectra were collected at 23.1°C, 42.4°C, and 51.5°C for various durations. Heating of the sample occurs by convection. Air is blown through a heating coil, located in the bottom region of the 12-mm probe, before reaching the sample . A thermistor monitors the temperature of the air blown onto the sample. The variable temperature unit controls the heating coil, so that the temperature of the air blowing on the sample equals the temperature set by the operator. Since the set temperature on the VT did not equal the temperature of the sample while inside the probe, the system had to be calibrated. The VT temperature setting was calibrated against the temperature-induced shifts of methanol and ethylene glycol by Brian B. Roman. The VT temperature setting was also calibrated using a calibrated thermistor probe (Cole Palmer) in a 50% Hct RBC sample with and without the proton decoupler running. The proton decoupler raises the temperature of the sample by depositing 1.044 watts of energy into the sample. When the proton decoupler was on, the actual temperature reading was taken approximately 3 seconds after the decoupler was turned off to avoid artefacts caused by local heating or radio frequency induced currents within the thermistor. During calibration and subsequent heating the VT air flow was adjusted to 25 ft^3/hr.

RBC samples were heated in the NMR probe by setting the VT to the corrected temperature using the equations shown in figure 11.

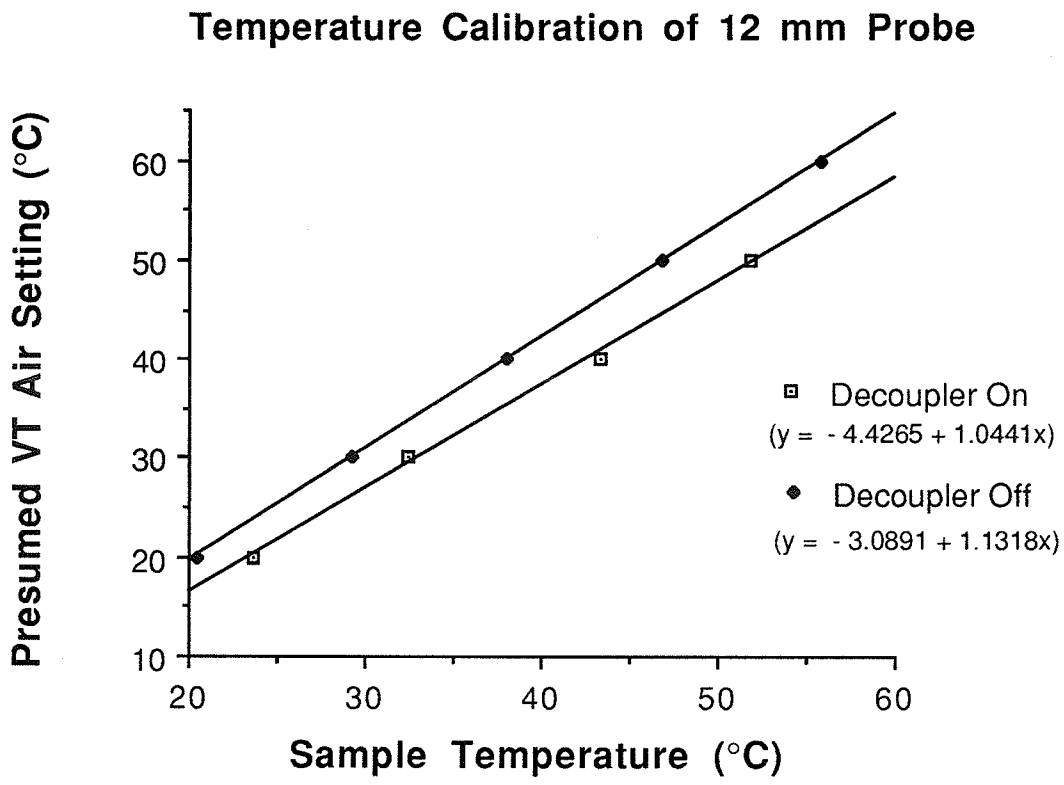

Figure 12. Temperature Calibration of 12 mm Probe.
Figure shows the temperature calibration curve for 12mm probe with the decoupler on and off. Temperature of a 50% RBC solution was determined using a thermistor probe .

Cell Sample Preparation

Chicken RBC suspensions at approximately 50% hematocrit (Hct) in MOPS buffered glucose-saline were added to a 12 mm sample tube. Aquarium fiber was then added to the tube to prevent cell settling. The aquarium fiber was carefully introduced after the RBC suspension was poured into the sample tube to prevent bubbles from getting trapped in the center of the aquarium fiber. Aquarium fiber was not used in later experiments and was determined to not affect spectra. In later experiments, the sample tube was removed from the magnet and agitated to prevent settling. The sample was agitated each time the temperature of the probe was changed. Cell suspensions were oxygenated immediately prior to each experiment by bubbling samples with 100% oxygen for at least 10 minutes. Cells were also

oxygenated at the end of some experiments to determine the effects of oxygenation of hemoglobin on the spectra and to decrease peak broadening. Narrower peaks allow for more accurate determination of peak positions.

For lysed-cell studies, suspension samples were placed at -10°C for 10 hours and then thawed at room temperature just prior to experiments. Before freezing, cells were suspended in a glucose-saline solution mimicking the internal milieu of the RBC at 325 milliosmolar containing 55 mM NaCl, 100 mM KCl, 5 mM glucose, and 10 mM HCO_3^- at pH = 7.2. After lysis, preparations were centrifuged and the supernatant was removed. This process removed the RBC nucleus and membrane components from the sample. Hemolyzed samples were heat-shocked following the same protocol as whole RBC samples.

NMR Heating Protocol

Spectra were first acquired at 23°C for 30 minutes to 3 hours. Because the acquisition of each spectrum required 30 minutes, it was assumed that the spectrum reflected the average state of the sample at 15 minutes. For later experiments, fewer aquisitions were collected so that the time required for data acquisition was only 15 minutes reflecting the metabolic state of cells at 7.5 minutes. For experiments in which cells received a HS, the temperature was raised to 42.4°C for 2 hours. The spectrometer tuning was adjusted each time the temperature was varied before the acquisitions of spectra was begun. The temperature was then raised to 51.5°C for one hour and then returned to 23°C. For control samples the temperature was raised to 51.5°C immediately from 23.°C. When the temperature of the spectrometer was changed, data acquisitions were halted to allow the sample temperature to equilibrate to the required temperature. It was determined during the temperature calibration of the 12-mm probe that the temperature of the sample did not reach the proper level until 15 minutes after the

VT display indicated that the VT air temperature was at the proper level. Therefore, acquisitions were not continued until 15 minutes after the VT display showed the required set temperature.

Internal Reference - Triethyl Phosphate (TEP).

Triethyl phosphate (TEP; $(CH_3CH_2O)_3PO$) was used as an internal frequency standard. It is a non-titratable compound that gives rise to a single, narrow ^{31}P NMR resonance peak when proton decoupled. The peaks corresponding to the intra- and extracellular TEP resonate so that they are not resolved; therefore, only one peak corresponding to TEP is observed. TEP was chosen since it can cross the membrane but does not react with the cellular phosphates. The use of an internal standard reduces magnetic susceptibility artifacts. The ^{31}P NMR signal of TEP in the presence of whole cells appears as a singlet, 0.44 ppm downfield of external 85% phosphoric acid. The chemical shift of TEP is insensitive to changes in RBC pH (Kirk, *et al.*, 1986). By recording the resonance frequency of the TEP peak relative to the absolute reference of the spectrometer, it was possible to determine that the position of this peak also did not change with temperature.

pH Measurements using Methylphosphonate

Since cell sensitivity to heat is altered by pH, it was necessary to monitor the changes in intracellular pH that occur during heating. With ^{31}P NMRS it is customary to rely on the position of inorganic phosphate (Pi) to determine intracellular pH. In fresh chicken RBC's the concentration of Pi is is too low to be detected by NMRS. Even if the concentration of Pi were artificially increased, the peak corresponding to Pi would resonate in a position which would overlap the peak corresponding to IP_5. For these reasons the position of the Pi peak cannot be used to monitor intracellular pH in chicken RBC's.

An alternative method to monitor intracellular pH is to introduce a non-physiologic probe. Methylphosphonate (MeP) (Aldrich) was chosen because it readily enters RBC's, its chemical shift is separate from the shifts of compounds intrinsic to the RBC, its pKa of 7.6 is near the physiologic pH range making its chemical shift very pH sensitive within this range, and MeP does not interact with hemoglobin or affect RBC glucose consumption (Labotka, 1984). In addition, MeP gives rise to a narrow peak when proton decoupling is applied.

Although, MeP was shown to readily cross the membrane in mammalian RBC's (Labotka and Kleps, 1983), it might not enter chicken RBC's. It was necessary to determine whether or not MeP enters the chicken RBC before we could proceed. Accordingly, I added 10^{-5} Molar $MnCl_2$ to the RBC suspension. Manganese is a broadening agent which cannot cross the RBC membrane; With Mn^{2+} added, all the signal from extracellular phosphate compounds become so broadened that they are considered NMRS invisible (Knubovets *et al.*, 1986). Following the addition of Mn^{2+}, the peaks corresponding to extracellular phosphates dissapeared. A peak corresponding to MeP remained indicating that MeP did enter the RBC, and I could procede to use MeP as a probe for pH.

pH Titration of MeP and IP_5

Titration curves were generated for both MeP and IP_5 at 23 and 42.4°C. The barium salt of IP_5 (CalBiochem) was dissolved in 0.5 M sulfuric acid to precipitate out the barium. The supernatant was added to sufficient solution mimicking the chicken RBC intracellular milieu to bring the final IP_5 concentration to 0.8 mM. This solution also contained 0.5 mM MeP and 0.5 mM TEP. The pH was adjusted by adding 1N NaOH or 1N HCl drop wise while monitoring the solution with a combination pH electrode attached to a Fisher pH meter. The pH probe and meter were calibrated with two standard solutions buffered to pH 7.01 and 4.0. A

thermistor attached to the pH meter monitored the temperature of the sample and standard solutions. The pH meter calibrations and solution adjustments were performed at both 23 and 42.4°C.

The initial pH reading of the IP_5 stock solution was adjusted to a pH near 6.7. A five ml aliquot was analyzed in the spectrometer at either 23 or 42.4°C using 830 acquisitions. The sample was returned to the stock solution and the pH was re-checked using the pH meter. The pH was changed by approximately 0.2 pH units before another 5 ml aliquot was removed for NMR analysis. Before each sample acquisition, the tuning of the magnet was checked for both ^{1}H and ^{31}P. The pH range used for the MeP and IP_5 calibrations ranged from 6.7 to 8.1 in approximately 0.2 pH unit increments.

The peak positions for both IP_5 and MeP relative to TEP were obtained from transformed spectra and plotted against pH to obtain pH titration curves. The intracellular pH of samples could then be determined by measuring the chemical shift of the peak corresponding to a samples intracellular MeP and using the relationship obtained from the titration curves.

Line Width Determinations

The peak width of TEP was determined for each spectrum by fitting the peak corresponding to TEP to a Lorentzian curve and taking the width of that curve at half peak height. These calculations were done using the Lorentzian fit subroutine of the MacNMR software. The peak corresponding to TEP was chosen to measure broadening, since it was the only peak that was sufficiently resolved during all stages of the heating protocols.

Results

Energetics.

RBC phosphorus metabolites were monitored using ^{31}P NMRS during both the non-heat-shocked control and heat shock protocols. Figure 8 (page 38) shows the survival curves for both non-heat-shocked and heat-shocked cells. Survival of the two cell populations is not significantly different after one hour at 51.5°C. Both cell populations experience approximately 7 % hemolysis. NMR spectra were not obtained during the longer exposures to 51.5°C because greater levels of hemolysis could greatly decrease the resolution of spectra, and make them more difficult to interpret.

Figure 13 shows a representative spectrum from chicken RBC's. The major phosphates present in these RBC's are resolved and labeled.

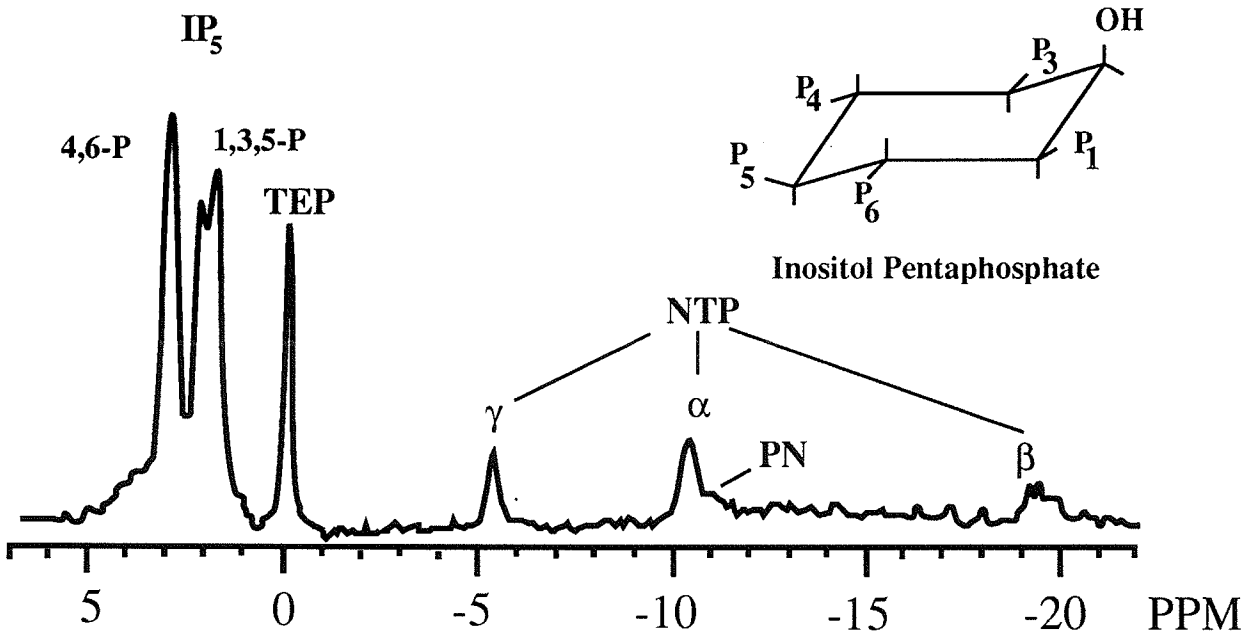

Figure 13. A Representative ^{31}P Spectrum From Chicken Red Cells at 23°C.
The peaks represent the major phosphate compounds found in chicken RBC's are shown. The abbreviations refer to;
IP_5: 1,3,4,5,6-myoinositol pentaphosphate
TEP: triethyl phosphate (added as an internal reference)
NTP(γ,α,β): nucleoside triphosphate (primarily ATP). The γ and α peaks contain contributions from nucleoside diphosphates.
PN: Pyridine nucleotides (NAD and NADP, both oxidized and reduced forms).

Inositol pentaphosphate is represented by the left-most peaks occurring in the spectrum. It consists of 3 partially resolved peaks. The peak farthest left represents the phosphates bound to the 4th and 6th carbon of inositol. These phosphates positions on the inositol are equally magnetically shielded so that they resonate at the same frequency. The remaining two overlapping peaks of IP_5 represent the phosphates bound to the 1st, 3rd, and 5th carbons of inositol. The 1-P and 3-P of IP_5 resonate at the same frequency, while the 5-P resonates between the peaks representing 4,6-P and 1,3-P.

The peak labeled TEP represents TEP which was added as an internal chemical shift reference. The position of this peak was always set to zero parts per million (ppm).

The peaks labeled NTP represent the peaks corresponding to the phosphates of nucleoside triphosphates. In the chicken RBC these peaks are primarily due to ATP. The peaks labeled γ and α also contain contributions from nucleoside diphosphates; therefore, only the peak labeled β represents the concentration of NTP. The peak labeled PN, which is really just a shoulder on the α peak, represents the pyridine nucleotides.

