Order Number 9210888

The effects of hyperthermia and ionizing radiation on chicken erythrocytes: A possible model for terminally-differentiated tissues

Lee, Susan Wei Shenn, Ph.D.

University of Illinois at Urbana-Champaign, 1991

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE EFFECTS OF HYPERTHERMIA AND IONIZING RADIATION ON CHICKEN ERYTHROCYTES : A POSSIBLE MODEL FOR TERMINALLY-DIFFERENTIATED TISSUES

BY

SUSAN WEI SHENN LEE

B.S., University of Illinois, 1985
M.S.,University of Illinois , 1987

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, 1991

Urbana, Illinois

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN

THE GRADUATE COLLEGE

JUNE 1991

WE HEREBY RECOMMEND THAT THE THESIS BY

SUSAN WEI SHENN LEE

ENTITLED THE EFFECTS OF HYPERTHERMIA AND IONIZING RADIATION ON CHICKEN ERYTHROCYTES: A POSSIBLE MODEL FOR TERMINALLY-DIFFERENTIATED TISSUES

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

H S Bracoff
Director of Thesis Research

John E Fahn
Head of Department

Committee on Final Examination†

H S Bracoff
Chairperson

Patricia A. Johnson M.D. Ph.D

Harold M. Swartz

C. Ladd Prosser

Janice Baker

† Required for doctor's degree but not for master's.

O-517

ACKNOWLEDGEMENTS

I want to thank my research advisor, Dr. Howard S. Ducoff, for his support and guidance during the course of these studies. His wisdom in both science and life is greatly appreciated.

My sincere gratitude also extends to Drs. J. Bahr, P. Johnson, C. L. Prosser and H. Swartz for serving on my doctoral committee.

I would like to thank my family and friends for their support and understanding. Special thanks go to my good friend and colleague, Ms. Melissa Woo, for her expertise in computer applications and for just being her.

Most of all I would like to thank my fiance, S. K. Dong, for his love and understanding even when I was being impossible.

TABLE OF CONTENTS

1. INTRODUCTION

Ionizing radiation has been used therapeutically at least since 1897, when Professor Freund successfully eliminated a hairy mole with X-rays (Hall, 1988). Although heat has a long history of clinical use, most of the cases reported are either poorly controlled or anecdotal. Only recently has heat been recognized as an effective therapeutic modality to treat neoplasms when used in conjunction with radiation. Due to this clinical application, the effects of heat and of radiation have been studied intensively.

Before clinical usefulness of heat was recognized, the cellular mechanisms of heat had long interested many scientists. The heat shock response was first described by Ritossa in 1962 when he noted that heat induced a set of well-defined puffing patterns on chromosomes of the salivary glands of *Drosophila busckii*. The puffs were shown to be sites of active transcription and their gene products were later identified in *Drosophila melanogaster* (Tissieres et al., 1974). The gene products are called heat shock proteins (HSP's). Since then HSP synthesis has been reported in *Escherichia coli* (Yamanori and Yura, 1980), yeast (McAlister and Finkelstein, 1980), plants (Altschuler and Mascarenhas, 1985; Lin et al., 1984), mammalian (Henle and Leeper, 1979) and avian cells (Kelley and Schlesinger, 1978).

HSP's are a family of proteins with different molecular weights. These proteins are well-conserved throughout evolution. HSP 70 (the 70 denotes the molecular weight of the protein subuint in kilodalton) is the most conserved among them. HSP 70 from yeast, slime mold, corn seedling roots, flies, rodent, chicken and human cells all crossreact with anti-chicken HSP 70 antibody (Schlesinger et al., 1982b). There is also evidence for similarities between the molecular mechanism regulating the heat shock response in procaryotic and eucaryotic organisms (Bardwell and Craig, 1984). Since HSP's are so well

conserved, it seems likely that they would play an important role in cellular function. One of the roles postulated is the development of thermotolerance, a transient resistance to heat due to a prior non-lethal heat treatment.

While the development of thermotolerance is well characterized in proliferative tissues using cultured mammalian cells as models, it is not well characterized in terminally-differentiated tissues. This may be due to the lack of a convenient endpoint to measure survival in terminally-differentiated cells. There are reports of HSP synthesis in nucleated avian red blood cells (RBC's), but the investigators (Dean and Atkinson, 1985; Banerji et al., 1984) did not address the biological question: do avian RBC's exhibit thermotolerance? Therefore, one of the major objectives of this study was to develop a model for thermotolerance in terminally-differentiated tissues using chicken RBC's.

The cytotoxic action of ionizing radiation is better understood than heat. Radiation acts at the level of DNA. This is clearly shown by the radionuclide experiments (Warters et al., 1977). Because radiation acts at the level of DNA, it is more effective in killing mitotically active cells such as intestinal crypt cells (Quastler, 1956). Radiation has two modes of action, direct ionizing and indirect action via the formation of free radicals, especially hydroxyl radicals from radiolysis of water; these cause DNA strand breaks, base damage and cross-links.

However, there are many studies suggesting that cell membranes are also targets for radiation damage (Alper, 1979; Yatvin et al., 1984; Suzuki and Akamatsu, 1980). This is very plausible since the major components of cellular membranes are phospholipids and proteins and they are susceptible to radiation damage. Free radicals formed by radiolysis of water can easily oxidize polyunsaturated fatty acyl chains found in phospholipids. This lipid peroxidation can in turn damage membrane-bound proteins and eventually lead to cell lysis.

Since clonogenicity was inappropriate for assaying chicken RBC's, a hemolytic assay was developed. The assay became a reliable measurement for scoring the effects of physical insults (e.g., heat and ionizing radiation) on chicken RBC's when cells were suspended in the proper medium conditions. Two other indices, namely potassium leakage and osmotic fragility, were also used to score [heat] effects on chicken RBC's.

In the present study, the effects of heat, radiation and their combination on chicken RBC's have been investigated. Using chicken RBC's as a model for terminally-differentiated tissues, the objectives of this thesis were to determine: 1) the inducibility of thermotolerance and the heat shock conditions that may affect the level of induction, 2) the sparing effect of [radiation] dose fractionation and 3) possible synergism between heat and ionizing radiation.

2. HISTORICAL REVIEW

The elucidation of the mechanisms of hyperthermic cell killing and of thermotolerance are critical in the treatment of cancer, for hyperthermia is currently used in conjunction with ionizing radiation in treating human cancers (Overgaard and Overgaard, 1972; Overgaard and Nielson, 1983). The rationale of using this combined therapy stems from the following complementary actions of heat and radiation (Hall, 1988):

(1) heat and radiation act on different cell populations. Cells in S phase is most sensitive to heat, whereas M and G_2 cells are most sensitive to radiation,

(2) chronic hypoxia enhances heat killing,but it reduces radiosensitivity,

(3) heat interferes with repair of radiation damage either by reducing the activity of repair enzymes or by making damaged DNA sites inaccessible to repair enzymes (Dewey, 1989).

2.1 Action Of Radiation

The action of radiation is well established. Its critical target is believed to be at the level of DNA. Auger electron emitters such as ^{125}I decay by emitting low energy electrons which results in high density of electron irradiation in the immediate surrounding of the decaying radionuclide (Kassis et al., 1985). Because of this property, ^{125}I labelled thymidine analogue (iododeoxyuridine) and ^{125}I labelled concanavalin A are used to preferentially irradiated DNA and plasma membrane, respectively. Radionuclide experiments such as this showed that ^{125}I-iododeoxyuridine is much more lethal to cells than ^{125}I-concanavalin A (Warters et al., 1977). This illustrates DNA as the primary target for radiation cell killing.

However, there are reports that indicate damage to cellular membranes can also lead to cell death. The mode of action is most likely through lipid peroxidation. Free radicals formed by radiolysis of water can oxidize polyunsaturated fatty acyl chains found in phospholipids, and the peroxidized lipids can cause damage to membrane-bound proteins. This leads to ion leakage, thus disturbing the delicate ionic balance of the cell and then killing the cell.

Regardless of the mechanism of radiation cell killing, cytocidal radiation damage can be classified into three categories:

(1) lethal damage, which is irreparable,

(2) sublethal damage (SLD), which is repairable and does not cause cell death unless more SLD accumulates,

(3) potentially lethal damage (PLD), which leads to cell death, but can be modified by postirradiation conditions.

These are all operational terms, since the nature of these damages is unknown. However, they are used to describe survival curves. The survival curve includes an initial shoulder, followed by a straighter portion. The shoulder represents ability to accumulate SLD, and as SLD accumulates, cell survival begins to drop. The straighter portion, or the slope portion, is represented by PLD.

The slope portion of the survival curve can be modified by postirradiation conditions. One of them is the liquid-holding technique. If after irradiation, cells were held in balanced salt solution rather than nutrient-rich medium for several hours before plating, cell survival is increased. This is attributed to PLD repair. While cells are in the balanced salt solution, they can repair the radiation damage they sustained before mitosis takes place. Cells kept in nutrient-rich medium enter mitosis before radiation damage is repaired resulting in more cell killing.

Repair of SLD was demonstrated by split-dose fractionation experiments (Jacobson, 1957; Elkind and Sutton, 1959). Cell survival is enhanced when a total dose is given in two or more fractions with an incubation period spaced between the fractions. Split-dose experiments were first done on asynchronous cell populations. Although the resulting survival curve showed sparing effect of dose fractionation (sdf), it was affected by differences in the radiosensitivity of cells at different stages of the cell cycle. Cell cycle progression was disrupted when cultured mammalian cells were maintained at room temperature. When the same dose fractionation experiment was performed (Elkind et al.1965) at room temperature, SLD repair was clearly demonstrated. SLD can also be characterized by the return of the shoulder (Elkind and Whitmore, 1967).

2.2 Action Of Heat

There is much uncertainty about the critical target(s) for heat killing. Several cellular sites have been suggested; they are plasma membrane components (e.g., lipids and/or membrane proteins), cellular proteins and DNA. Heat induces plasma membrane blebbing and the degree of blebbing correlates with cell survival (Borrelli et al., 1986a). Also, substances that increase membrane fluidity such as ethanol and procaine enhance hyperthermic cell killing (Massicotte-Nolan et al., 1981; Yatvin, 1977). However, plasma membrane blebbing doesn't appear to affect membrane function when ion, sugar and amino acid transport are measured (Borrelli et al., 1986b). This does not imply that plasma membrane is unaffected by heat. Heat may interfere with anchoring of cytoskeleton to plasma membrane and this may be the event associated with membrane blebbing (Dewey, 1989).

Another proposed target for heat killing is cellular proteins. The activation energy for hyperthermic cell killing is about 140 kcal/mole (for temperatures above 43°C) which is

similar to that for protein denaturation and inhibition of protein synthesis (Dewey et al., 1977). For temperatures below 43°C, the activation energy for heat cell killing is 365 kcal/mole. This suggests that there is more than one mechanism of heat killing depending on the temperature range.

DNA is also a proposed target. Heat has been shown to induce a few single-strand or double-strand DNA breaks as well as depurination of DNA (Roti Roti and Laszlo, 1988; Warters and Brizgys, 1987). The activation energy for depurination is about 140 kcal/mole which is similar to the activation energy required for hyperthermic cell killing. Nonetheless, the evidence for DNA as the critical target for heat killing is not very strong. Bromodeoxyuridine incorporation sensitizes cell to ionizing radiation but not to heat (Dewey et al., 1971a and b). It is possible that heat does not damage DNA per se but it affects cellular enzymes or structural proteins that are associated with DNA which leads to the eventual cell death.

Heat induces distinct patterns of chromosomal puffs in *Drosophila* (Ritossa, 1962). The puffing patterns are sites of active transcription and their gene products are called heat shock proteins (Tissieres et al., 1974). Almost all organisms examined to date synthesize heat shock proteins (HSP's) in response to elevated temperature and develop thermotolerance (reviewed by Schlesinger et al., 1982a and by Atkinson and Walden, 1985). The only cells reported that do not exhibit detectable HSP synthesis while developing heat resistance are those in very early stages of *Xenopus* embryogenesis (Heikkila et al., 1985), *Tetrahymena thermophila* (Hallberg, 1986) and growing pollen tubes of *Tradescantia* when temperature is increased gradually (Altschuler and Mascarenhas, 1982).

HSP's are a family of proteins with different molecular weights. They are named according to their subunit molecular weights (e.g., heat shock protein subunits weighing

70 kilodaltons is termed HSP 70). These proteins are well conserved throughout evolution but HSP 70 is the most conserved. HSP 70 from yeast, slime molds, corn seedling roots, flies, rodents, chickens and human all crossreact with anti-chicken HSP 70 antibody (Schlesinger et al., 1982b). This shows that the 3-dimensional structure of HSP 70 from different organisms is quite similar suggesting that HSP 70 must play an important role in cellular function.

Since the first report of heat-induced heat resistance in mammalian cells (Gerner and Schneider, 1975), the same phenomenon has been observed in many other cell systems. Because the synthesis and decay of HSP's appear to correlate with the development of thermotolerance (Mivechi and Li, 1985; Landry et al., 1982; Tomasovic and Koval, 1985; Li and Hahn, 1983), one of the functions of HSP's that has been postulated is the development of thermotolerance. Other lines of evidence supporting this hypothesis include: agents such as ethanol and arsenite induce HSP's synthesis as well as thermotolerance (Li, 1983); HSP's synthesized using amino acid analogs are nonfunctional and even sensitize cells to thermal killing (Li and Laszlo, 1985). Rat fibroblasts injected with anti-HSP 70 antibody were unable to survive heat stress (Riabowol et al., 1988).

There are several HSP's with different molecular weights, but HSP 70 appears to be important in terms of protecting cells against heat. Li (1985) showed that low molecular weight HSP's do not correlate with cell survival under heat stress, but HSP 70 does. In the study by Riabowol et al. (1988), they showed that injecting anti-HSP 28 did not affect cell survival under heat stress.

HSP 70 is present in normal cells as well as heat-shocked cells. Thus, HSP 70 has been implicated in normal cell function. It is believed to be involved in the translocation of proteins through intracellular membranes during normal cell growth; this is done probably by an "unfoldase" activity (Deshaies et al., 1988). HSP 70 has been shown to contain

ATPase activity. It is probably through this action that HSP 70 stabilizes proteins against heat denaturation or promotes renaturation of proteins that have been denatured by heat (Welch and Suhan, 1986; Mizzen and Welch, 1988). There is good evidence for these actions of HSP 70 in procaryotes and lower eucaryotes. Whether, the actions of HSP 70 in higher eucaryotes are similar is uncertain.

Because cell membranes are believed to be important in cells' sensitivity to hyperthermia (Yatvin, 1977; Lepock, 1982), their modification under heat stress has been extensively studied. Bacteria respond to heat by changing the degree of saturation of their fatty acid chains in the membrane and mammalian cells change their membrane fluidity by altering their cholesterol to phospholipid ratio (Leyko and Bartosz, 1986). However, cholesterol to phospholipid ratio is not altered in many mammalian cell systems that are in a thermotolerant state (Anderson and Parker, 1982; Konings and Ruifrok, 1985; Anderson et al., 1988). It has been suggested that thermotolerance is brought about by HSP 70's association with cytoskeletal components during thermotolerant state (Ohtsuka et al., 1986).

A ubiquitin-linked protein degradation system has been proposed as the trigger mechanism for HSP's synthesis (Munro and Pelham, 1985). During heat shock and other environmental stresses, abnormal proteins are not degraded efficiently because of limiting ubiquitination. As abnormal proteins accumulate in cells, the heat shock response is triggered. A non-ubiquitinated heat-shock transcription factor (HSTF) promotes the transcription of heat shock genes (Parker and Topol, 1984a and 1984b). This mechanism is self-regulating: as free ubiquitin becomes available, the ubiquitinated HSTF becomes inactivated and shuts off heat shock transcription.

The molecular mechanism of heat shock response varies from organism to organism (Burdon, 1982). The ubiquitin-linked degradation system explains the control at

transcription level. Translation control of HSP synthesis has been cited in *Drosophila* and chicken reticulocytes (Lindquist, 1980; Banerji et al., 1984). An autoregulation hypothesis has been put forward to explain translational control of heat shock response. Chicken reticulocytes contains high levels of cytoplasmic HSP 70 under normal conditions. Under physiological temperature, HSP 70 binds to pre-existing HSP 70 mRNA to block its translation. Upon heat shocked, HSP 70 dissociates with mRNA. This allows ribosome to move along its mRNA and translation can proceed efficiently. In *Drosophila* cells, autoregulation involves high levels of HSP 70, which are made during heat shock, to destabilize its mRNA and hence, turning off the synthesis of HSP 70 (Lindquist, 1980).

The level of abnormal proteins appears to play an important role in triggering the heat shock response in procaryotes and lower eucaryotes. The data for higher eucaryotes are limited. The possible role of DNA damage as a trigger in eucaryotes is being investigated (Anderson et al., 1988). They have shown that DNA damage does not trigger HSP synthesis in Chinese hamster ovary (CHO) cells. However, the authors explained that DNA damage produced in their study is not representative of the DNA damage produced by heat. They are trying to carry out experiments using DNA lesions that are more similar to heat-induced DNA damage, namely, abasic DNA sites. Meanwhile, DNA base damage produced by hydrogen peroxide has been shown to induce HSP 70 synthesis in CHO cells (Spitz et al., 1987). At this stage, the triggering mechanism in higher eucaryotes is not resolved. It may turn out to be similar to that of procaryotes. It would not be surprising for a universal response such as the heat shock response to have similar triggering molecular mechanisms.

2.3 Combined Action Of Heat And Radiation

The potential advantages of combining heat and radiation for the treatment of neoplasms were discussed in the beginning of this chapter. However, the biggest challenge for this therapy to become a success is to find a treatment sequence (i.e., preirradiation heating vs. postirradiation heating) that will yield the largest therapeutic gain. Depending on which of the three complementary actions of heat and radiation one believes most important, the treatment sequence will differ. According to the repair theory, one would need to give heat first in order to prevent repair of radiation damage. If radiation precedes hyperthermia, some radiation damage will be repaired before patients are set up for hyperthermia treatment, since the half time for fast radiation repair in plateau-phase mammalian cells is about 10 minutes at 37°C (Malcolm and Little, 1979). According to either the cell cycle sensitivity or chronic hypoxia theory, the sequence of treatment doesn't matter much. For the cells cycle sensitivity theory, the treatment sequence should follow one another shortly to prevent sensitive populations from entering the resistant phase of the cell cycle. As for chronic hypoxia theory, timing does not play an important role since tumor reoxygenation takes weeks to accomplish.

There are many reports on the efficacy of cell killing by combining heat and radiation. In most cases, the combined treatment resulted in greater cell killing than either agents alone but the degree of potentiation varies from system to system (Raaphorst et al., 1979; Ben-Hur et al., 1974; Harris et al., 1976). However, there is no consensus on which treatment sequence provides the greatest amount of cell killing. Some reported more killing for preirradiation heating (Li et al., 1976), while others reported more killing for postirradiation heating (Raaphorst et al., 1988). No difference in the treatment sequence has also been observed (Boone et al., 1976; Murthy et al., 1977).

The synergism between heat and radiation could theoretically be either heat potentiation of radiation damage or radiation potentiation of heat damage. To distinguish between the two, the kinetics of cell or organism death caused by different treatment sequences were carefully studied (Hofer, 1987; Lai and Ducoff, 1977). Their data suggested that the synergism between heat and radiation was heat potentiation of radiation killing rather than radiation potentiation of heat killing.

The mechanism of hyperthermic radiosensitization is not yet resolved. Since postirradiation heating prevents SLD repair for both exponential (Ben-Hur et al., 1974) and plateau phase cells (Harris et al., 1976), inhibition of SLD repair has been proposed as the cause of heat potentiation of radiation. Nevertheless, SLD repair appears to be unaffected by preirradiation heating (Murthy et al., 1977; Raaphorst et al., 1979), but preirradiation heating does potentiate radiation cell killing. This contradicts the notion that heat potentiation of radiation cell killing is due to inhibition of SLD repair. It is possible that hyperthermic radiosensitization involves more than one mechanism.

2.4 Red Blood Cells And Hemolysis

Most studies on heat and/or radiation use proliferative cells as the model system. While they are quite appropriate for tumor cells, they are not representative of all normal biological systems. Most cells in organ systems stay in the quiescent phase unless they have been damaged and regeneration is required; some cells are terminally-differentiated. This thesis was designed to study the effects of heat and/or radiation on avian red blood cells (RBC's) in order to develop a possible model for terminally-differentiated tissues.