Figure 14 shows a region between -1 and 5 ppm of the characterisitic spectrum of 2,3-DPG from rat RBC's and IP_5 from chicken RBC's along with these compounds molecular structure. The spectrum of 2,3-DPG was obtained for comparison purposes. Interestingly we found that the phophorus groups in these two molecules are comparably magnetically shielded so that their ^{31}P NMR spectra are similar.

Both the peaks representing intra- and extracellular inorganic phosphate (Pi) are resolved in the 2,3-DPG spectrum. From figure 14 it is apparent that the peaks corresponding to Pi cannot be resolved in the spectrum of chicken RBC's, since the position of the peaks representing IP_5 overlap the resonance position for Pi. The peak representing Pi could only be resolved in chicken RBC's if the concentration of Pi increased dramatically.

Representative Spectra of 2,3-DPG and IP_5

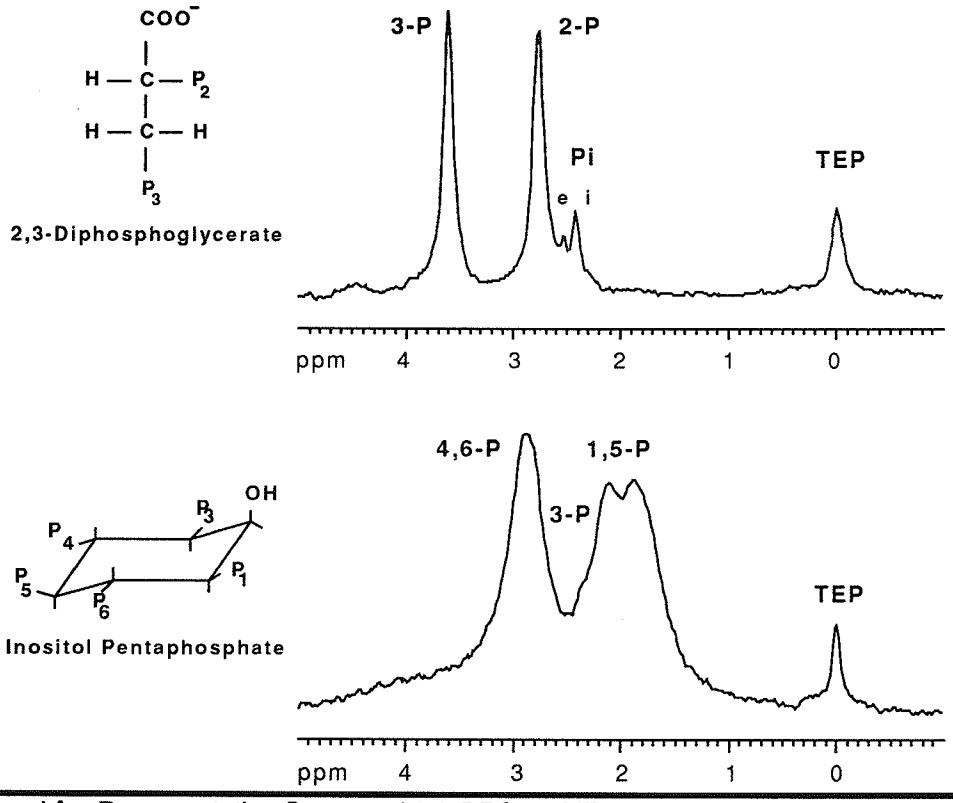

Figure 14. Representative Spectra of 2,3-DPG and IP_5.
This figure shows the region between -1 and 5 ppm of spectrum obtained from rat RBC's and chicken RBC's respectively. TEP was used as the chemical shift reference and its resonance position was adjusted to 0 ppm.

It is not clear whether the smaller peak of IP_5 labeled 3-P, which appears as a shoulder on the peak labeled 1,5-P, is actually the peak representing 3-P or is the peak for Pi. The peak asssignments were made according to the assignments of Johson and Tate (1969). Better resolution of IP_5 is attainable when examining chicken RBC lysates or pure IP_5 samples. Using such samples the 5-P peak is identifiable and the 1,3-P and 4,6-P peaks appear as doublets (not shown).

Figures 15 and 16 are stacked plots of representative chicken RBC spectra showing the changes that are observed while the cells undergo the two heating protocols. No changes were observed in the ^{31}P spectra while cells were maintained within the spectrometer at 23°C for at least 4 hours prior to heating.

Figure 15 shows spectra during 23°C incubation and challenge at 51.5°C. Spectrum A represents the initial state of cells at 23°C. Spectrum B represents the state of cells at approximately 105 min at 23°C. This spectrum required 30 min to collect and was started on the 90th min of the total time that cells were being maintained at 23°C. Because 30 min was required, the spectrum represents the state of cells at 15 min of the total aquisition. The spectra representing the state of cells at 45 and 75 min at 23°C are the same as spectra A and B. Spectrum C represents the state of cells at approximately 45 min of heating at 51.5°C. This spectrum was started after the cells were at 51.5°C for 30 min. The spectrum representing the state of cells at 15 min of heating at 51.5°C is a cross between spectra A and C (not shown). Spectrum D represents the state of cells 45 min after returning cells to 23°C.

The peak representing β NTP has dissapeared completely in spectrum C indicating that NTP was hydrolyzed (see fig. 17). The peaks corresponding to IP_5 increased in height and decreased in width during exposure to 51.5°C, so that their relative areas remained constant. The peak area of the 1,3-P of IP_5 was slightly greater when cells were returned to 23°C, but this was due to an increase in Pi (see fig. 18).

Although only 3 peaks corresponding to IP_5 were apparent in all spectra, 5 peaks corresponding to IP_5 are present in addition to one corresponding to Pi. The summation of these 6 independent peaks gave rise to the strange spectral changes observed in figures 15 and 16. It was necessary to deconvolute overlapping IP_5 peaks using the program NMR1 before changes in the relative areas and positions of these peaks were determined.

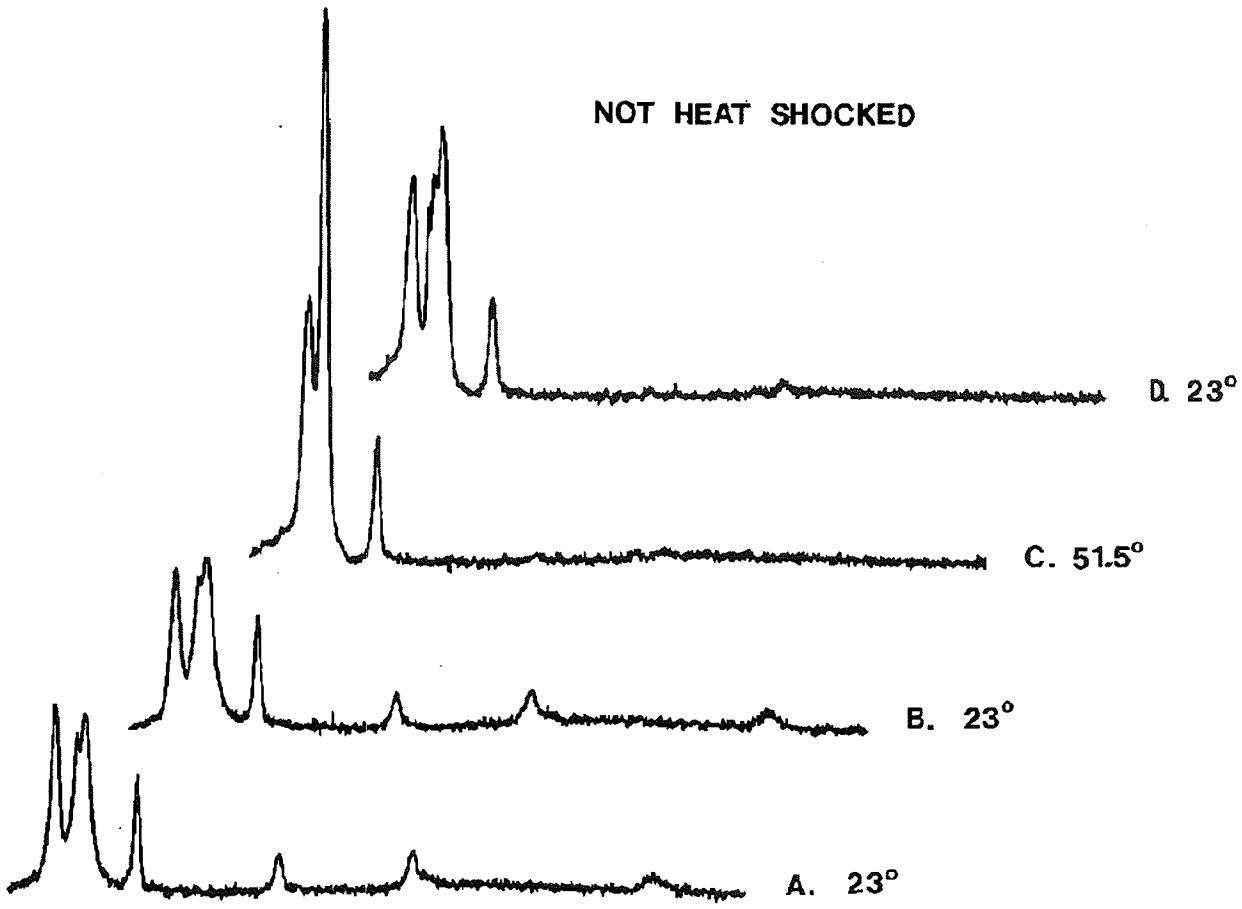

Figure 15. Stacked Plot of Representative Spectra From Non-Heat-Shocked Red Cells.
A represents initial state of cells at 23°C.
B represents state of cells after 105 minutes at 23°C.
C represents state of cells after 45 minutes at 51.5°C.
D represents state of cells after returning to 23°C for 45 minutes.
Cells were maintained at 23°C for several hours without significant changes in peak positions or relative concentrations of metabolites. During challenge at 51.5°C there was a shift to the right of the 4,6-P peak of IP_5. Additionally, there was a decrease in the relative area of the peaks representing NTP and an increase in the relative area of peaks representing IP_5. When cells were returned to 23°C, the shifted peak of IP_5 returned to its original positions at 23°C (see figure 20).

Figure 16 shows a stacked plot of spectra representing the state of chicken RBC's at selected times during a HS at 42.4°C for 2 hr followed by heating at 51.5°C for 1 hr. Spectrum A represents the state of cells at 23°C. This spectrum is identical to figure 15A except that the concentration of TEP is slightly greater. Spectrum B of figure 16 represents the state of cells after 105 minute at 42.4°C. In this spectrum the relative concentrations of compounds remained unchanged and

the peaks are slightly broadened. There was also a time-dependent shift to the left in the peaks representing IP_5. Spectra representing 15, 45, and 75 min at 42.4°C show the gradual shift in the IP_5 peaks (not shown; see figure 20).

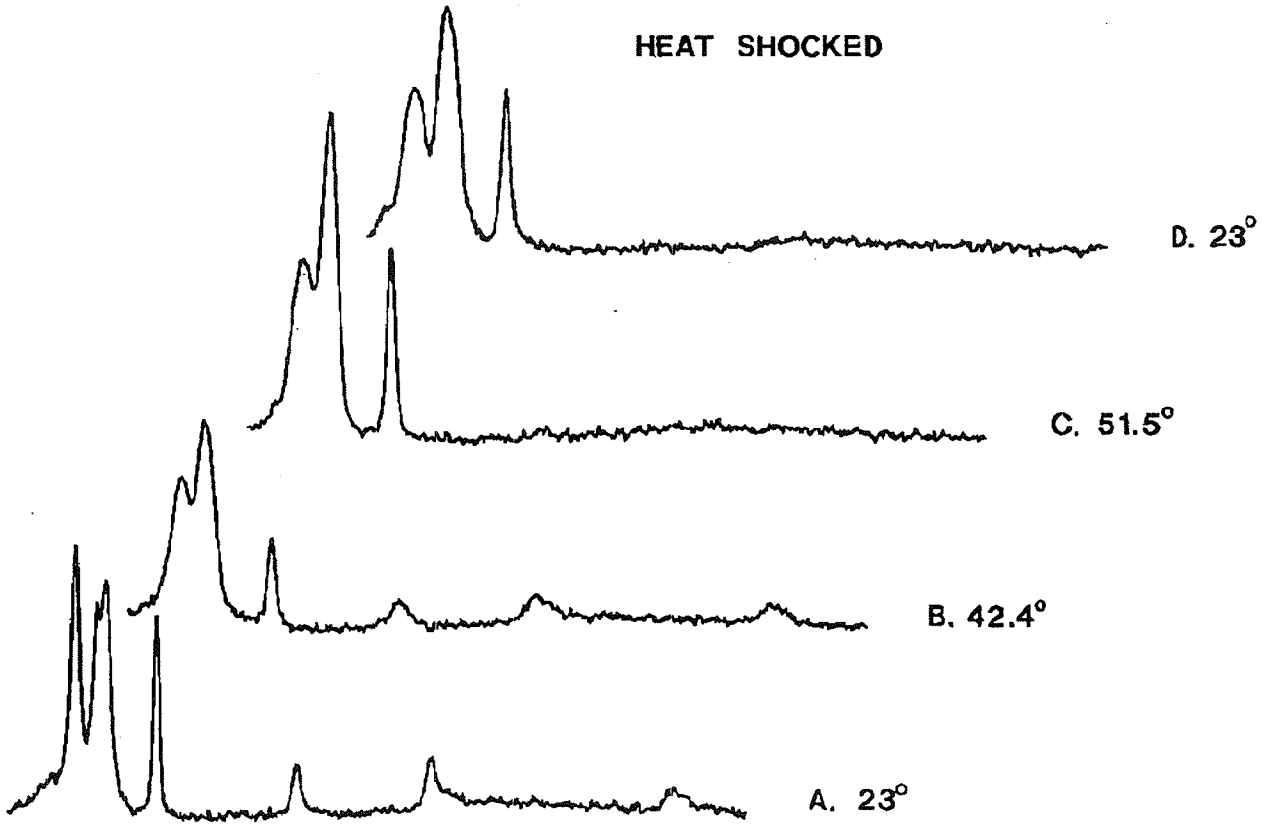

Figure 16. Stacked Plot of Representative Spectra From Heat-Shocked Red Cells.
A represents initial state of cells at 23°C.
B represents state of cells after 105 min at 42.4°C.
C represents state of cells after 45 min at 51.5°C.
D represents state of cells after returning to 23°C for 45 min.
During heat shock at 42.4°C, a time-dependent shift to the left occurred for the peaks representing IP_5. The relative concentrations of all compounds remained unchanged and all peaks broadened. During challenge at 51.5°C, there was a shift to the right of the peak representing IP_5. Additionally, there was a decrease in relative area of the peaks representing NTP (see fig. 17). When cells were returned to 23°C, the 4,6-P peak of IP_5 remained significantly shifted to the left of its original position at 23°C (see figure 20).

Spectrum C represents the state of cells after 45 min at 51.5°C. The peaks representing NTP completely disappeared (see fig. 17) and the peaks representing IP_5 shift to the right (see fig. 20). All remaining peaks appeared broadened. Spectrum D represents the state of cells after returning to 23°C for 45 min. The

peak corresponding to the 4,6-P of IP_5 remains significantly shifted to the left of its original position at 23°C. In addition, all remaining peaks are significnatly broadened (see figure 26).

Nucleoside Phosphate Levels

Figure 17 compares the relative NTP concentrations in RBC's during the two heating protocols. These concentrations were obtained by calculating the area of the peak corresponding to β NTP relative to the total area of all peaks representing the naturally occurring phosphate metabolites in chicken RBC's. In chicken RBC's the major NTP is ATP. During the two hour 42.4°C heat shock, cells maintained initial nucleoside phosphate levels. Complete loss of signal from the nucleoside phosphate peaks occurred within one hour of exposure to 51.5°C either with or without previous protection via heat shock (Figure 17). Therefore, ATP levels were not protected by induction of thermotolerance. Samples were examined one and two days after heating with and without a heat shock to determine if ATP levels would recover. No recovery in ATP levels was detected in either the heat-shocked or non-heat-shocked sample.