Avian RBC's differ from mammalian RBC's in several aspects. Avian RBC's are nucleated, they are not biconcave shaped, and avian hemoglobin has lower affinity for oxygen than its mammalian counterpart (Vandecasserie et al., 1971). There is continued

controversy about the genetic activity of avian RBC's. Mature hen erythrocytes have been shown to synthesize mRNA but the mRNA is not translocated to the cytoplasm and not translated (Zentgraf et al., 1975). However, there are reports of heat shock protein synthesis in avian red cell preparations (Dean and Atkinson, 1985; Banerji et al., 1984).

While these investigators demonstrated heat shock protein synthesis in avian RBC's, they did not address the topic of thermotolerance. Thermotolerance is defined as a transient resistance to heat induced by a prior non-lethal heating. In avian RBC's heat resistance cannot be measured using the usual clonogenic assay. An appropriated biological endpoint is hemolysis. Osmotic hemolysis has traditionally been used to study RBC's (Jacobs et al., 1936). In my assay hemolysis was recorded in isotonic medium. This has the advantage that the damage scored is due to the physical agent not the combinations of the agent and hypotonicity. In my study and in one by Kondo et al. (1989), hemolysis induced by heat or by radiation in isotonic solution is scored promptly as well as several days after treatment. Spontaneous hemolysis induced by heat (or by radiation) does not manifest until at least 24 hours later. This is different from osmotic fragility induced by heating which is manifested immediately after treatment (Jacobs et al., 1936; Sutherland et al., 1967). This suggests heat-induced osmotic fragility and heat-induced spontaneous hemolysis in isotonic solution are not the same events.

3. MATERIALS AND METHODS

3.1. Blood Preparation

2 ml of chicken blood was obtained on heparin from wing vein or by heart puncture. The blood was washed three times in an isotonic medium[1] consisting of 140 mM NaCl, 10 mM KCl, 10 mM glucose, 1.5 mM $MgCl_2$, 10 mM HEPES buffer, pH adjusted to 7.4 (by adding 1M NaOH dropwise) to remove plasma and white buffy coat. After washing, the packed cell volume was 0.7 ml. The cells were then resuspended to 1.4 % hematocrit. Blood cell suspensions were further diluted to the appropriate concentrations as described in later sections.

3.2. Hyperthermia Treatment

A Neslab Exacal water bath with the ability to maintain a constant temperature by continuous agitation was used; bath temperature was monitored periodically throughout the course of the heat treatment using a thermometer calibrated against a NBS-traceable mercury thermometer; temperature was maintained within ± 0.1°C. Since heat was to be applied to chicken RBC's suspended in a 15-ml polystyrene centrifuge tube, temperature of such a tube was monitored after immersion. Ten min or less sufficed for a tube containing 10 ml of cell suspension at room temperature to equilibrate with the desired bath temperature (range from 42°C to 51.5°C). Therefore, 9.5 ml of medium was preheated for ten min before adding 0.5 ml of the 1.4 % hematocrit suspension to the tube, providing a final blood cell suspension 0.07% hematocrit. A hematocrit of 0.07% was used for all experiments unless indicated otherwise.

---

[1] We would like to thank Dr. John Willis for suggesting this isotonic medium.

Thermal dose-response curves were constructed by exposing chicken RBC's to either 50.5°C or 51.5°C for 0 to 180 min. After heating, cells were put on ice for 5 min to stop further heat damage to the cells. Some samples were then immediately spun down for absorbance measurement. Other samples were placed in an incubator (35 ± 2 °C) until they were spun down and scored 1, 2 or 3 days after heat treatment and the 5-min cooling.

For the heat shock experiments, chicken RBC's were subjected to two different regimens: 1) heat shocked at 42.6°C for 0.5 0.75, 1.0, 1.5 or 2 hr, or 2) heat shocked at 43.5°C for 15 min and then placed in a 35 ± 2°C incubator for 0, 1 or 2 hr. Following these heat shock treatments, tubes containing the cell suspension were challenged at 51.5°C for 40 min. Non-shocked controls received the same heat challenge but no prior heating.

3.3. Radiation Treatment

A ^{137}Cs source in a vertical cylinder was used. Polystyrene vials containing 5 ml of 0.7% hematocrit were placed on a turntable located 4.8 cm from the source. The turntable was designed to ensure even radiation exposure to all cells in the vials. The dose rate used was about 10 Gy per minute. The dose rate was determined by $FeSO_4$ dosimetry (Fricke and Hart, 1965) and corrected for radioactive decay every six months. The irradiation was performed at room temperature and in room air, and after irradiation, the cells were diluted to 0.07% hematocrit for absorbance reading.

Radiation dose-response curves were constructed by giving chicken RBC's 0 to 106 gray of ionizing radiation. In the split-dose experiments, chicken RBC's received a total of 100 Gy delivered in either 2 or 4 (multifractionation) equal fractions. Samples that received 2 equal fractions were incubated for 0 to 4 hours at 35 ± 2°C or for 60 minutes at 22°C between the fractions; the multifractionated samples were incubated for 60 minutes at 35 ± 2°C between fractions.

3.4. Combined Heat And Radiation Treatment

Chicken RBC's received 60 Gy and 40 minutes of 51.5°C for the combined treatment. The concentration used was 0.7% hematocrit. After the combined heat and radiation treatment, the samples were diluted to 0.07% hematocrit for absorbance reading. There were three different treatment sequences: 1) preirradiation heating, 2) postirradiation heating, and 3) radiation split-dose followed by heating. Kinetics experiments were preformed for postirradiation heating. After irradiation cells were incubated at 35 ± 2°C for 0 to 24 hours before they were exposed to hyperthermia.

3.5. Osmotic Fragility

Chicken RBC's were placed in serially-diluted saline (ranging from 44 mM NaCl to 155 mM NaCl) for 10 minutes during hyperthermic treatment (5°C to 51.5°C) in order to test the osmotic fragility of cells. Osmotic fragility was also used as an endpoint to test for heat-induced heat resistance. Cells were heat shocked for 10 minutes at 51.5°C, incubated at 35 ± 2°C for 1 to 7 hours, then challenged for 30 minutes at 51.5°C. Cells were tested for osmotic fragility both immediately and 1 day after the heat challenge.

3.6. Survival Measurement

After centrifugation (900g for 5 min), heated samples were read at 410 nm against the supernatant of a non-heated control using a Beckman model DB prism spectrophotometer. A sample completely hemolyzed by water was read against the non-heated control to obtain its absorbance, 0% nonhemolyzed cells (NHC). The percentage of NHC was calculated as follows:

$\%\ NHC = 100\% - m(Ab_x)$, m= slope,

Ab_x= sample absorbance

The slope was obtained by:

(100% NHC - 0% NHC) ÷ (absorbance of 100% NHC - absorbance of 0% NHC)

A slope was determined for each experiment in order to account for any fluctuation which might occur.

3.7. Potassium Leakage

Potassium leakage to the extracellular solution was measured with a potassium ion electrode, Orion model 93-19. A potassium-free medium (the same isotonic-glucose medium described in 2.1 except KCl was replaced by NaCl) and 0.42 % hematocrit were used for these experiments so that low levels of extracellular potassium concentrations could be measured. The extracellular potassium concentration was determined from a standard curve of electrode potential versus potassium concentration.

Chicken RBC's were heated at 51.5°C for 20 to 120 min to determine heat-induced potassium leakage. K^+ leakage was scored immediately and 1 day after heating. To compare the amount of K^+ leakage during actual heating and the amount of leakage following heat treatment, cells were exposed to either 20 min at 51.5°C followed by 40 min at 35°C or to continuous heating for 60 min at 51.5°C.

Heat shock experiments were performed by heating RBC's for 60 min at 42.6°C followed immediately by heat challenge at 51.5°C for various durations. K^+ leakage was recorded at 0, 24, and 48 hr after heat challenge.

3.8. Statistical Analysis

All of the values were plotted or tabulated as mean ± standard error of the mean. Student's t-test for normally distributed populations and unknown, but assumed equal population variances were used to calculate the level of significance. The null hypothesis was rejected when $p \leq 0.05$.

4. EFFECTS OF HYPERTHERMIA

The mode of action of heat is not well understood. Plasma membrane components, cellular proteins and DNA have all been proposed as the critical target for hyperthermic cell killing. Heat has been also shown to induce heat shock protein synthesis in a variety of cell systems and heat shock proteins appear to protect cells against further heating (see chapter 2 for review). The effects of heat are often studied using proliferating cells. One of the advantages of using proliferating cells is the well-established endpoint, clonogenic survival.

In this chapter, the effects of heat on avian erythrocytes will be examined. Avian red blood cells are selected because they are nucleated and have been shown to synthesize limited amounts of mRNA (Zentgraf et al., 1975) as well as to respond to heat shock by synthesizing heat shock proteins (Dean and Atkinson, 1985).

4.1 Heat Dose-response

Heat-induced Hemolysis. Heat dose-response was measured for two different temperatures, 50.5°C and 51.5°C. Results are shown in Tables 1 and 2, respectively. When hemolysis was scored immediately after 50 to 180 min of heating at either 50.5°C or 51.5°C, minimal amounts of hemolysis were observed; heating at 51.5°C did not produce significantly more hemolysis than 50.5°C except for the 180-min heating. However, when hemolysis was scored one, two or three days after heating, 51.5°C did produce significantly more hemolysis than 50.5°C. Heat-induced hemolysis as a function of heating duration at each of the two temperatures is plotted in Figures 1 and 2. Cells heated at 51.5°C for 40 and 80 min exhibited a slower rate of hemolysis than those heated for 120 and 180 min. Cells heated for 180 and 120 min hemolyzed completely in one and two

Table 1. Dose response of chicken RBC's at 50.5 ° C.

Heating Duration (minutes)	% Nonhemolyzed Cells			
	day 0	day 1	day 2	day 3
50	99.1 ± 0.26	96.4 ± 1.15	81.4 ± 0.77	64.2 ± 1.24
80	95.5 ± 1.01	88.4 ± 1.11	62.5 ± 6.06	47.8 ± 8.39
120	92.8 ± 1.13	69.0 ± 2.08	38.5 ± 3.85	17.7 ± 5.15
180	91.3 ± 1.78	19.3 ± 2.88	5.7 ± 1.54	CH*

*complete hemolysis

Table 2. Dose response of chicken RBC's at 51.5 ° C.