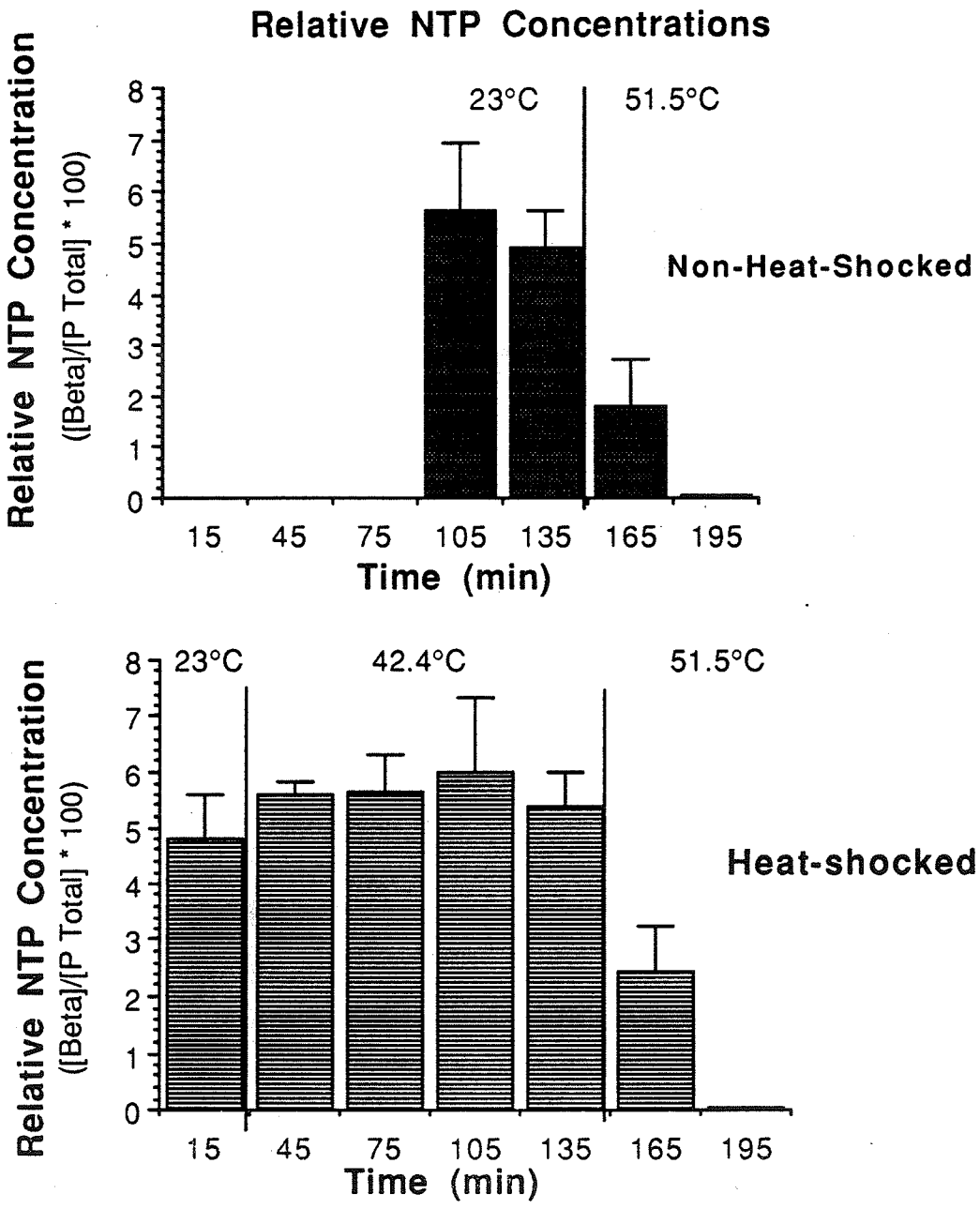

Figure 17. Relative NTP Concentrations.
This figure compares the relative concentrations of NTP in heat-shocked and non-heat-shocked red cells. NTP levels do not change during 42.4°C heat shock. Also there are no significant differences between NTP levels in heat-shocked and non-heat-shocked cells at 51.5°C.

Chemical Shift

Changes in temperature were accompanied by immediate changes in the resonance frequencies of all observable metabolites. Figure 18 shows the

changes that occurred in the peaks representing IP_5 during the contol (non-heat shock) protocol. Each spectrum in this figure required 15 min to acquire so they represent the state of cells 7.5 min later in time than the times for the spectra in figure 15. The shift in all peaks, especially the peak corresponding to 4,6-P, and slight broadening of all peaks can be seen. The spectrum representing the state of cells when returned to 23°C reveals a peak that represents Pi. The position of the peak representing Pi seems to occur at approximately 2 ppm in the spectrum representing the state of cells in the last 7.5 min of 1 hr heating at 51.5°C. The shift towards the right during exposure to 51.5°C indicates a more acidic state of cells during heating at 51.5°C. The final spectrum of this figures also shows the state of cells after re-oxygenation following heating. The re-oxygenation of cells following the control protocol doesn't affect the spectum at all.

Chicken RBC IP_5 Spectra (Not Heat-Shocked)

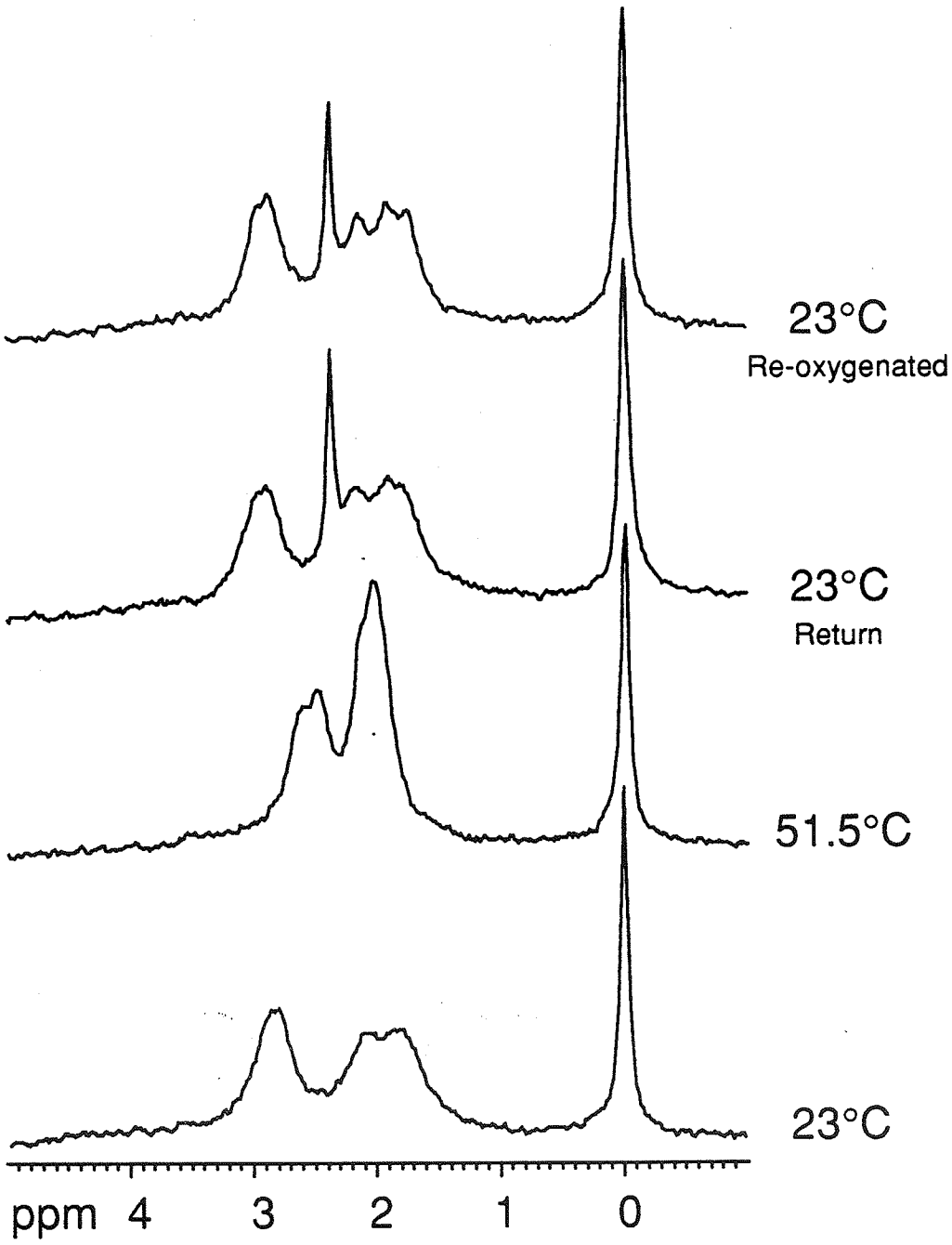

Figure 18. Chicken RBC IP_5 Spectra (Not Heat-Shocked).
This figure shows the peaks representing IP5 changing over time during the control (non-heat shock) protocol. A peak representing Pi emerges during heating at 51.5°C.

Figure 19 shows the changes that occurred in the peaks representing IP_5 during the heat shock protocol . The spectra in this figure were also aquired in 15 min intervals and represent the state of cells 7.5 min later than the times in figure 16. During HS, a leftward shift of the peaks representing IP_5 can be seen along with broadening of all peaks. The final specturm of this figure shows the state of cells following heating and re-oxygenation. It shows that re-oxygenation narrowed all peaks and returned the peaks to near their initial positions at 23°C. In addition, a peak representing Pi was resolved indicating that a dramatic increase in Pi occurred during heating.

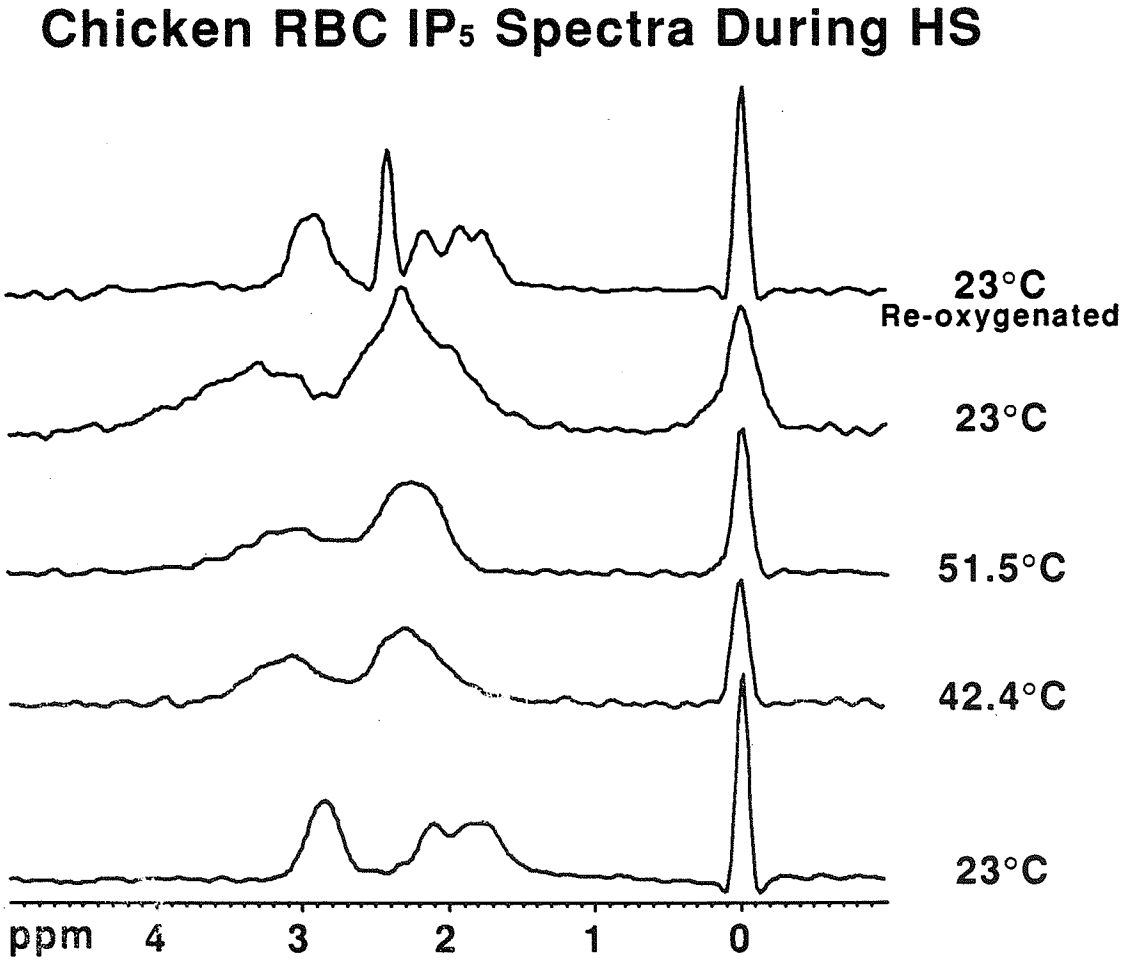

Figure 19. Chicken RBC IP_5 Spetra During Heat Shock.
This figure shows the peaks representing IP_5 changing over time during the heat shock protocol. The final spectrum represents the state of cells after heating following re-oxygentation. The peak representing Pi was resolved following re-oxygenation.

Figure 20 shows the chemical shift of the peak corresponding to 4,6-P in whole RBC's during heat shock and non-heat shock protocols and in RBC lysates during the heat shock protocol. During heat shock of whole RBC's there were time-dependent shifts of the peaks corresponding to IP_5 which continued throughout the exposure period. The characteristic which differentiated spectra obtained from heat-shocked cells from spectra obtained from non-heat-shocked cells was a shift in the peak corresponding to the 4,6-P of IP_5 and broadening of all peaks. Figure 20 shows that the initial resonance position relative to TEP of the peak corresponding to 4,6-P is approximately 3.0 ppm at 23°C. During the two hour heat shock a time dependent shift to the left occurs. When cells are heated at 51.5°C there is a time dependent shift to the right (decreasing ppm values). In addition, the peak corresponding to 4,6-P in heat-shocked cells is in a different position during 51.5°C exposure than the same peak in non-heat-shocked cells (figure 20). After returning cells to 23°C, the 4,6-P peak in spectra from heat-shocked cells is broadened and significantly shifted 0.419 ppm ($p<0.005$) to the left of its original position at 23°C while the peak in non-heat-shocked cells returned to its original position at 23°C (figure 20). These results suggest that IP_5 may be involved in a permanent change occurring in the chicken RBC during heat shock.

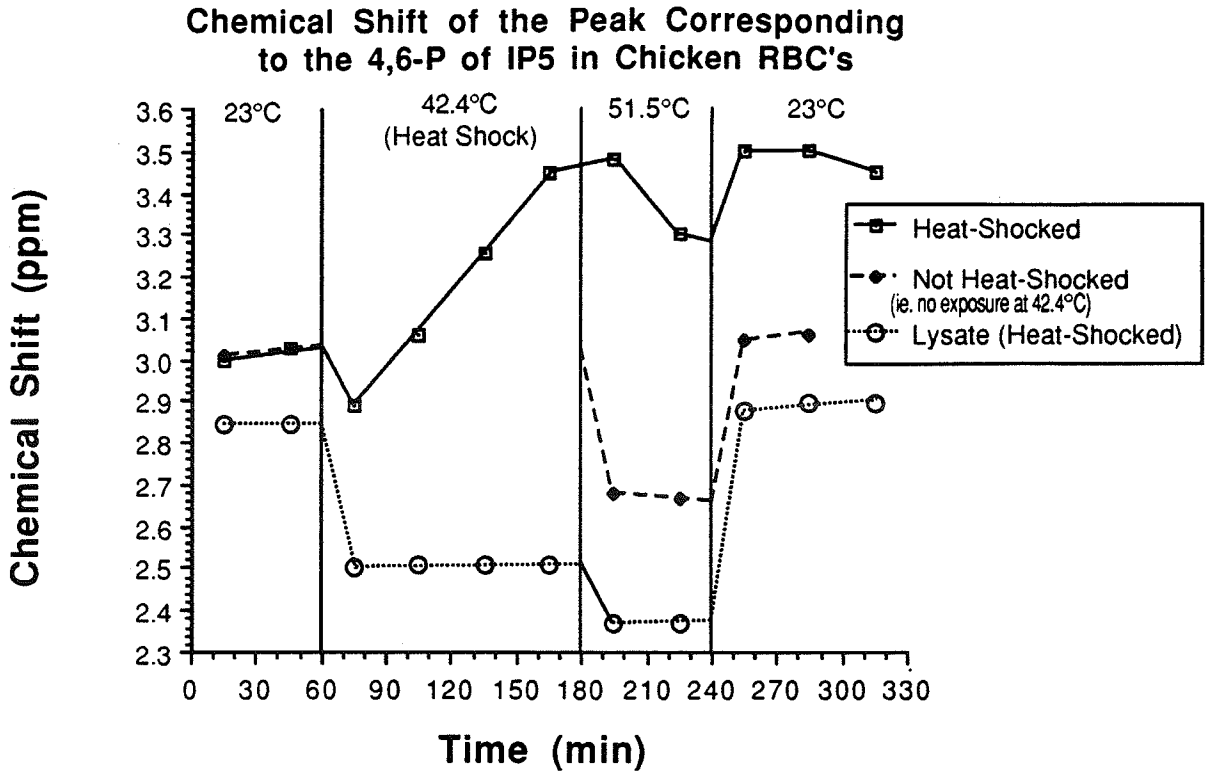

Figure 20. Chemical Shift of the Peak Corresponding to the 4,6-P of IP_5 in Chicken RBC's. The chemical shift of the 4,6-P of IP_5 is shown in heat-shocked and non-heat-shocked chicken cells, and lysed cell preparations. Shifts occur in the position of the 4,6-P peak of IP_5. However, upon return to 23°C, this peak returns to its original position in non-heat-shocked cells and lysates, but remains significantly shifted 0.419 ± 0.056 ppm ($p<0.005$) to the left of its original position in the heat-shocked cells.

To determine whether the RBC membrane or nucleus is involved in this shift, the cells were lysed and the membrane and nucleus were removed. Cell lysis did not affect the resonance positions of the IP_5 peaks at 23°C. No time-dependent changes in resonance positions were observed during heat shock of lysed cells, and the 4,6-P peak returned to its original position when returned to 23°C (Figure 20). This suggests that the observed change in the position of the 4,6-P peak resulting from heat shock requires an intact nucleus or membrane

pH Changes

Titration Curve of MeP

The shift of MeP relative to TEP was monitored in order to examine any changes in RBC intracellular pH. First, a chemical shift titration curve in the absence of cells was generated at 23 and 42.4°C. Figure 21 shows the chemical shift of MeP relative to TEP at various pH values within the pH range of 6.8-8.0 at 23°C in a glucose-saline solution mimicking the RBC intracellular milieu. A linear regression was performed on the middle linear portion of the curve to generate an equation used to calculate pH from chemical shift values. The peak corresponding to MeP shifts to the left as pH is decreased (greater ppm values, see figure 13, pg. 62).

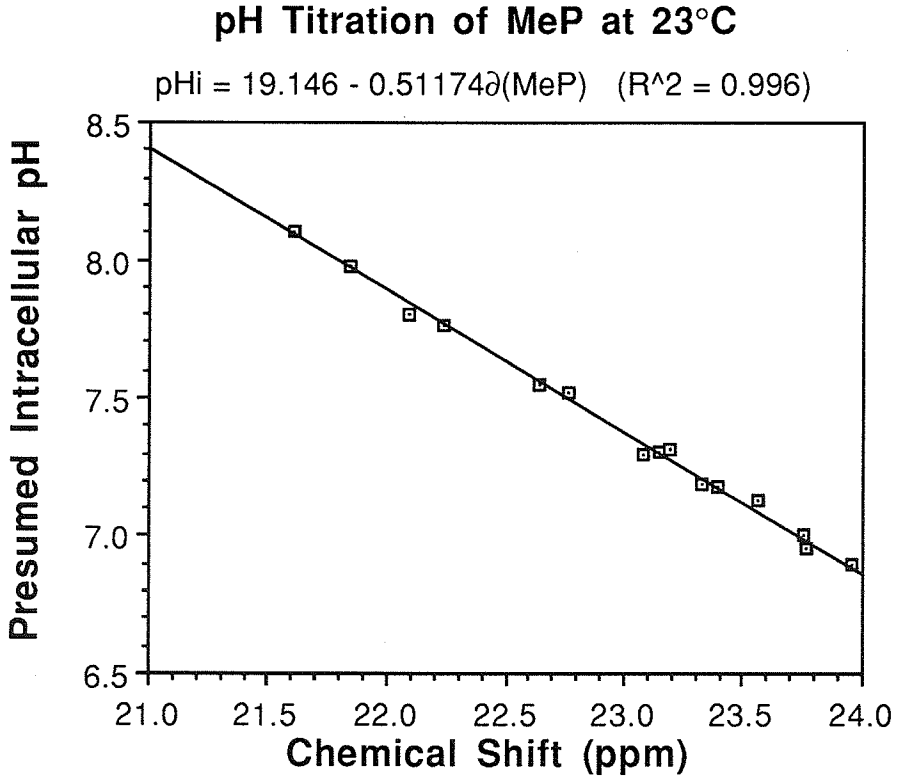

Figure 21. pH Titration of MeP at 23°C.
The linear portion of the pH titration curve for the shift of MeP relative to TEP is shown. This curve was generated with 5 mM MeP and 5 mM TEP in a glucose-saline solution mimicking the RBC intracellular milieu at 23°C.

The titration curve of MeP obtained at 42.4°C (not shown) was almost identical to the curve obtained at 23°C. The equation representing the relationship between chemical shift and pH at 42.4°C was determined to be:

$$pH = 19.141 - 0.51154\ \delta_{(MeP)} \quad (R^2 = 0.998)$$

This indicates that the shift of MeP relative to pH is independent of temperature between 23 and 42.4°C.

pH Titration of IP_5

The titration curve of a pure sample of IP_5 purchased from CalBiochem was determined at 23°C and 42.4°C in a glucose-saline solution mimicking the intracellular milieu of chicken RBC's described earlier. Figure 22 shows the chemical shift of the peak corresponding to the 4,6-P of IP_5 over a pH range of 6.8 to 8.2 at both 23 and 42.4°C. As the pH decreases the 4,6-P peak shifted to the right, in the opposite direction that the MeP peak shifted. The shift of this peak as pH changes was independent of temperature between 23 and 42.4°C.

Intracellular pH Determined by Shift of MeP

The RBC intracellular pH was determined by monitoring the shift of MeP relative to TEP. The shift of the 4,6-P peak of IP_5 would not be reliable as a pH monitor since it interacts with other intracellular components of the RBC possibly affecting its chemical shift. Initially it was difficult to resolve the peak corresponding to intracellular MeP. The location of the intracellular peak was resolved after the addition of 10^{-5} Molar $MnCl_2$ to the RBC suspension. The Mn^{2+} served to eliminate all the extracellular signal. At 23°C the intracellular MeP resonated slightly to the left of the extracellular signal indicating that the intracellular environment was approximately 0.13 pH units more acidic than the extracellular environment (not shown). Without the addition of Mn^{2+}, the peak corresponding to extracellular MeP was significantly larger than and overlapped the peak corresponding to

intracellular MeP at 23°C, so that accurate intracellular pH measurements at 23°C could not be determined. By increasing the RBC Hct to greater than 60% and by waiting for 1 hr after the addition of MeP [1 hr is required for the MeP to equilibrate across the cell membrane (Labotka and Kleps, 1983) a small peak corresponding to intracellular MeP could be resolved at 42.4°C and 51.5°C.

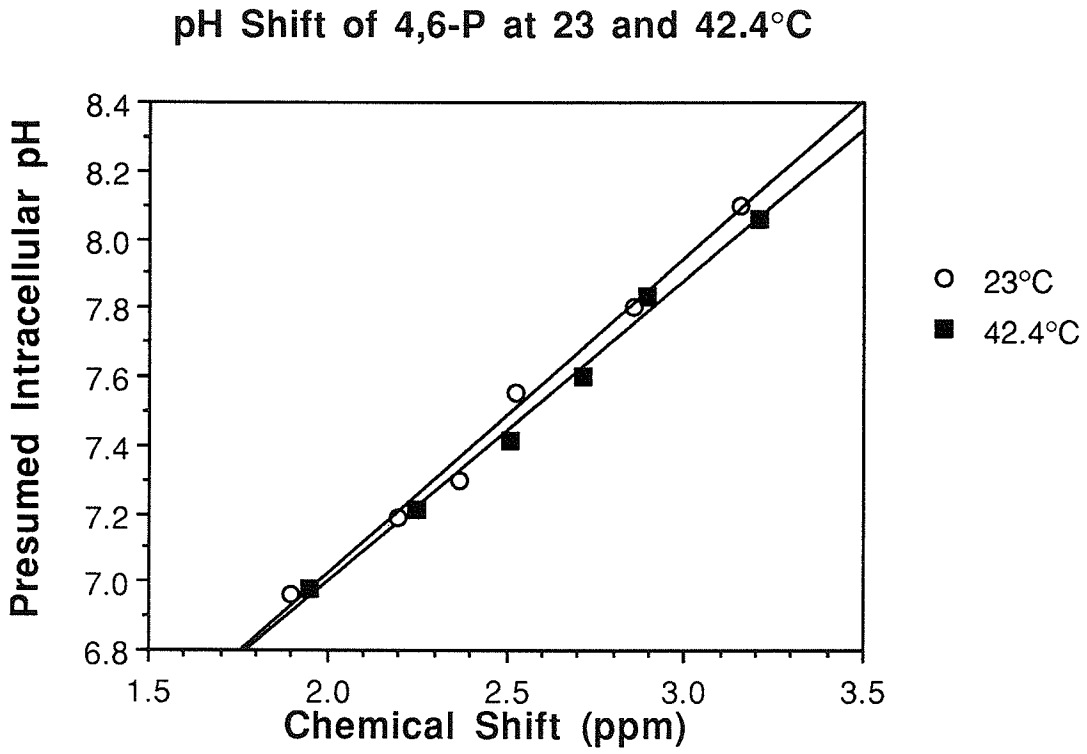

Figure 22. pH shift of 4,6-P at 23 and 42.4°C.
Figure shows the pH titration curve of the peak representing the 4,6-P of IP5 at 23 and 42.4°C. The pH shift of the 4,6-P peak is essentially the same at 23 and 42.4°C.

Intracellular pH During Heating

The intracellular pH of chicken RBC's was monitored during heating using the position of MeP relative to TEP and the titration curve generated. Because the resonance position of the peak corresponding to intracellular MeP was difficult to resolve when the cells were initially at 23°C, the intracellular pH was taken to be 0.13 pH units more acidic than the extracellular pH. The extracellular pH was easy

to monitor using the chemical sift of the peak corresponding to extracellular MeP. Petersen *et al.* (1987) showed that the intracellular human RBC pH was approximately 0.15 pH units more acidic than extracellular pH when extracellular pH is 7.3. In addition, I found using Mn^{2+} to broaden the peak corresponding to extracellular MeP, that the intracellular RBC environment was 0.13 pH units more acidic than the extracellular environment when cells were in a solution at pH 7.3 at 23°C.

Figure 23 shows the intracellular pH of chicken RBC's during the control (non-heat shock) protocol. This figure indicates that the intracellular pH varies with temperature but remains constant while the cells are maintained at a fixed temperature within the time frame of these experiments.

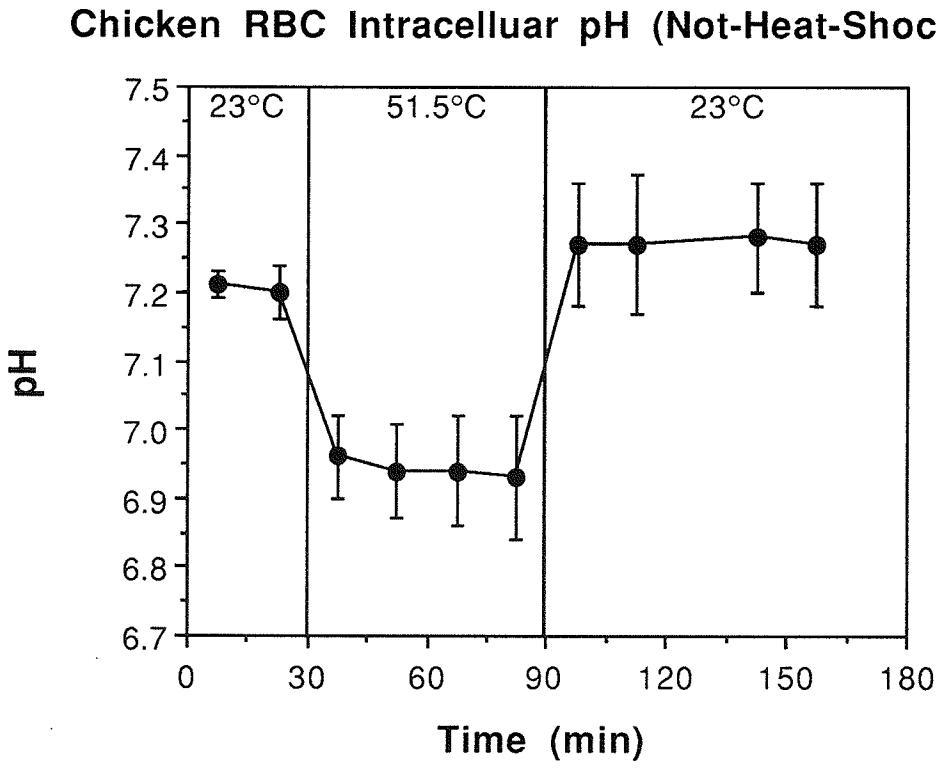

Figure 23. Chicken RBC Intracellular pH (Not Heat-Shocked).
The figure indicates that pH changes with temperature but remains stable at fixed temperature.

Figure 24 shows the intracellular pH of chicken RBC's during the heat shock protocol. This figure also indicates that intracellular pH changes with temperature. A slight acid shift, approximately 0.4 pH units occurred during the 42.4°C heat shock, but it is not significant.

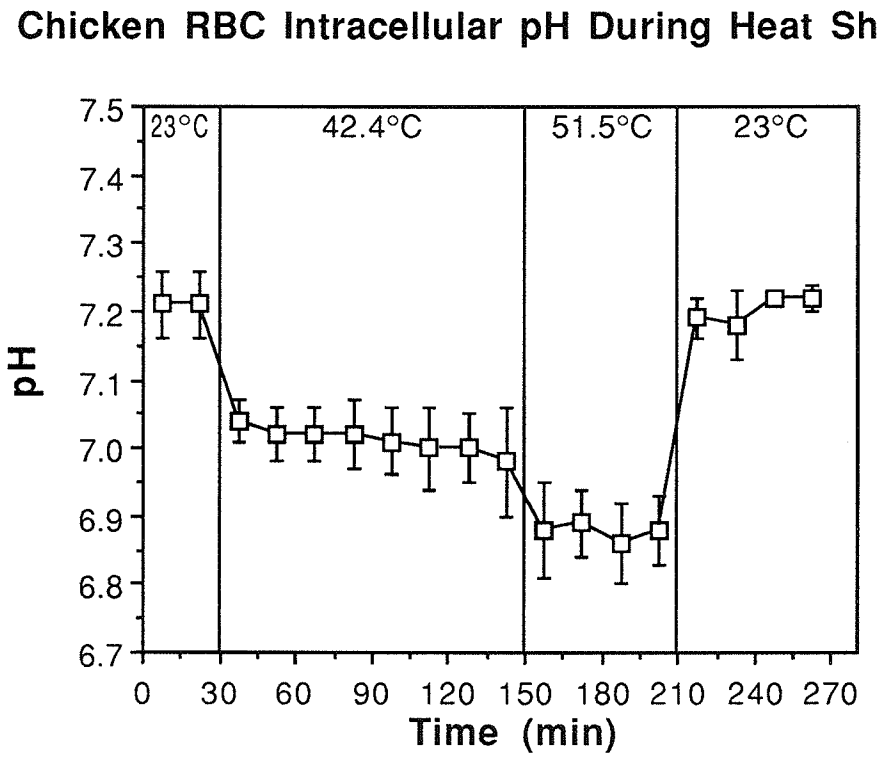

Figure 24. Chicken RBC Intracellular pH During Heat Shock.
The intracellular pH of RBC's during the heat shock protocol is shown. The pH becames more acidic as temperature increased. There might be a slight acid shift during 42.4°C heat shock, but it is insignificant. Plotted as mean ± S.E.M. (n=4)

There was also no significant difference between the intracellular RBC pH during either the heat shock or non-heat shock protocols. The intracellular pH at a specific temperature was the same for cells during either protocol. The intracellular pH of heat-shocked cells might be shifted 0.5 pH units more acid during heating at 51.5°C than non-heat-shocked cells during heating at 51.5°C.