Heating Duration (minutes)	% Nonhemolyzed Cells			
	day 0	day 1	day 2	day 3
50	98.2 ± 0.44	90.6 ± 2.69	59.0 ± 6.28	NR†
80	97.3 ± 0.69	76.6 ± 2.69	44.9 ± 3.41	31.0 ± 3.16
120	93.8 ± 1.61	31.4 ± 0.94	CH	NR
180	86.9 ± 1.82	CH	NR	NR

*complete hemolysis
†no reading

Figure 1. Heat-induced hemolysis in chicken RBC's at 51.5°C for various heating durations. 40 min (open squares), 80 min (closed squares), 120 min (open circles) and 180 min (closed circles). Each data point represents a mean of 4 experiments in each of which triplicate samples were used.

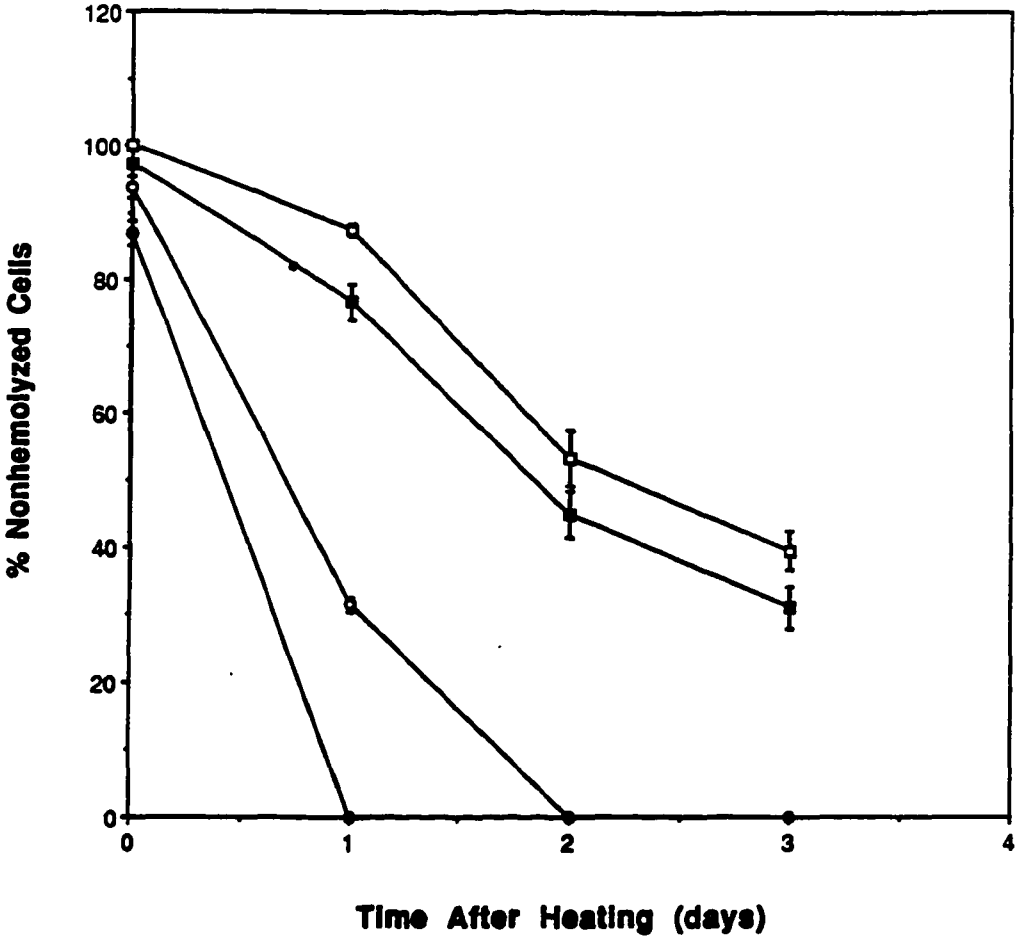
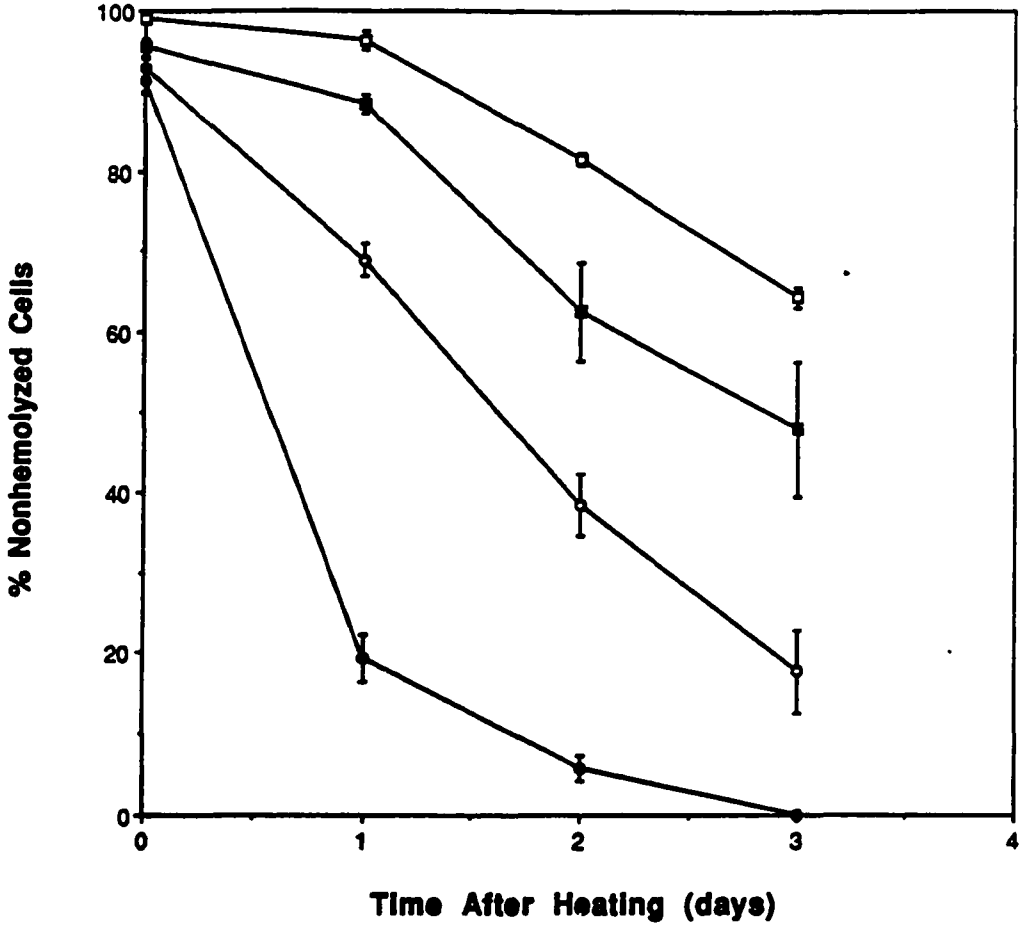

120
100
80
60
40
20
0
% Nonhemolyzed Cells
0
1
2
3
4
Time After Heating (days)

Figure 2. Heat-induced hemolysis in chicken RBC's at 50.5°C for various heating durations. 50 min (open squares), 80 min (closed squares), 120 min (open circles) and 180 min (closed circles). Each data point represents a mean of 4 experiments in each of which triplicate samples were used.


100
80
60
40
20
0
% Nonhemolyzed Cells
0
1
2
3
4
Time After Heating (days)

days, respectively. At 50.5°C, the response was similar except that the amount of hemolysis was less for each corresponding heating durations and only the 180-min heating resulted in complete hemolysis; shorter durations left measurable amounts of nonhemolyzed cells three days after heating.

When the initial thermal response experiments were performed, all samples were kept at room temperature prior to the heating. Room temperature fluctuated from day to day, and preheating temperature was not carefully controlled. Therefore, similar heat dose-response experiments were repeated but with preheating temperatures tightly controlled. Chicken RBC's were kept for 60 min in incubators set at 5°C, 22°C, 35°C and 39.5°C before exposure to 51.5°C for various times. The results are shown in Figure 3. Preheating temperature did not alter the heat dose-response of chicken RBC's significantly. This was still true when they were scored for hemolysis 1, 2 and 3 days later. Therefore, thermal response of chicken RBC's was quite stable regardless of the preheating temperature.

Heat-induced Potassium Leakage. Potassium leakage was examined as another possible endpoint for the effects of heat and to examine whether potassium leakage correlated with hemolysis. The two endpoints had very different kinetics. Potassium leakage induced by heat was apparent immediately after heating, and increased with heating duration (See Figure 4). All samples were kept for 24 hr in a 35°C incubator to observe for any additional post-heating K^+ leakage (Also Figure 4). The levels of K^+ leakage found after the 24-hr incubation was the same regardless of heating duration.

The data in Figure 4 indicated that K^+ leakage measured immediately after heating increased with heating duration. It was not clear, however, whether the increased K^+ leakage observed for the longer heating durations was the result of prior heating or an actual increase of potassium leakage induced by the additional heating. To distinguish between the two possibilities, cells were heated for either 20 min at 51.5°C and then

Figure 3. The effect of preheating temperature on heat dose-response of chicken RBC's. The cells were incubated at 5°C, 22°C, 35°C and 39.5°C for 60 min before they were heated at 51.5°C for various durations. Hemolysis was scored immediately after heating (panel A), 1 day after heating (panel B), 2 days after heating (panel C) and 3 days after heating (panel D). Each data point represents a mean of 3 experiments in each of which triplicate samples were used. Student's t-test was performed to compare the effect of various preheating temperature has on the thermal response of chicken RBC's and $p > 0.10$.