Line Width

The line width of TEP was calculated for each spectrum during each RBC treatment. The width of all peaks changed during heating, but the width of TEP was easiest to monitor. The line width of the peaks corresponds to the oxygenation of hemoglobin (Labotka, 1984). A broadening of line width indicates that Hb is becoming deoxygenated.

Figure 25 shows the line width of TEP during a non-heat shock protocol with broad band proton decoupling. This figure shows that there was a slight increase in the line width of the TEP peak during and following heating at 51.5°C while cells were returned to 23°C. This figure shows the results of only 3 experiments; however, five additional experiments run without proton decoupling verify that there was a broadening of approximately 5 Hz in the peak representing TEP during and following 1 hour of heating at 51.5°C. These experiments were not averaged with the data in figure 25, because the line width of TEP without proton decoupling were significantly broader (15 Hz) than with proton decoupling. Heating at 51.5°C for periods longer than 1 hour did indicate that gradual broadening of the TEP peak began to occur after 1 hour (data not shown).

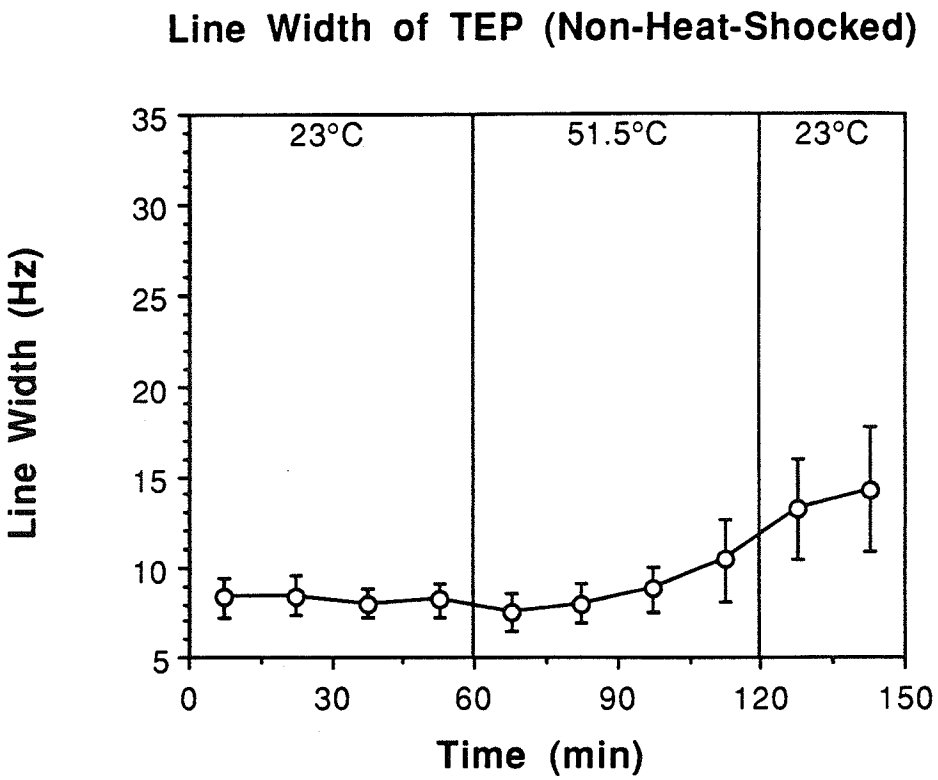

Figure 25. Line Width of TEP (Non-Heat-Shocked).
Figure shows the line width of TEP during heating of chicken RBC's for 1 hour. The line width of the peak representing TEP increased slightly during and following heating at 51.5°C. Data accumulated from experiments run without decoupling confirmed that the line width of the TEP peak increases approximately 5 Hz during heating (data not shown). Plotted as mean ±S.E.M. (n=3)

Figure 26 shows the line width of TEP during a heat shock protocol using broad band proton decoupling. This figure indicates that the line width of TEP began to broaden after 1 hour at 42.4°C and continued to broaden during and following heating. When the cells were returned to 23°C, the peak representing TEP was broadened approximately 15 Hz from the starting width at 23°C prior to any heating. Again these results were confirmed by experiments that were run without proton decoupling.

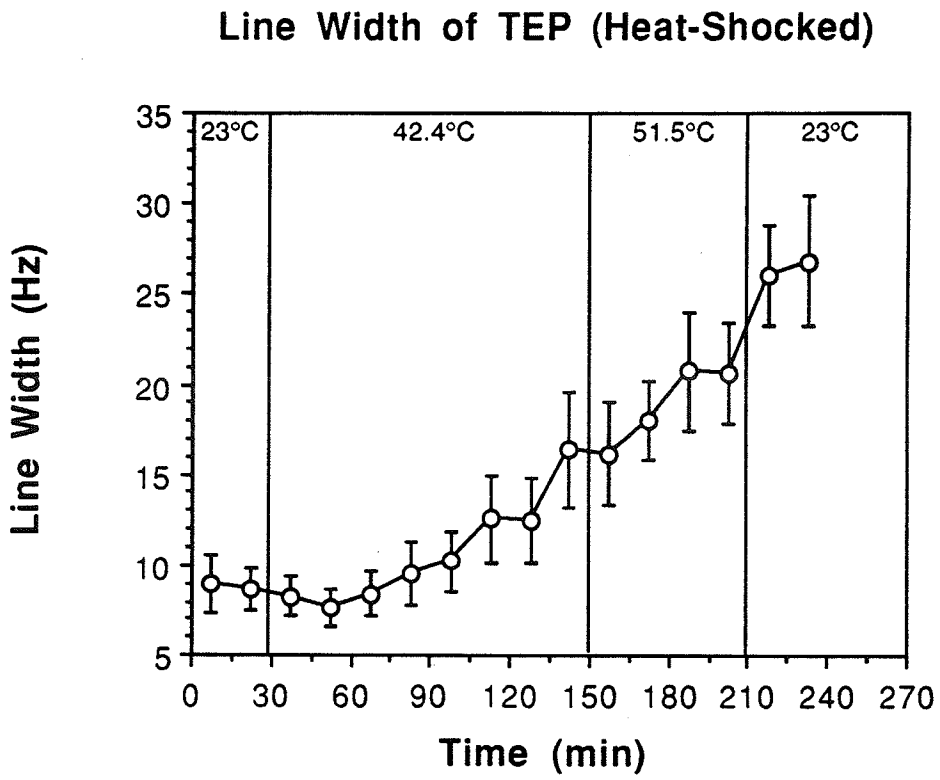

Figure 26. Line width of TEP (Heat -Shocked).
The line width of TEP began to broaden after approximately 45 minutes at 42.4°C and continued to broaden during heating. The final line width of TEP at 23°C was approximately 15 Hz broader than at 23°C prior to any heating. Plotted as mean ± S.E.M. (n=4)

Discussion

The levels of phosphorus metabolites during heating and heat shock were investigated using ^{31}P NMRS. No correlation was found relating the induction of thermotolerance to changes in levels of NTP or other phosphorus metabolites. Differences were found in thermotolerant cells in the position of the peak corresponding to the 4,6-P of IP_5, in the line width of TEP, and possibly in the intracellular pH during heating at 51.5°C.

Chemical Shift

The observations from data acquired using ^{31}P NMRS show that during heating at 42.4°C there was a shift to the left, at a constant rate, of the peak corresponding to the 4,6-Phosphates of IP_5. When the cells were returned to 23°C

following the heat shock protocol, the 4,6-P peak was shifted 0.419 ppm to the left of its original position. In preliminary experiments, reoxygenation of erythrocytes following a heating protocol that was sufficient to broaden all peaks and shift the 4,6-P peak of IP_5 0.4 ppm leftward, restored the peaks to their original widths and returned the 4,6-P peak to near its original position at 23°C (figure 19). This suggests that the differences observed between thermotolerant heat-shocked and non-thermotolerant non-heat-shocked cells using ^{31}P NMRS are reversible and related to the oxygenated state of Hb.

Line Width Broadening

In addition to changes in the resonance position of the peak corresponding to 4,6-P during heating, broadening occurred, at a constant rate, of all peaks as monitored by the broadening of the peak representing TEP. Following the heat shock protocol, this peak was broadened by 15 Hz relative to its initial line width before heating. Also, during heating at 51.5°C, the line width of TEP in heat-shocked cells was 10 Hz broader than in non-heat-shocked cells.

In NMRS the broadening of a peak could result from a number of factors including changes in the mobility of the compound which is represented by the peak or decreases in the homogeneity of the magnetic field of the spectrometer. Changes in a compounds mobility usually result from either changes in temperature or from changes in the compounds interactions with other molecules. An increase in temperature alone, however, usually causes all the peaks occurring in a spectrum to narrow due to an increase in the mobility of the compounds being observed. Additionally, changes in the mobility of compounds, which are suspended in saline, would occur immediately as the temperature is altered. This was not observed in this investigation. Results indicated that as temperature was increased, a broadening of all peaks, rather than narrowing, occurred at a constant

rate. No immediate changes occurred in line widths when the temperature was changed except when cells were returned to 23°C following the heat shock protocol.

The binding of a mobile compound to another molecule also affects the line with of the peak(s) representing that compound. Generally, upon binding of a metabolite to a macromolecule, it becomes immobile and the peak(s) representing the metabolite become so broad that they can no longer be observed in the spectrum and the metabolite becomes NMRS invisible. This does not occur in this investigation; however, the peak corresponding to the 4,6-P of IP_5 does appear to broaden slightly more than the peak corresponding to TEP. This could not be quantified, since the peak corresponding to 4,6-P could not be resolved sufficiently from the other peaks of IP_5. The apparent greater increase in linewidth along with its shifting, may indicate that these two phosphates are interacting with other smaller molecules or ions occurring inside the RBC.

Broadening may also result when the magnetic field of the spectrometer becomes less homogenous. The deoxygenation of Hb causes inhomogeneities of the magnetic field. Deoxyhemoglobin has a high-spin state Fe^{2+} which is paramagnetic. As hemoglobin becomes paramagnetic, a magnetic susceptibility gradient forms across the cell membrane and magnetic field inhomogeneities arise. This results in broadening of all peaks (Labotka, 1984). In this study only the peak corresponding to TEP was resolved sufficiently to monitor broadening quantitatively.

The observed broadening of all peaks is most likely due to the deoxygenation of Hb as was shown by Labotka (1984). Therefore, the broadening of the peak representing TEP might be useful as a method to monitor the oxygenated state of Hb. Several physiological properties are dependent on the

oxygenated state of hemoglobin including pH, since deoxyhemoglobin is a stronger base than oxyhemoglobin. Hemoglobin has a pK near 7.93 while oxyhemoglobin has a lower pK of 6.68. Under *in vivo* conditions as oxyhemoglobin releases oxygen to the surrounding tissues there is a rapid influx of H^+ from the increased CO_2. The excess H^+ are taken up by the deoxyhemoglobin preventing a change in pH. This buffering effect of Hb is called the isohydric shift.

Within our saline solution, during heating, Hb becomes deoxygenated without a great influx of H^+ from surrounding tissues. Therefore, it was expected that Hb would bind the existing H^+ in solution shifting the intracellular pH of the RBC alkaline.

pH

The results observed, however, indicate that pH shifts towards acid as temperature is increased and deoxygenation of Hb occurs. This intracellular pH shift is likely due to pH changes in the MOPS buffered solution which also shifts acid as temperature is increased. Because the intracellular pH of the RBC is coupled to the extracellular pH, an acid shift of the extracellular solution would cause the RBC intracellular pH to follow. The intracellular pH seemed to not be dependent on the oxygenated state of Hb, since intracellular pH did not shift upon reoxygenating cells following heating at 51.5°C (data not shown).

The pH-dependent processes within the erythrocyte include rate of glycolysis, hexose monophosphate shunt activity, and hemoblobin-oxygen affinity. Therefore, as Hb becomes deoxygenated due to the increase in temperature, intracellular pH decreases causing more oxygen to be released.

The peak broadening occurring in thermotolerant cells seems to be a result of Hb becoming deoxygenated, but the chemical shift of the peak representing 4,6-P is not explained. By monitoring the shift of MeP it is known that the pH shifts acid

during increases in temperature. As indicated by the titration curve of the peak representing 4,6-P of IP_5 (fig. 22; pg. 79) an acid shift in pH would cause the chemical shift of this peak to shift to the right, in the direction opposite of that observed. This indicates that the shift of the 4,6-P peak resulted from factors other than changes in pH.

The observed deoxygenation of Hb may also cause a downfield shift (towards the left, greater ppm values) in the resonant frequencies of all the intracellular and extracellular phosphates, including TEP (Labotka, 1984). This makes it very difficult to relate chemical shifts of peaks representing metabolites in RBC's to changes in pH alone. Shifts resulting from changes in bulk magnetic susceptibility were corrected for by using TEP as an internal reference compound whose resonance position shifts along with the cellular phosphates as susceptibility differences occur.

Since the shift of the 4,6-P peak correlates with the broadening of peaks, it seems that the shift of the 4,6-P peak of IP_5 is a result of deoxygenation of hemoglobin causing IP_5 to bind to another molecule or ion as Hb becomes deoxygenated. While chicken RBC's are heat shocked at 42.4°C, hemoglobin becomes progressively deoxygenated. As deoxygenation of Hb occurs some IP_5 binds in the central core of the deoxyhemoglobin while some IP_5 binds to another component of the RBC resulting in the leftward shift, at a constant rate, of the peak corresponding to the 4,6-P of IP_5. When cells are returned to 23°C the Hb remains deoxygenated indicated by the broadened state of all peaks, and IP_5 remains bound to the other component indicated by the maintained leftward shift of 4,6-P.

As Hb becomes deoxygenated and IP_5 binds to its central cavity, one would expect that the concentration of IP_5, as monitored by ^{31}P NMRS, to decrease. The binding of a metabolite to a macromolecule would make the metabolite NMRS

invisible. No decrease in the relative peak areas of the peaks representing IP_5 were observed, but the relative peak areas were very difficult to quantify since they overlapped. These relative peak areas might be decreasing, but could not be accurately detected. Therefore, it is not likely that binding to Hb would cause the observed shift of the 4,6-P peak.

Perhaps a direct property of the IP_5 is responsible for the observed thermotolerance. Remember that rat RBC's, which contain 2,3-DPG rather then IP_5, did not develop thermotolerance. IP_5 occurs in high concentrations in avian erythrocytes accounting for approximately 60 % of total RBC metabolic phosphate (Johnson and Tate,1969), but the pathway for its formation is not known. In avian RBC, IP_5 functions as a modulator of hemoglobin oxygen affinity as 2,3-DPG does in mammalian RBC's. IP_5 acts by binding to the same position in the central cavity of deoxy-Hb as 2,3-DPG, stabilizing the deoxygenated form of hemoglobin by cross-linking the beta chains of Hb (Arnone and Perutz, 1974). On oxygenation, IP_5 is extruded since the central cavity where it is bound becomes too small (Stryer, 1981).

Recently, IP_5 has also been found in small quantities (10-100 μM) in mammalian tissues (Szwegold *et al.*, 1987). It has been found in brain cells having a dose dependent effect on heart rate and blood pressure (Vallejo *et al.*, 1987). This along with the finding of a membrane phosphatase that hydrolysizes IP_5 suggests that IP_5 may have a role as an extracellular messenger (Vallejo *et al.*, 1987). The inositol phosphates have been shown to have intracellular "second messenger" roles in many cell types, causing activation of protein kinase C and elevating cytosolic calcium (Berridge and Irvine, 1984).

IP_5 also has antioxidant properties resulting from its ability to chelate iron (Graf, 1986). In this way IP_5 acts to suppress adverse iron-driven oxidative

reactions within the RBC. RBC's contain high levels of ferrous ions, oxygen and polyunsaturated fatty acids, but they remain resistant to oxidative damage. Both catalase and glutathione peroxidase functions in the RBC to remove hydrogen peroxide. Oxidative stress on the RBC can lead to hemolysis (Gutteridge, 1987). The shift of the 4,6-P peak might be due to binding of IP_5 to free iron which could be released from ferritin or Hb during heating of RBC's. Once bound to IP_5 the iron becomes completely unreactive preventing oxidative damage from occurring while heating RBC's at 51.5°C. Future experiments should be performed to investigate how changes in iron concentration affect the chemical shift of the peaks representing IP_5.

A final possible explanation for the observed thermotolerance is that during HS, cells metabolize much of the free oxygen in solution so that the partial pressure of oxygen during heating at 51.5°C is much higher in non-heat-shocked cells during heating than in the heat-shocked cells. The higher oxygen concentrations would result in the non-heat-shocked cells receiving greater oxidative damage than the heat-shocked cells. The respiratory activity of avian RBC's was shown to increase 80% with a 10°C rise in temperature (i.e., a Q_{10} of 1.8) (Besch, 1965). In preliminary experiments, the partial pressure of oxygen in RBC suspension following HS was approximately 5 times less (87 mm Hg) than in non-heat-shocked RBC suspension (436 mm Hg). The partial pressure of oxygen solution never fell below normal levels occurring in plasma, and the partial pressure of carbon dioxide never exceded normal plasma levels during either heating protocol. Future experiments should ivestigate how oxygen levels affect hemolysis during heating.

Chapter 4

Ultrasound Investigations

The goal of this study was to determine whether the effects of ultrasound radiation, below cavitational levels, could be detected and compared to damage produced by hyperthermia. The effects of U.S. heating have been investigated in proliferative tissues, but little has been done to investigate U.S. effects on terminally-differentiated tissues, where it is difficult to score damage. Ultrasound induced damage in nervous tissues has been determined by the detection of lesions (Borrelli, 1984), while cellular damage has been detected by the uptake of radiolabelled compounds across membranes (Bundy, 1978). In this investigation we used hemolysis of rat erythrocytes as an end-point. Rat RBC's were used, because their heat response indicated that they were more sensitive to stress than chicken RBC's.

Biological Effects of Ultrasound.

The effects of moderate U.S. intensity levels on terminally-differentiated tissues have been very difficult and time consuming to study due to the difficulty in scoring damage. Some examples of the investigations on terminally-differentiated tissues follow.

Fry *et al.* (1970) investigated the level of focused pulsed U.S. required to produce a lesion in the cat brain. It was determined that at lower intensities, 100 W/cm^2, and long exposure times (> 1 sec), lesions were produced by thermal mechanisms. At the highest intensity levels, above 2000 W/cm^2 and short exposure times (< 40 milliseconds) lesions resulted from cavitation. In the intermediate range, 300 W/cm^2 (1 second exposure) to 1500 W/cm^2 (0.01 second exposure) peak intensity levels, mechanical mechanisms affected the structure of the membranes.

Borrelli (1984) showed that tissue damage occurred in the cat brain following 1 MHz focused ultrasound having a spatial peak temporal average (SPTA) intensity of 300 W/cm^2 for exposure times up to 5.0 seconds. Chemical synapses were found to be the first structures affected by ultrasound. In addition, U.S. exposure caused disruption of the mitochondria in nonmyelinated structures, condensation of chromatin in neurons, damage of glial cells, swelling in the endothelial wall of capillaries, and alterations in the structure of myelinated axons. Brain regions with a higher white matter content required a lower dosage of US to induce tissue damage due to higher tissue temperatures induced in the white matter. Borrelli postulated that acoustic cavitation was involved in producing the tissue damage, since pressurizing the irradiation chamber reduced the magnitude of the effects produced by ultrasonic exposures. He suggested that the primary site of ultrasonic damage is in the selective permeability of the biological membranes at the macromolecular level. The changing ionic content of the tissue structure may have triggered the morphological changes observed in his study.

Erythrocytes are the simplest, terminally-differentiated cells that can be used to study membrane damage. There have been several investigations of the effects of U.S. in RBC's. Bundy *et al.* (1978) investigated the uptake of radiolabeled leucine in avian RBC's following 30-minute continuous wave 1 MHz exposures of U.S. having a spatial average intensity of 0.6 W/cm^2. Immediately after exposure there was no detectable hemolysis; however, they found that U.S. exposure reduced the nonmediated uptake of leucine by 5 percent. They hypothesized that a non-thermal mechanical mechanism, such as acoustic microstreaming, could reduce the RBC membrane pore size, inhibiting nonmediated leucine uptake.

Pinamonti *et al.* (1982) investigated oxygen affinity and alterations in membrane proteins including Na^+/K^+ ATPase activity following pulsed sonication

of human RBC's in whole blood. They found that following exposures of 8 MHz U.S. at a temporal peak intensity of 31.5 W/cm^2 and a SPTA intensity of 0.00207 2.07 mW/cm^2, the oxygen affinity of whole RBC samples decreased, while the oxygen affinity of Hb solutions did not change. In addition, ATPase activity was completely lost, and the major membrane antigens (A and B), assayed by agglutination with anti-sera, were reversably removed. They suggested that the pulsed U.S. exposures modified the cell membrane resulting in loss of membrane ATPase activity, thus affecting the intracellular milieu so that the oxygen affinity of Hb was reduced.

Wong and Watmough (1983) found that human RBC's suspended in saline were hemolyzed by 5 min CW 0.75 MHz exposure at a threshold U.S. intensity of 0.5 W/cm^2. Complete hemolysis occurred following 2.0 W/cm^2 exposures. They found a correlation between the threshold for cavitation, assayed by potassium iodide reduction, and hemolysis, indicating that the observed damage resulted from cavitational effects.

Hrazdira and Adler (1983) investigated the electrophoretic mobility of erythrocytes following 30 min pulsed U.S. exposures of 0.64 W/cm^2 (spatial average temporal peak) at frequencies of 2 and 6 MHz. The SPTA intensity used was estimated to be 2.6 X 10^{-3} W/cm^2. They found that the electrophoretic mobility of RBC's increased when cells were assayed 10 min after sonication. They suggested that this increase was due to an increase in the electrical potential across the membrane. They suggested that microstreaming caused changes in the ion distribution across the membrane affecting the membrane potential.

Gluckner and Milsch (1983) found that complete destruction of the erythrocyte occurred following 20 KHz sonication at 200 W/cm^2 for 15 sec. They found no differences in the electron spin resonance spectra of non-sonicated and

sonicated cells indicating that the hemoglobin molecule was not affected and no long-lived free radicals were produced following sonication.

The effects of free radicals produced during U.S. cavitation on the hemolysis of RBC's was investigated by Kondo *et al.* (1989). They found that cells would hemolyze to the same extent following 50 KHz U.S. exposure whether hydroxyl free radicals were formed or not. In addition, they found that sonication with or without the formation of free radicals produced hemolysis without affecting membrane fluidity, membrane permeability, cell deformability or cell shape. They concluded that hemolysis induced by sonication resulted from the mechanical shearing stress accompanying cavitation.

Presently a relationship between the intensities of U.S. exposure and extent of tissue damage is not known. This study will utilize hemolysis of RBC's to determine whether non-thermal effects of U.S. could be detected in a terminally-differentiated tissue and compared to effects of heat.

Materials and Methods

U.S. Procedure.

Rat RBC's were suspended to 0.7 % Hct in the same 300 milliosmolar glucose-saline that was used in all the heat response studies. The solution was oxygenated for at least 15 minutes prior to each experiment before the addition of RBC's. Approximately 16 ml of RBC suspension was added to the pyrex holders shown in figure 27. Saran wrap covered the two large ends of the holders using rubber O-rings to hold it in place. Saran wrap is an ideal acoustic coupler. The pyrex holders with saran wrap were autoclaved prior to each experiment. RBC suspension was added through the small outlet of the holder using a sterile Pasteur pipette. This outlet was then covered with parafilm.

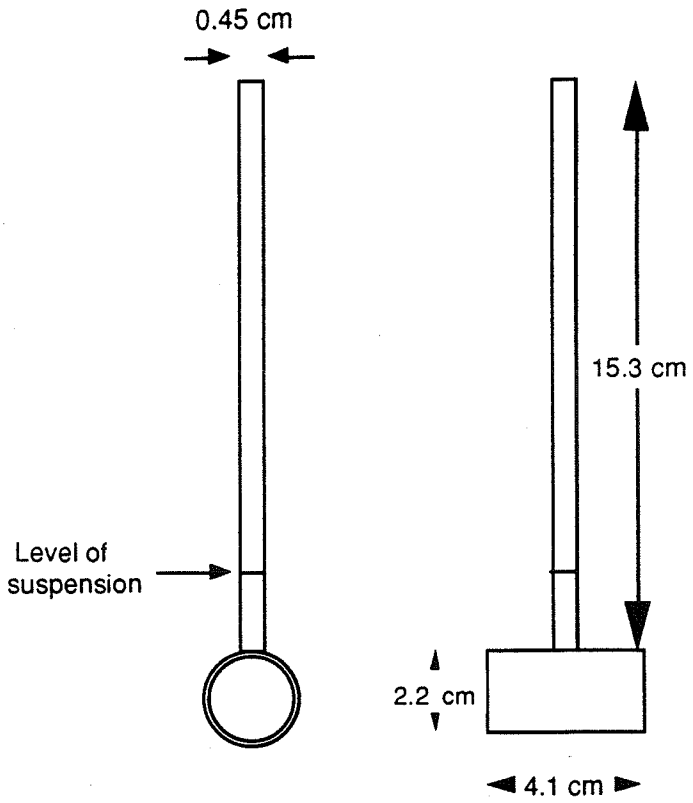

Figure 27. Diagram of Pyrex Holder Used to Sonicate RBC's.
The dimensions of the holder are as indicated. The two 2.2 cm open ends were covered with saran wrap held in place with rubber O-rings which fit snugly over the glass.

Transducer and Equipment.

Samples were irradiated using a piezoelectric focussed transducer mounted in a stainless steel housing (PZT-4). The transducer has a 5 cm aperture with a 15 cm radius of curvature. The beam pattern in the plane perpendicular to the beam axis has a half power beam width of approximately 4.5 mm determined in water. The actual beam pattern present in the RBC sample may differ from that measured in water due to differences in the acoustic properties between the two media.

The transducer was driven at 0.990 MHz using a Hewlett-Packard 8660A Synthesized Signal Generator. The transducer output was calibrated each week using the elastic sphere radiometer method (Dunn *et al.* 1977). The applied voltage necessary to generate a specific intensity, spatial peak temporal peak

(SPTP) was determined using the following equation:

$$\text{Voltage} = V_{cal} * \sqrt{\frac{\text{Intensity}}{I_{cal}}}$$

Where V_{cal} is the calibrated voltage value when a current (I_{cal})of 19.7 amperes is applied to the transducer.

The exposure times were regulated using a Computer Measurement Company Counter which activated a gate for the amplifier (Amplifier Research Model 1000L). Exposure times ranged from 1 second to 60 seconds.

The transducer was positioned by first placing a pointer over the face of the transducer. The tip of the pointer represented the location of the focal volume center and was positioned in the center of the RBC sample volume. The sample volume was 15.6 cm^3 as can be calculated from figure 27 (page 94).

U.S. Experimental Protocol.

Sample-filled holders were placed in a 27.3°C degassed water bath during ultrasound exposure. Samples were irradiated at 5, 10, 20, 30, 40, 50, and 60 W/cm^2. Exposure times ranged from one to two seconds at high intensities (60, 40, W/cm^2), 5 seconds at 30 W/cm^2, to 60 seconds at lower intensities. At 60 W/cm^2 peak exposures, the beam was pulsed at one second intervals with a 10 second wait between pulses to prevent overheating of the transducer. At 40 and 50 W/cm^2 peak exposures, the beam was pulsed for 2 sec intervals with a 10 sec wait. During this wait period the samples were mixed by depressing a pipette bulb placed on the open end of the pyrex sample holder.

During several sonications the temperature of the cell suspension was monitored using a copper/constantine thermocouple kindly supplied by Dr. James Heath. The initial temperature of the RBC suspension was taken using the thermocouple. The tip of the thermocouple was then withdrawn from the RBC sample and remained in the narrow shaft of the pyrex holder during sonication.

Exposure of the thermocouple may cause local heating of the sample at the RBC suspension-metal interface. Following exposure, the thermocouple was lowered into the sample and temperature readings were compared to readings taken before exposure. The temperature increased 0.1°C following 10 pulses of 60 W/cm^2 temporal peak intensity U.S. Local increases in temperature may have taken place near the RBC membrane interface or near cavitational bubbles, but these could not be measured and did not contribute to a significant increase in the temperature of the RBC suspension.

After exposure the samples were transferred to 15 ml polystyrene centrifuge tubes. Aliquots from each sample were withdrawn 20 minutes after sonication, diluted ten fold to 0.07 % Hct, and assayed for hemolysis as described in chapter 2 (pg 22-24). The remaining sample was maintained at 22°C $\pm$ 0.1°C at 0.7% Hct for various times before aliquots were removed and assayed. Following most U.S. exposures no immediate hemolysis was observed. These samples were heated at 50.5°C for 60 minutes following sonication to determine wheter the U.S. affected the cells sensitivity to heat.

Split Heating Protocol.

In order to try and seperate out the effect resulting from U.S. alone from samples that were sonicated and heated, survival values were compared to survival curves generated following split heating at 50.5°C. The heating procedures were the same as described in chapter 2 (pg. 24-25). Briefly cells were heated at 50.5°C in a water bath capable of maintaining temperature to within 0.1°C in the region of exposure. Polystyrene centrifuge tubes containing 9.0 ml of isotonic, MOPS buffered, glucose-saline solution were placed in the bath at least 15 minutes prior to the addition of 1.0 ml of RBC suspension at 0.7%Hct. Sample tubes were exposed to 50.5°C for 15 to 75 min; the 75 min exposure was added

first. The 60 min exposure was added 15 min later. Tubes exposed at the shorter durations were maintained at 22°C until the time for their exposure. After 75 min of adding the first sample, all exposed sample tubes were removed from the bath. The tubes were immediately placed in 15°C water for 5 min to cool. Sample tubes were then maintained at 22°C for 20 min. All sample tubes were then placed back into the 50.5°C water bath and allowed to equilibrate for 7 min before timing for the 60 min exposure was begun. Following heating, sample tubes were placed in a 15°C water bath for 5 min to cool cells before centrifuging and scoring hemolysis. Hemolysis was scored as described in chapter 2 (pg 22-24).

Iso-effect Curve Generation.

The survival following sonication alone was compared to survival following continuous heating at 50.5°C to determine the time of heating required to produce equivalent survival (iso-effect) following sonication. The comparison of survival values was also made between samples that were split-heated at 50.5°C and sonicated and heated at 50.5°C. Iso-effect curves were generated by calculating heating times required to produce the same given survival value as following 1 to 10 pulses of U.S. at 60 W/cm^2 The heating time for a continuous heat exposure was calculated from the equations describing survival following continuous heating at 50.5°C shown in fig. 4 (pg. 33). For survival greater than 92 %, the equation used to calculate heating time was:

$$\text{Time (min)} = \frac{\text{\% survival - 100.22}}{0.145}.$$

For survival less than 92 %, the equation used to calculate heating time was:

$$\text{Time (min)} = \frac{\text{\% survival - 149.5}}{0.961}.$$

These equations were obtained from the two phases of the survival curve following contiuous heating at 50.5°C shown in fig. 4. Iso-effect heating times were only calculated for survival following 4 and 5 pulses of 60 W/cm^2 U.S. exposures.