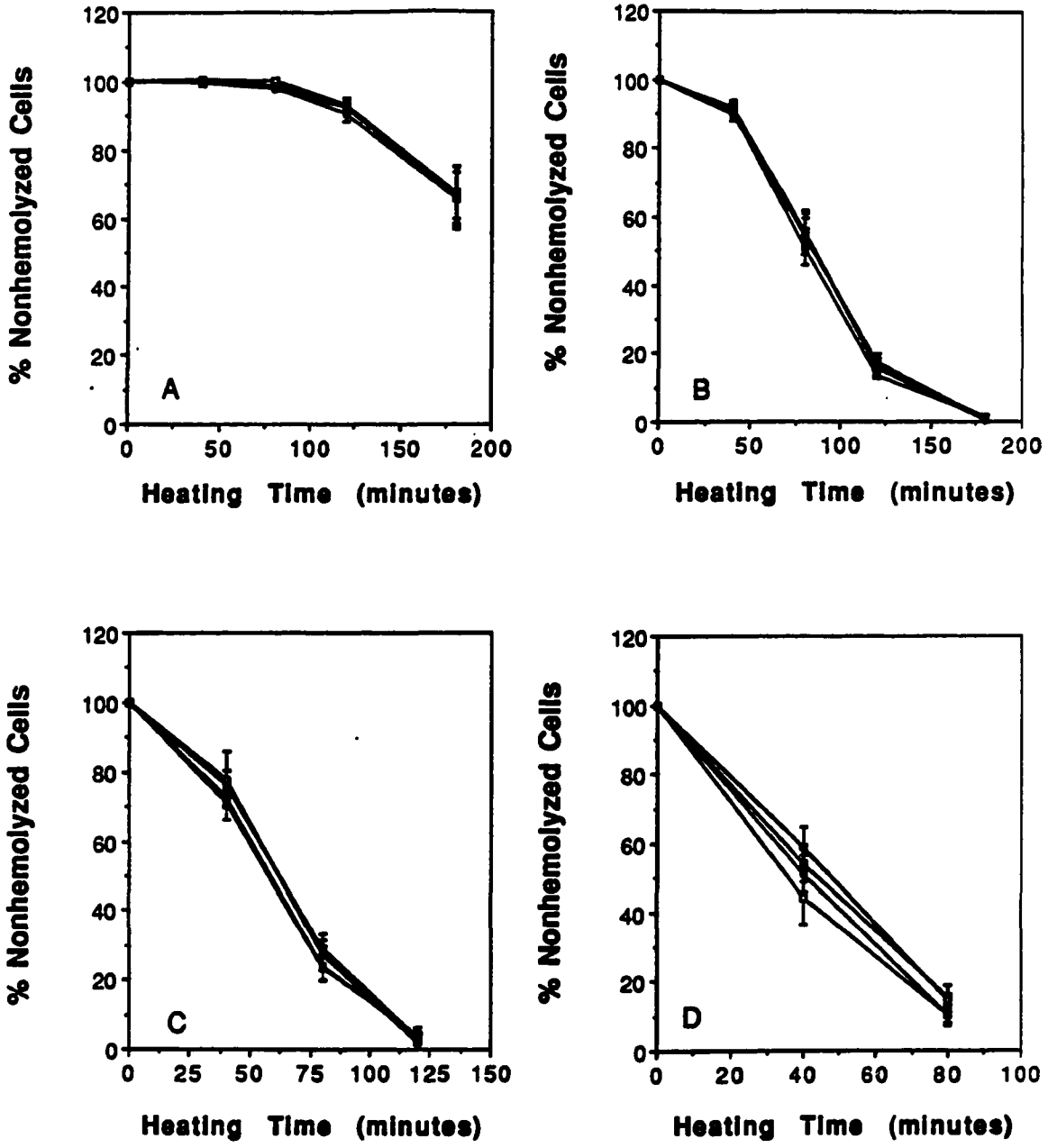
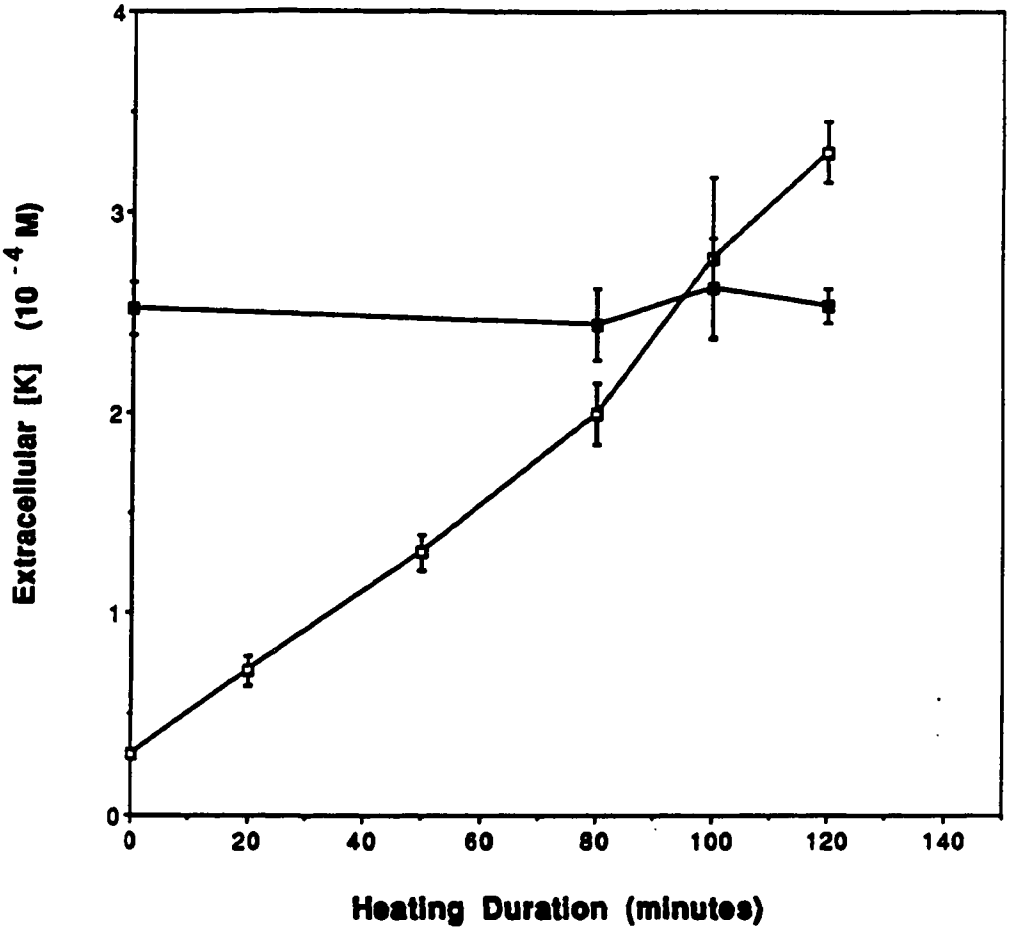

% Nonhemolyzed Cells
120
100
80
60
40
20
0
A
0
50
100
150
200
Heating Time (minutes)
% Nonhemolyzed Cells
120
100
80
60
40
20
0
B
0
50
100
150
200
Heating Time (minutes)
% Nonhemolyzed Cells
120
100
80
60
40
20
0
C
0
25
50
75
100
125
150
Heating Time (minutes)
% Nonhemolyzed Cells
120
100
80
60
40
20
0
D
0
20
40
60
80
100
Heating Time (minutes)

Figure 4. Heat-induced potassium leakage of chicken RBC's at 51.5°C for various heating durations. Immediately after heating (open squares) and 24 hr after heating (closed squares). Each data point represents a mean of 4 experiments in each of which duplicate samples were used.


Extracellular [K] (10 -4 M)
0
1
2
3
4
0
20
40
60
80
100
120
140
Heating Duration (minutes)

incubated for 40 min at 35°C or 60 min at 51.5°C. The results (See Figure 5) showed that continuous heating produced more ion leakage, and that very little leakage occurred following heating (e.g., in 40 min at 35°C).

Osmotic Fragility Of Chicken Erythrocytes. Effects of heat on osmotic fragility of chicken RBC's was tested by subjecting the cells to four different temperatures: 5°C, 22°C, 39.5°C or 51.5°C (See Figure 6). When temperature was below 40°C, the sensitivity of chicken RBC's to hypotonic saline appeared to be inversely related to temperature, but at 51.5°C, osmotic fragility of the cells returned to the level exhibited by cells treated at 22°C. No conclusive remarks on the effects of heat on osmotic fragility of chicken RBC's could be made based on this study since the four curves overlap with each other for hypotonic solutions containing ≥ 60 mM NaCl.

4.2 Induction Of Thermotolerance

Heat-induced Hemolysis As The Endpoint. I used different heat shock treatments to test for possible development of heat-induced heat resistance (i.e., thermotolerance). Thermotolerance was observed when chicken RBC's were either heated at 42.6°C for 0.5 to 2.0 hr and immediately heat challenged at 51.5°C for 40 min or heated at 43.1°C for 15 min, then incubated at 35°C for 0 to 2 hr and finally heat challenged at 51.5°C for 40 min. The heat shock temperatures were selected because 43°C was used by Banerji et al. (1984) and Dean and Atkinson (1985) to investigate the induction of heat shock protein synthesis in chicken reticulocytes and quail red cell preparations, respectively. The heat shock protocols were designed to determine whether either chronic (i.e., heat shock temperature below 43°C and heat challenged immediately following heat shock) or acute (i.e., heat shock temperature above 43°C, incubated at physiological

Figure 5. K^+ leakage induced by continuous heating for 60 min at 51.5°C versus K^+ leakage induced by heating for 20 min at 51.5°C and incubating for 40 min at 35°C. Chicken RBC's were heated at 51.5°C for either 60 min or 20 min plus 40 min incubation at 35°C. Each data point represents a mean of 4 experiments in each of which duplicate samples were used. Student's t-test was performed to compare the 20-min at 51.5°C plus 40 min at 35°C treatment with the 60-min at 51.5°C treatment and $p < 0.05$.

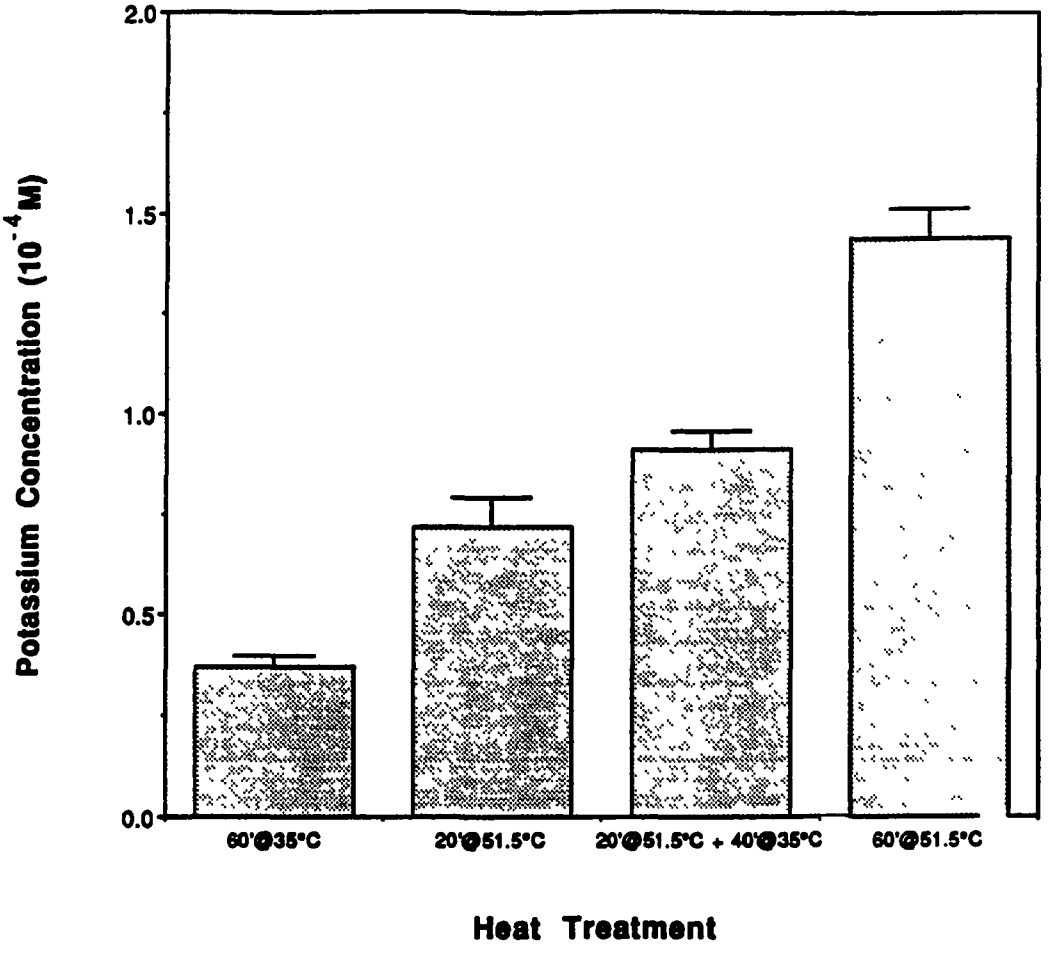
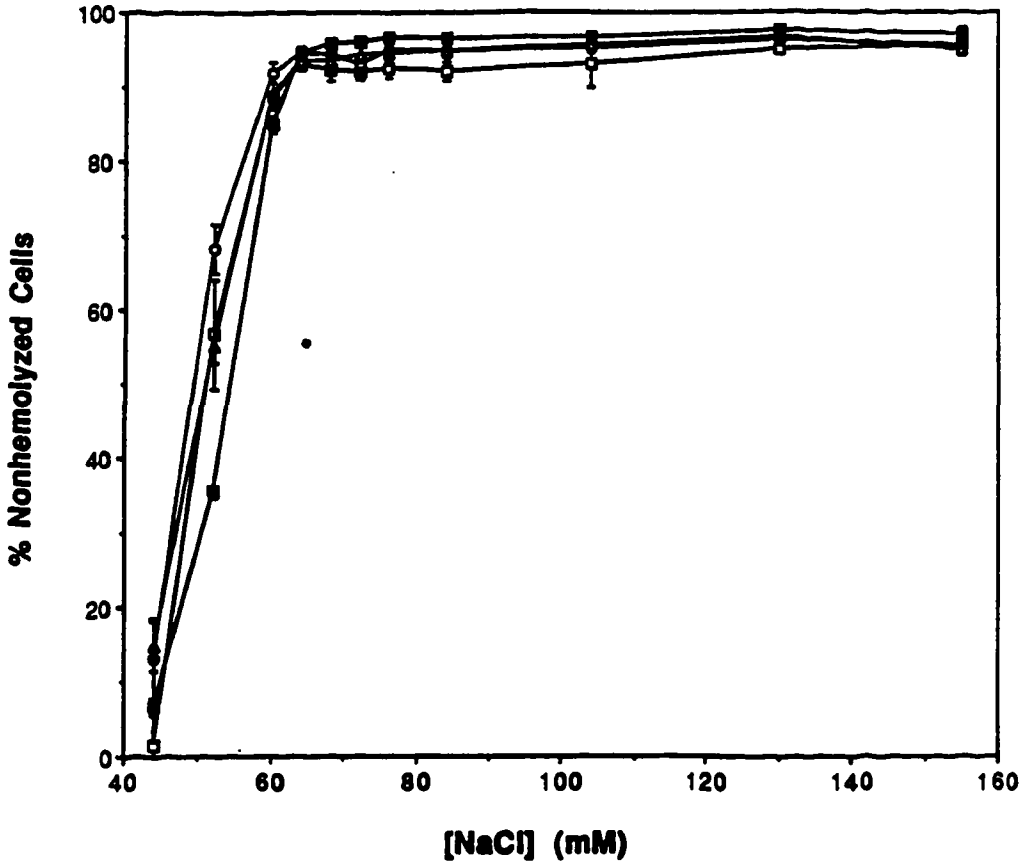

2.0
1.5
1.0
0.5
0.0
Potassium Concentration (10^{-4}M)
60'@35°C
20'@51.5°C
20'@51.5°C + 40'@35°C
60'@51.5°C
Heat Treatment

Figure 6. Osmotic fragility of chicken RBC's at various temperatures. The cells were incubated for 10 min at the chosen temperature. 5°C (closed squares), 22°C (open triangles), 39.5°C (open circles) and 51.5°C (open squares). Each data point represents a mean of 3 experiments in each of which duplicate samples were used.


100
80
60
40
20
0
% Nonhemolyzed Cells
40
60
80
100
120
140
160
[NaCl] (mM)

temperature for some time prior to heat challenge) heat shock can induce thermotolerance in chicken RBC's.

The results with the 42.6°C group are plotted in Figure 7. Immediately following the heat challenge, there was virtually no hemolysis in any of the groups. Some degree of thermotolerance was apparent one day after the heat challenge: the heat-shocked samples had more nonhemolyzed cells than the non-heat-shocked groups. Two days after the challenge, thermotolerance was clearly discernable in the samples heat shocked for 0.5, 0.75, 1.0 and 1.5 hr, but the 2-hr heat-shocked cells had hemolyzed more than the non-heat-shocked cells. Three days after challenge, the 0.75, 1.0 and 1.5 hr heat-shocked samples still contained more nonhemolyzed cells than the non-heat-shocked groups, but neither the 0.5- nor the 2-hr-samples were statistically different from the non-heat-shocked groups. Of all the heat shock durations tested, treatment between 1.0 and 1.5 hr appeared to give the best protection to the cells (See Figure 8).

Figure 9 presents the results for cells heated for 15 min at 43.1°C, and then incubated at 35°C for 0, 1 or 2 hr prior to heat challenge for 40 min at 51.5°C. Immediately after heat challenge, no difference was observed. However, the percentage of nonhemolyzed cell recorded at two or three days after the heat challenge was significantly higher in the heat-shocked groups than in the non-heat-shocked controls.

Potassium Leakage As The Endpoint. Thermotolerance was not observed with potassium leakage used as the endpoint (See Table 3). This might be attributed to the lack of potassium in the suspending medium because under potassium-free conditions, thermotolerance was also not observed with hemolysis as the endpoint (See Table 4). The results implied that for the protocols tested here potassium leakage was not a suitable endpoint to score thermotolerance.

Figure 7. Chicken RBC's heat shocked at 42.6°C for various durations, and then immediately heat challenged at 51.5°C for 40 min. Non-heat-shocked controls (open circles), heat-shocked for 0.5 hr (closed circles), heat-shocked for 0.75 hr (open triangles), heat-shocked for 1 hr (closed triangles), heat-shocked for 1.5 hr (open squares) and heat-shocked for 2.0 hr (closed squares). Each data point represents a mean of 3 to 4 experiments in each of which triplicate samples were used. Student's t-test was performed to compare non-heat-shocked controls and the heat-shocked samples 2 or 3 days after the heat challenge and $p < 0.001$.

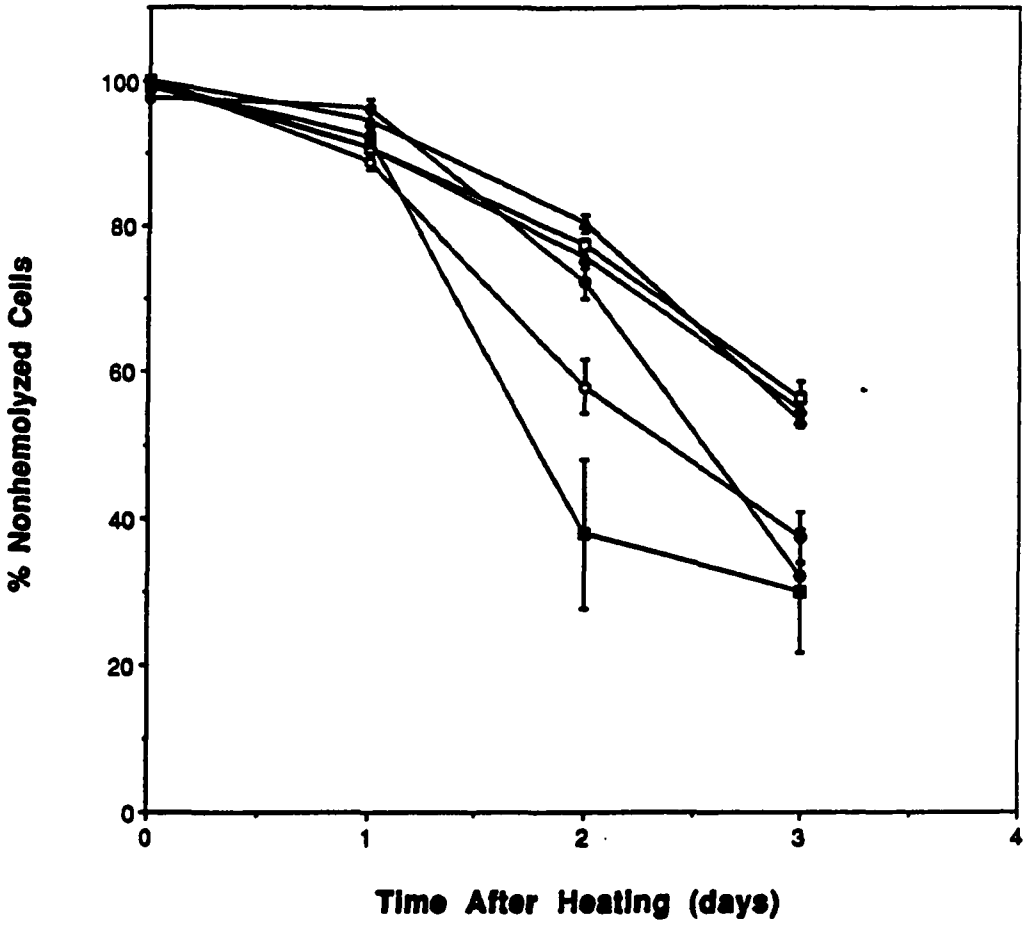

% Nonhemolyzed Cells
100
80
60
40
20
0
0
1
2
3
4
Time After Heating (days)

Figure 8. Chicken RBC's heat shocked at 42.6°C for 0 to 2 hr and immediately followed by the heat challenge. Two days after the 51.5°C heat challenged. Each data point represents a mean of 3 to 4 experiments in each of which triplicate samples were used. Student's t-test was performed to compare non-heat-shocked controls with the heat-shocked samples and $p < 0.001$.