The heating time of initial heating during a split heat exposure was calculated from the regressions describing survival following split-heating at 50.5°C shown in fig. 30 (pg. 104). The points of fig. 30 were fit to a polynomial described by the equation:

$$\% \text{ Survival} = 93.96 - 4.738 * (\text{Time}) - 0.0007095 * (\text{Time})^2.$$

This equation was solved using the quadratic formula so that heating times could be calculated from experimentally obtained U.S. survival values.

Number of U.S. pulses was plotted against the calculated time values for heating, obtained from both the continuous and split exposures, to produce equivalent survival.

Kinetic Studies.

The kinetics of hemolysis following heating and following U.S. were determined and compared. Kinetics of hemolysis resulting from heating was determined by heating RBC's at 50.5°C for 30 min using the procedure described earlier. Cells were cooled by placing in 15°C water for 5 min. Cells were maintained at 22°C at 0.7% Hct and assayed for hemolysis for up to 54 hr at approximately 12 hr intervals. Hemolysis was assayed with and without heating at 50.5°C for 60 min. When heating, tubes containing 9 ml of the above saline were placed in the water bath 15 min before adding 1 ml of sample. Cells were cooled before hemolysis was assayed.

The kinetics following 4 and 5 pulses of 60 W/cm^2 U.S. were performed in the same way. Cells were sonicated and then maintained at 22°C at 0.7% Hct. Controls which were samples that were not sonicated were also maintained at

22°C and served as references in the spectrophotometer. Samples were heated for 60 min at 50.5°C after incubating at 22°C for various times up to 70 hr.

Results

Ultrasound Survival.

Figure 28 shows the survival curve of rat RBC's irradiated with one second pulses of 60 W/cm^2 U.S. with and without following sonication with heating for 60 min at 50.5°C. Samples were pulsed for 1 sec followed by a 10 sec wait resulting in a SPTA intensity of 5.45 W/cm^2. Sonication at 60 W/cm^2 had a significant effect on the survival of cells both with and without heating at 50.5°C. The survival of cells following U.S. sonication at 60 W/cm^2 alone and sonication followed by heating at 50.5°C had similar slopes indicating that RBC sensitivity to heat remained the same following U.S. exposures. Cells were heated following U.S. exposure, since initial trials at lower intensity levels resulted with low levels of hemolysis following U.S. exposures alone.

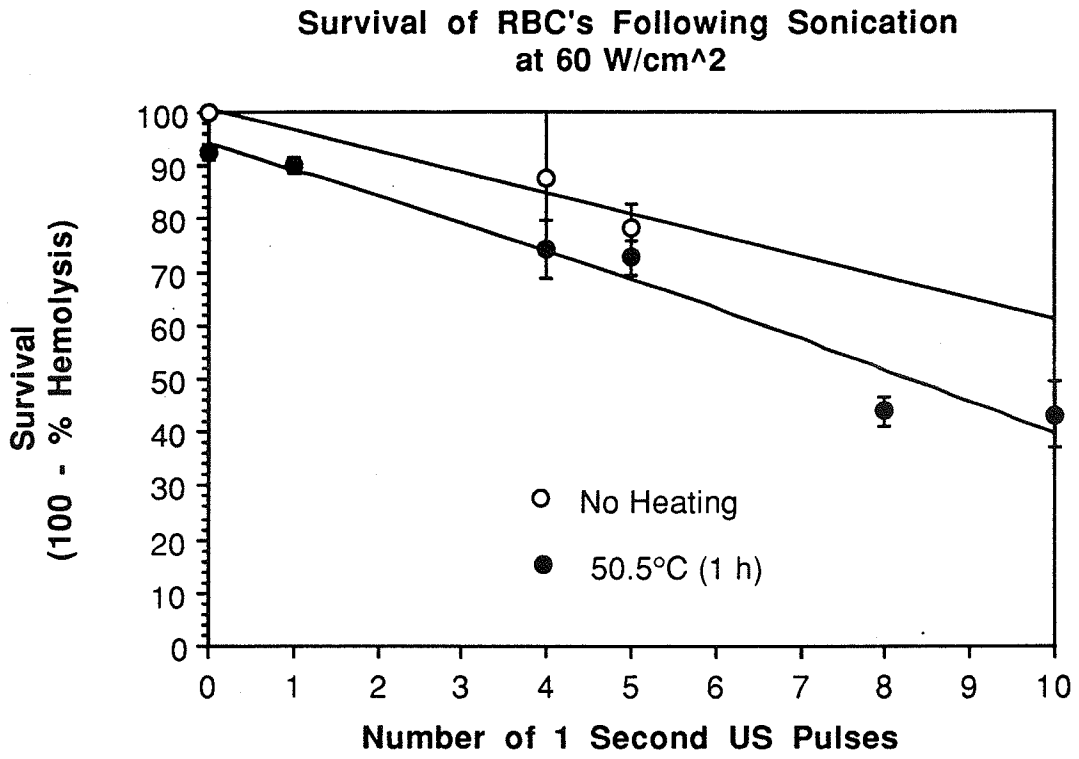

Figure 28. Survival of RBC's Following Sonication at 60 W/cm^2.
Cells were exposed to 60 W/cm^2 U.S. and then assayed immediately or following heating at 50.5°C for 60 minutes. Survival was scored immediately after heating. Plotted as mean ± SEM (n=4).

Figure 29 shows survival curves of rat RBC's after U.S. exposures at 40, 50, and 60 W/cm^2 followed by heating at 50.5°C for 1 hr. Again the SPTA intensity of the 60 W/cm^2 peak exposure was 5.45 W/cm^2. The 40 and 50 W/cm^2 peak exposures were pulsed for 2 sec with a 10 sec wait. Their SPTA intensities were 6.67 and 8.33 W/cm^2, respectively.

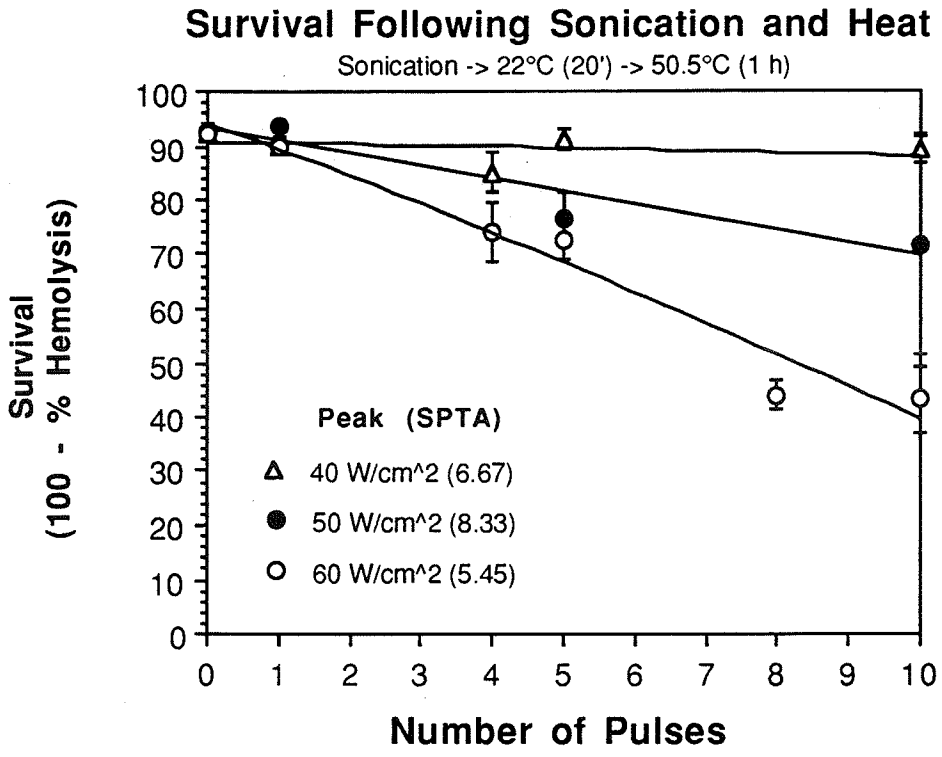

Figure 29. Survival Following Sonication and Heat.
The survival immediately after sonication at peak intensities of 40, 50, and 60 W/cm^2 and heating for 1 hr at 50.5°C is shown. The temporal average intensities are; Δ=6.67 W/cm^2, O=8.33 W/cm^2, and •=5.45 W/cm^2.

Figure 29 shows that there was no correlation between cell survival and temporal average intensity; however, there is a correlation between peak intensity and survival. The higher peak intensities caused more hemolysis. SPTA intensities below 40 W/cm ^{2} didn't cause any immediate or delayed hemolysis even after 60 sec exposure times (not shown). Observations of cell suspensions during sonication showed that cell suspensions were disrupted. During exposure at all intensities, streaming of cells could be seen. At the highest intensity level, 60 W/cm^2, slight bubbling of cell suspensions was also observed indicating that cavitation was taking place.

Because U.S. survival curves were generated by first sonicating RBC suspensions and then heating at 50.5°C for 1 hr, 20 minutes after sonication, survival curves following a split heat exposure separated by 20 minutes were generated. This would allow for comparing the hemolysis produced following sonication alone with hemolysis produced by heating. In this way the heat induced hemolysis component is subtracted from the U.S. plus heat induced hemolysis component.

Figure 30 shows the survival of RBC's following continuous and split-heating at 50.5°C. For split heating, cells were exposed to 50.5°C for 0 to 75 min and then incubated at 22°C for 20 min. Following incubation, cells were exposed to 50.5°C for 1 hr using the same methods used with sonicated cells. The survival curve following split-heating at 50.5°C was different from the survival curve following heating at 50.5°C continuously. Cells that were split-heated survived better than cells that were heated continuously when comparing total heating times. This difference was significant only up to a total heating time of 105 min. For longer total heating times differences in survival were not significant.

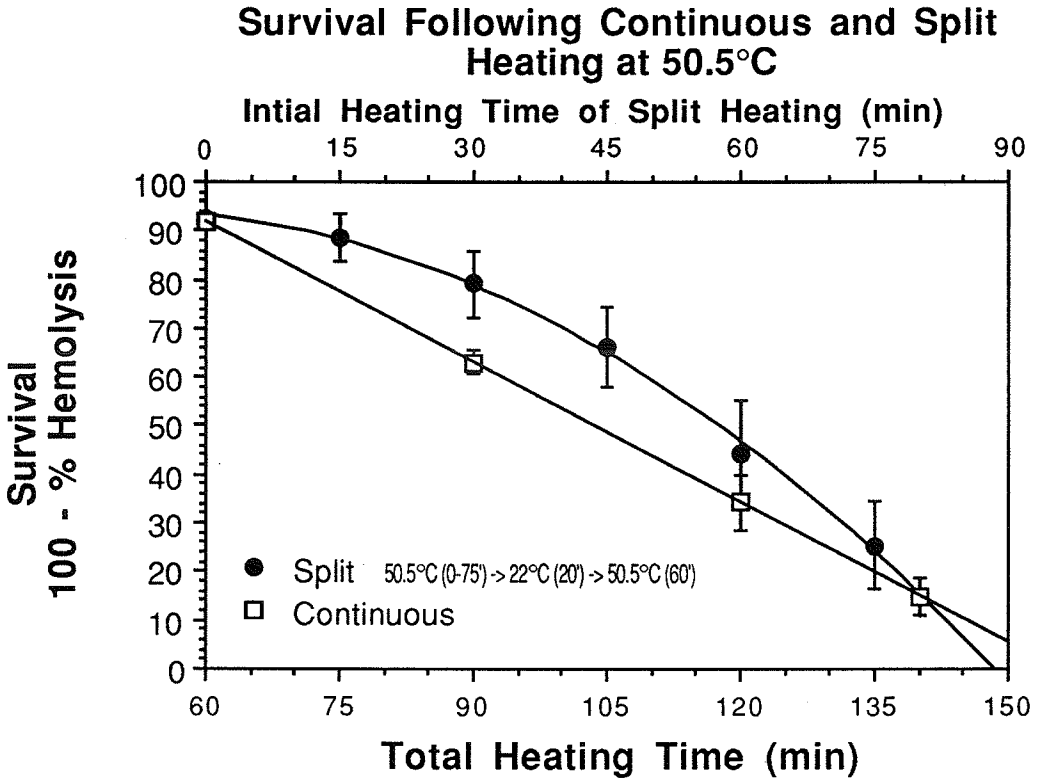

Figure 30. Survival of RBC's Following Continuous and Split Heating at 50.5°C. Survival following continuous and split-heating at 50.5°C is shown. Split heat exposures were separated by 20 min with the initial heating lasting between 0 and 75 min. Total heating time (0 -> 75 + 60 min) is shown along with initial heating time for samples that were split heated. Plotted as Mean ± SEM (n=5).

Figure 31 shows the relationship between pulses of 60W/cm^2 U.S. alone and continuous heating time at 50.5°C, and pulses of 60W/cm^2 U.S. followed by heating at 50.5°C and split-heating time at 50.5°C to produce equivalent hemolysis. This figure shows the iso-effect between heat and U.S.

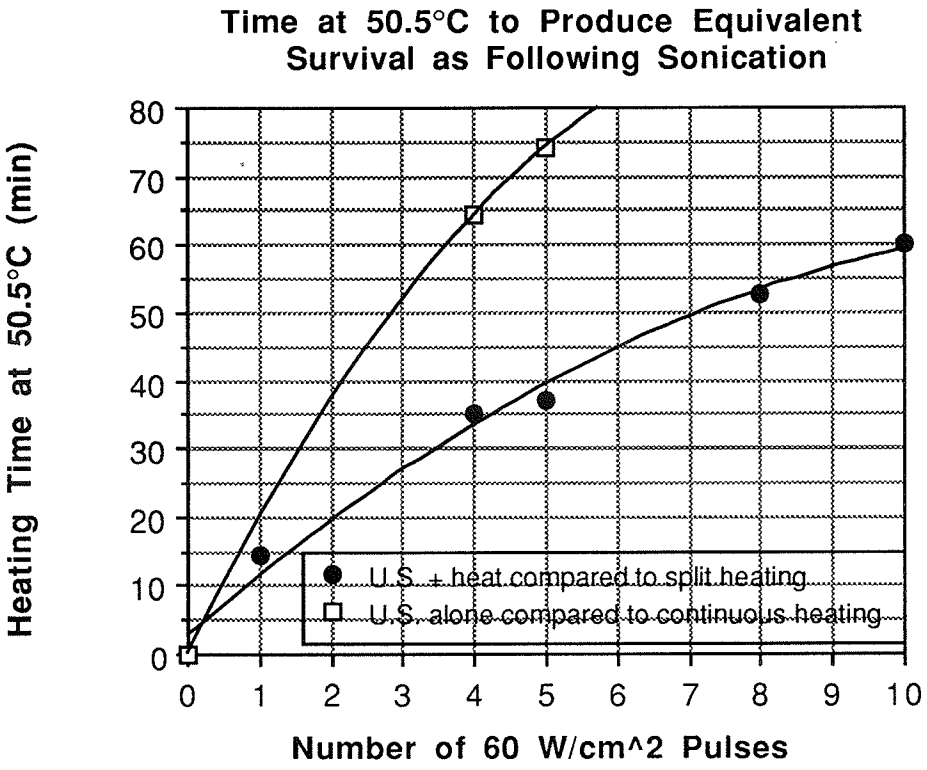

Figure 31. Time at 50.5°C to Produce Equivalent Survival as Following Sonication. Equivalent time at 50.5°C to produce the same survival following sonication at 60 W/cm^2. Two curves are shown. One curve shows the equivalence of U.S. + heat to split heating at 50.5°C, while the other curve shows the equivalence of U.S. alone and continuous heating at 50.5°C. Heating times for this figure were calculated from regression equations describing survival following split heating or continuous heating.