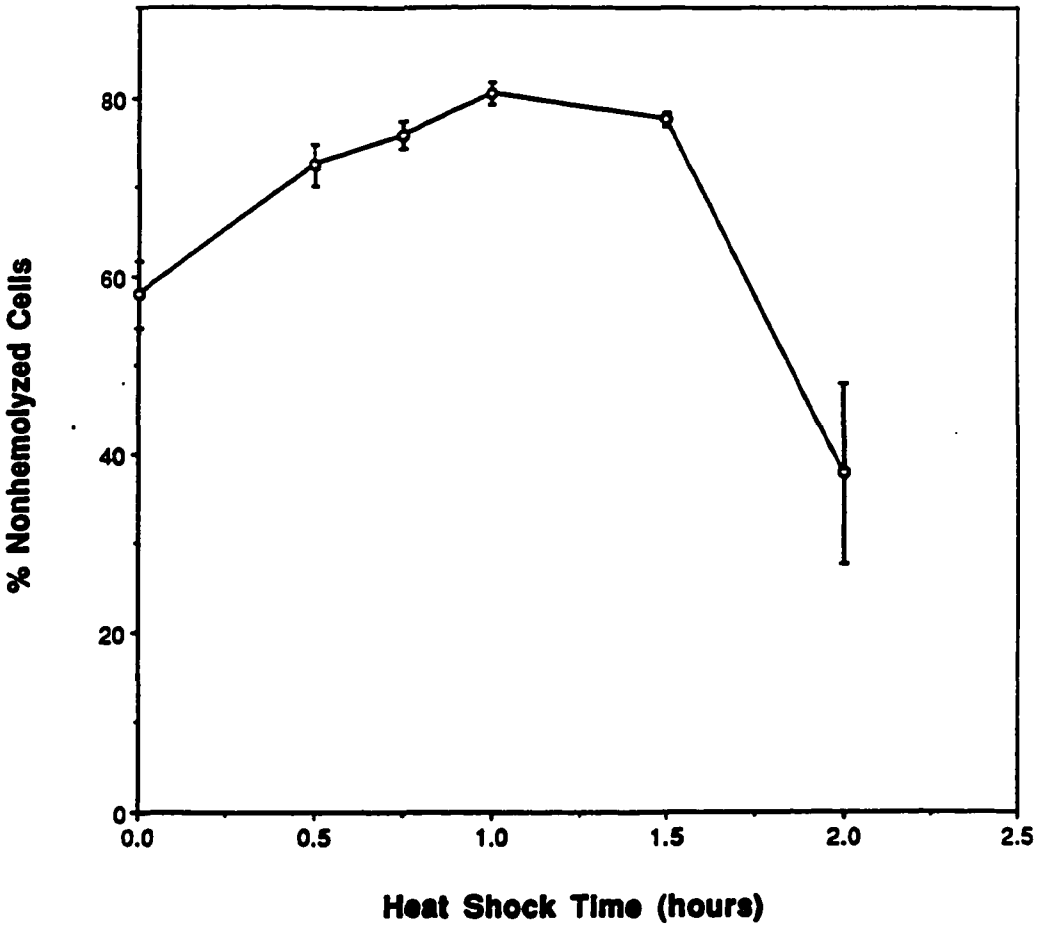

% Nonhemolyzed Cells
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
Heat Shock Time (hours)

Figure 9. Chicken RBC's heat shocked at 43.1°C for 15 min, and then incubated at 35°C for various times before receiving heat challenge at 51.5°C for 40 min. Non-heat-shocked controls (closed squares), heat-shocked with 0 hr incubation (open squares), heat-shocked with 1 hr incubation (open circles) and heat-shocked with 2 hr incubation (open triangles). Each data point represents a mean of 3 experiments in each of which triplicate samples were used. Student's t-test was performed to compare non-heat-shocked controls with the heat-shocked samples at 2, 3 or 4 days after the heat challenge and $p < 0.05$.

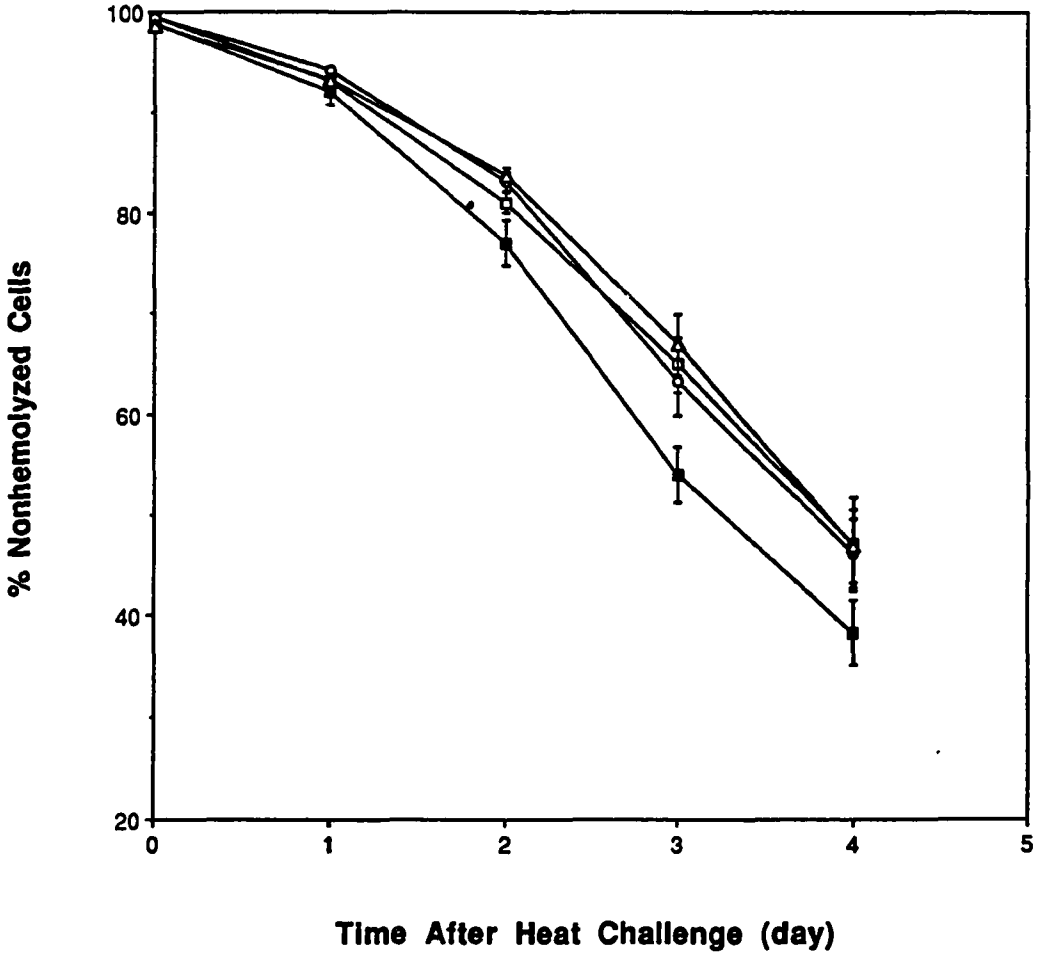

100
80
60
40
20
0
1
2
3
4
5
% Nonhemolyzed Cells
Time After Heat Challenge (day)

Table 3. Potassium leakage of chicken RBC's. Cells heat shocked at 42.6 ° C for 1 hour and then heat challenged at 51.5 ° C for various times.

Heat Challenge Duration (minutes)	Extracellular $[K^+]$ (10^{-4} M)					
	Day 0		Day 1		Day 2	
	NHS*	HS†	NHS	HS	NHS	HS
40	1.53 ± 0.15	1.95 ± 0.22	2.59 ± 0.58	2.59 ± 0.58	3.00 ± 0.0	3.04 ± 0.0
80	2.63 ± 0.05	2.72 ± 0.06	2.44 ± 0.25	2.56 ± 0.42	3.00 ± 0.0	3.00 ± 0.0
120	3.30 ± 0.15	3.30 ± 0.15	2.54 ± 0.12	2.60 ± 0.04	3.00 ± 0.0	3.00 ± 0.0

*non-heat-shocked controls

† heat-shocked samples

Each data point represents a mean of 3 experiments and for each experiment, duplicate samples were used.

$p > 0.10$

Table 4. Chicken RBC's suspended in K^+-free medium. Cells heat shocked at 42.6 ° C for 1 hour and then heat challenged at 51.5 ° C for various times.

Heat Challenge Duration (minutes)	% Nonhemolyzed Cells					
	Day 0		Day 1		Day 2	
	NHS*	HS†	NHS	HS	NHS	HS
40	98.7 ± 0.0	99.4 ± 0.04	90.7 ± 0.71	92.9 ± 0.18	80.9 ± 4.28	81.6 ± 4.38
80	99.6 ± 0.11	99.7 ± 0.0	64.9 ± 1.66	72.4 ± 4.60	46.9 ± 5.66	52.8 ± 8.56
120	95.8 ± 0.85	96.1 ± 0.50	32.4 ± 1.26	38.1 ± 4.28	24.5 ± 2.26	28.3 ± 4.63

*non-heat-shocked controls

† heat-shocked samples

Each data point represents a mean of 2 experiments and for each experiment, duplicate samples were used.