Next the kinetics of damage following heat or U.S. were compared to determine whether each modality caused similar damage to the RBC. If both U.S. and heat affected the same target(s) of the RBC identically, then it would be expected that equivalent doses would cause damage to occur at the same rate during incubation at 22°C. Figure 32 shows the survival of RBC's heated at 50.5°C for 30 minutes followed by incubation at 22°C for 0 to 54 hours, with and without heating again at 50.5°C for 1 hour. The figure indicates that hemolysis continues to occur during incubation at 22°C and total hemolysis takes approximately 55 hours. The difference between the two curves remains relatively constant suggesting that

there is no significant change in the sensitivity of cells to 50.5°C heating during the incubation period up to 30 hr.

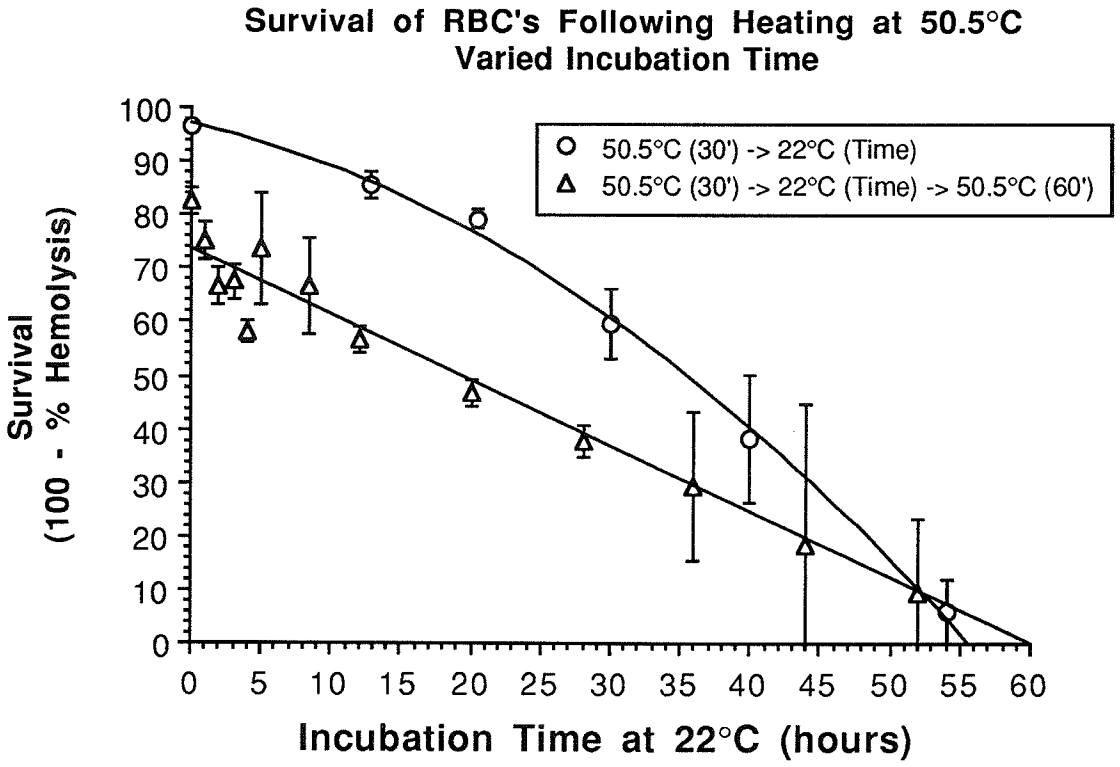

Figure 32. Survival of RBC's Following Heating at 50.5°C: Varied Incubation Time. Survival curve of RBC's heated at 50.5°C for 30 minutes followed by incubation at 22°C for 0 to 52 h, with (Δ) and without (O) heating again at 50.5°C for 1 hour. The figure indicates that hemolysis continues to occur during the incubation period. The sensitivity of cells to subsequent heating at 50.5°C does not change significantly over time. Plotted as Mean ± SEM.

Figure 33 shows the survival of RBC's following either 1 or 5 pulses of U.S. and incubation at 22°C for up to 70 hours before heating at 50.5°C for 1 hour. Ideally, 1.5 or 3 pulses of 60 W/cm^2 U.S. (fig 31) caused the same amount of hemolysis as 30 minutes at 50.5°C, but the kinetics following 1 and 5 pulses of U.S. sonication were similar for hemolysis following 1,4,5, and 8 pulses of U.S. (not shown).

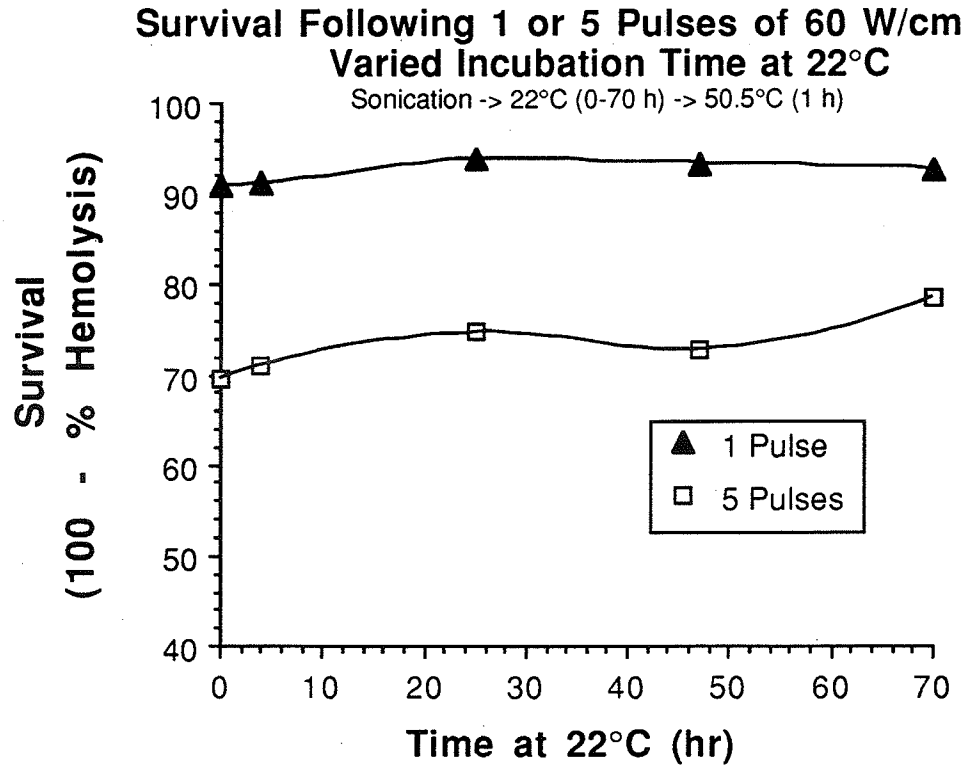

Figure 34. Survival Following 1 or 5 Pulses of 60 W/cm^2 U.S. Varied Incubation Time at 22°C. Survival of RBC's following either 1 or 5 pulses of U.S. followed incubation at 22°C for up to 70 hours. The extent of hemolysis following sonication and heat is not affected with incubation at 22°C unlike after heating for 30 minutes at 50.5°C shown in figure 33.

Comparing figures 32 and 33 indicate that the kinetics of damage following 50.5°C heating are makedly different from damage following sonication and heating. This suggests a qualitatively different effect of U.S. on membranes than the effect of heating at 50.5°C.

Discussion

The effect of U.S. on the survival of rat RBC's was compared to the effect of heat on RBC survival. By comparing the hemolysis produced following continuous heating at 50.5°C with hemolysis following sonication alone and the hemolysis following split heating at 50.5°C with hemolysis following sonication and heating at 50.5°C, iso-effect curves were generated. The kinetics of hemolysis following each

modality were significantly different indicating that each modality affected the RBC differently.

U.S. Survival.

The survival of RBC's, assayed by hemolysis, following U.S. exposure indicated that rat RBC's required greater than 50 W/cm^2 SPTP intensities of U.S. at 0.99 MHz to significantly affect hemolysis. RBC sensitivity to heating didn't change following sonication at the exposures used in this study. Hemolysis in RBC's increased as the SPTP intensity increased and as the number of pulses increased. The intensity levels used in these experiments to cause hemolysis were significantly higher than those used by other investigators. Pinamonti et al. (1982) used 8 MHz 31.5 W/cm^2 SPTA intensity and found that ATPase activity was lost and membrane antigens were removed, while Wong and Watmough (1983) found a correlation between cavitation at a threshold U.S. intensiy of 0.5 W/cm^2 and hemolysis. The theroretical threshold U.S. intensity required to cause cavitation in water is approximately 0.35 W/cm^2 (Akopyan, 1983). Factors affecting cavitation include U.S. frequency, pulse length, duty cycle; ambient pressure and temperature; and the presence of gasseous micronuclei in the sample. Crum and Fowlkes (1986) detected cavitation in water using a chemiluminescence technique following exposure of U.S. at a frequency of 1 MHz and peak intensities in the range of 10 to 30 W/cm^2. Their detection technique relied on the formation of free radicals which oxidized luminol to produce light emissions. The onset of cavitation was not investigated in this study; however, bubbling in the sample following 60 W/cm^2 is a good indication that cavitation was taking place.

The great local temperatures and pressures produced during cavitation produce free radicals, and shock waves, while the cavitation bubbles produce hydrodynamic shearing stress. The hydroxyl radical formed by cavitation was

shown to cause lipid peroxidation in liposomal membranes at frequencies of 20 KHz and 3.5 MHz (Jana, *et al.*, 1990). The lipid peroxidation of RBC membranes may affect the integrity of the membrane and result in the eventual hemolysis of cells; however, Kondo *et al.* (1989) showed that production of free radicals did not affect hemolysis. They found that hemolysis following cavitiation occurred whether hydroxyl free radicals were formed or not. They suggested that hemolysis was caused by the mechanical shearing stress accompanying cavitation.

Split Heating.

Results indicate that hemolysis following split heating at 50.5°C occurs to a lesser degree than following continuous heating for the same total heating time when the initial heating time of the split heating is less than 60 min (fig. 30, pg. 104). This relates back to the two phased kinetics of hemolysis following heating shown in figure 4 (pg. 33). Figure 4 indicated a threshold for hemolysis below 60 minutes of heating. Figure 30 suggests that if heating is suspended before 60 minutes, then there is a possible reversal or repair of initial damage delaying hemolysis. Split heating where the initial exposure at 50.5°C is less than 60 min, requires longer total time exposures to produce the same hemolysis that occurs following continuous heating, but for longer than 60 min initial exposures, produces the same hemolysis as following continuous heating at 50.5°C.

Iso-Effect Curve.

Figure 31 shows two iso-effect curves obtained by comparing survival following U.S. exposure alone to survival following continuous heating at 50.5°C and survival following U.S. exposure and heating to survival following split heating. The two curves are significantly different. The curve following continuous heating is greater because of the shoulder occurring for the first 60 min on the survival

curve during continuous heating at 50.5°C. This suggests that the effects of heat on the RBC are different from the effects of U.S. on the RBC.

Kinetics.

The kinetics of damage by the two modalities were also compared. Following sonication, the extent of hemolysis remains constant over time up to 70 hours, while the extent of hemolysis following heating increases over time. Following 30 min at 50.5°C, the sensitivity of cells to heating at 50.5°C remains fairly constant up to 45 hours at 22°C. The figures suggest that damage following heating is lethal and eventually causes the lysis of all cells. Damage following U.S. only affects cells initially by causing hemolysis, but does not affect cells later. These results are consistent with the results of Kondo, *et al.* (1989). Hemolysis increased as exposure time increased, and hemolysis remained constant during post-treatment incubation.

The three known damaging effects of U.S. are due to heating, microstreaming, and cavitation. The ultrasound irradiation of these RBC suspension is not likely to cause a change in temperature due to the low absorption coefficient of the cell suspension. Measurements of the RBC suspensions verified that the temperature of the suspension did not increase significantly over the duration of sonication. The observed hemolysis may be caused by cavitational effects or micro-streaming. During sonication at 10 to 60 W/cm^2 disruption of the cell suspension is observed. In addition, at 60 W/cm^2 bubble formation is seen indicating cavitation. The results of this study and the results of Kondo *et al.* (1989) suggest that U.S. affects RBC's differently from heat when hemolysis is used as an end-point.

Conclusions

In this study the effects of heat and of ultrasound were investigated in the terminally-differentiated erythrocyte. RBC's from rat and chicken were utilized. To determine extent of damage in the RBC, an assay was developed using hemolysis as an endpoint. It was determined that the absorption coefficient was most stable at 410 nm so the optical density of RBC supernatant was determined at 410 nm.

The heat induced hemoysis of both chicken and rat RBC's was determined at various temperatures ranging from 45 to 51.5°C. Rat RBC's were found to be more heat sensitive than chicken RBC's. The critical damage in rat RBC's was found to occur between 30 and 60 min of heating at 50.5°C. At some point between these times the slope of the survival curve increases possibly indicating that a non-lethal lesion in the RBC has transformed into a lethal lesion as suggested by Jung (1986). This is confirmed by monitoring the kinetics of hemolysis following heating for 30 or 60 min at 50.5°C and by generating split heat survival curves. Seventy five percent of cells heated for 60 min hemolyzed while only 15 % of cells heated for 30 min hemolyzed 20 hr following heating. The target of heat damage might be spectrin or a stabilizing protein of spectrin.

The pH of the suspension saline was found to affect survival. Cells suspended in glucose-saline between pH 7.2 and 7.4 survived equally well, while cells suspended at pH 6.8 and 8.0 were more sensitive to heating.

Induction of thermotolerance was achieved in fresh chicken RBC's by chronically heating cells at 42.4°C for 2 hr. This temperature was chosen because investigations with other cells lines indicated that chronically heating cells 1.5-2.0°C above normal physiological temperature caused the induction of thermotolerance, and avian RBC preparations were shown to synthesize HSPs when heated above 42.0°C. Induction of thermotolerance in fresh rat RBC's was

not achieved. Chronic heat shock of rat RBC's at 40.6°C did not cause induction of thermotolerance, but rat RBC's that were refrigerated overnight at 4°C could be made thermotolerant by acutely heating at 48.6°C for 6 min followed by incubation at 37°C for 1 to 3 hr. This tolerance was probably an artefact of the overnight refrigeration. Because rat RBC's did not develop thermotolerance following chronic HS at 40.6°C, they may not be able to achieve thermotolerance. Therefore, possibly some characteristic of the chicken RBC's that is not present in rat RBC's, such as the presence of a nucleus or IP_5, is causing themotolerance. Otherwise preincubation temperatures below 38°C had no effect on the subsequent survival of RBC's following heating.

The heat response of rat RBC's of different age was determined by separating cells according to age using ultracentrifugation. Preliminary data suggested that all cell populations survived the heat equally except for the youngest, least dense fraction. Since it has been shown that the least dense fraction is often reticulocyte enriched, reticulocytes might be more heat resistant than mature erythrocytes. The many changes which a RBC undergoes as it ages, does not affect the heat induced hemolysis of RBC's.

Investigations of the changes occurring in the phosphorus metabolites during heating using ^{31}P NMRS indicated a few differences between heat-shocked and non-heat-shocked cells. There was no difference in the levels of nucleoside triphosphates to differentiate thermotolerant cells as initially expected. In addition, the pH of thermotolerant and control cells were essentially the same during all heatings. Differences were observed in the line width of peaks indicating that heat-shocked cells are more deoxygenated following HS than control cells, and a shift in the peak representing the 4,6-P of IP_5. The deoxygenated state of Hb may indicated that heat-shocked cells have less free oxygen available to react causing

less oxidative damage in the RBC. The shift in the peak representing 4,6-P of IP_5 may indicate that IP_5 is binding to free iron in the RBC preventing iron from catalysing oxidative damage in the RBC. The observed difference between heat-shocked and non-heat-shocked control cells are not observed in cell lysates undergoing a HS protocol. This may indicate that the RBC membrane or nucleus is involved in the observed changes and the induction of thermotolerance.

Investigations using U.S. to induce hemolysis in RBC's showed that the extent of damage produced in the RBC could be monitored and compared to effects of heat, but that the effects caused by each modality were different. Also, the kinetics of damage following the two modalities were different indicating that the two modalities act on different targets or affect the same targets differently.

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