$p > 0.10$

Osmotic Fragility As The Endpoint. Heat-induced heat resistance was observed using osmotic fragility as the endpoint. After the initial heat shock (51.5°C for 10 minutes), the cells were incubated in isotonic medium at 39.5°C for 1 to 7 hours before the final heat challenge (51.5°C for 30 minutes). The controls were heat challenged without prior heat shock, although they were incubated at 39.5°C for the corresponding times (i.e., 1 to 7 hours).

Immediately after the heat challenge, both the control and heat-shocked cells were tested for their osmotic fragility. The results are shown in Figure 10. The heat-shocked cells were consistently more resistant to osmotic hemolysis than the controls. However, the length of the incubation time did not appear to affect the degree of resistance (See Figure 11). The incubation times also did not affect the osmotic fragility of the control cells (See Figure 12). When the cells were incubated overnight in isotonic solution, they became very sensitive to osmotic hemolysis. The prior heat shock not only did not protect them against osmotic forces, but made them even more sensitive (See Table 5).

Since the heat-induced heat resistance measured by osmotic fragility was transient, further experimentation was performed to examine the effect of the heat shock (51.5°C for 10 minutes) itself on the cells. Three hours [at 39.5°C] after the heat shock, the cells were more resistant to osmotic hemolysis than the cells exposed immediately to dilute saline following the heat shock, but 24 hours later, the cells were more sensitive to dilute saline than the ones tested immediately after heat shock (See Figure 13).

4.3 Discussion

Assay. Since erythrocytes are incapable of further division, their clonogenicity cannot be assayed. Accordingly I developed a hemolytic assay to score the effects of heat on chicken RBC's. When chicken RBC's were suspended in a HEPES-buffered isotonic-

Figure 10. Heat-induced heat resistance using osmotic hemolysis as the endpoint. Cells were heat shocked at 51.5°C for 10 min, and then incubated in isotonic medium at 39.5°C for 1 to 7 hr before receiving the heat challenge at 51.5°C for 30 min. Immediately after the heat challenge, the cells were tested for osmotic fragility. The non-heat-shocked controls were plotted using open symbols and the heat-shocked samples were plotted using closed symbols. 1 hr incubation (panel A), 3 hr incubation (panel B), 5 hr incubation (panel C) and 7 hr incubation (panel D). Each data point represents a mean of 4 experiments in each of which duplicate samples were used. Student's t-test was performed to compare non-heat-shocked controls with the heat-shocked samples and $p < 0.05$.

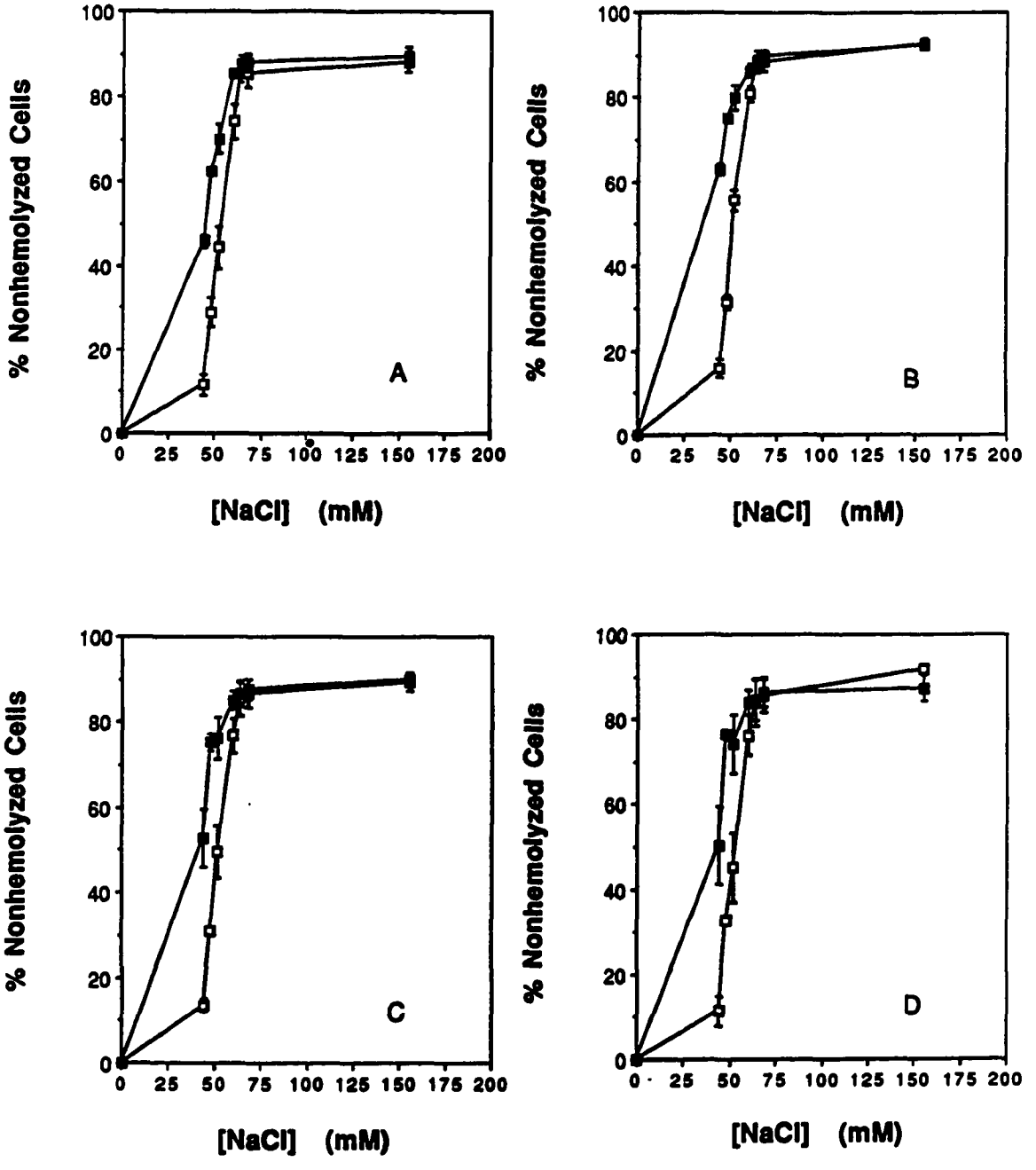

% Nonhemolyzed Cells
100
80
60
40
20
0
0 25 50 75 100 125 150 175 200
[NaCl] (mM)
A
B
C
D

Figure 11. The effect of incubation time on the osmotic fragility of heat-shocked cells. 1 hr incubation (open squares), 3 hr incubation (closed squares), 5 hr incubation (open circles) and 7 hr incubation (closed circles). Each data point represents a mean of 4 experiments in each of which duplicate samples were used. Student's t-test was performed to compare the different incubation times and $p > 0.10$

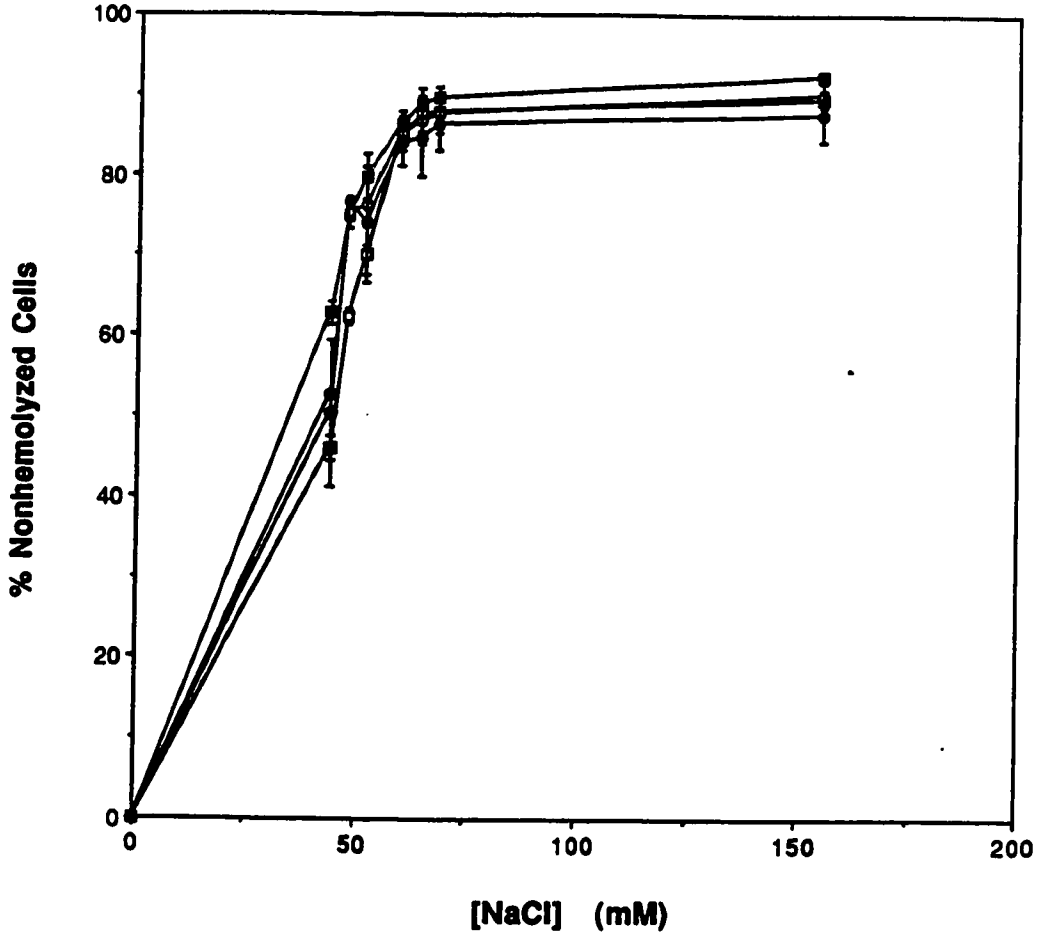

100
80
60
40
20
0
% Nonhemolyzed Cells
0
50
100
150
200
[NaCl] (mM)

Figure 12. The effect of incubation time on the osmotic fragility of non-heat-shocked controls. 1 hr incubation (open squares), 3 hr incubation (closed squares), 5 hr incubation (open circles) and 7 hr incubation (closed circles). Each data point represents a mean of 4 experiments in each of which duplicate samples were used. Student's t-test was performed to compare the different incubation times and $p > 0.10$.

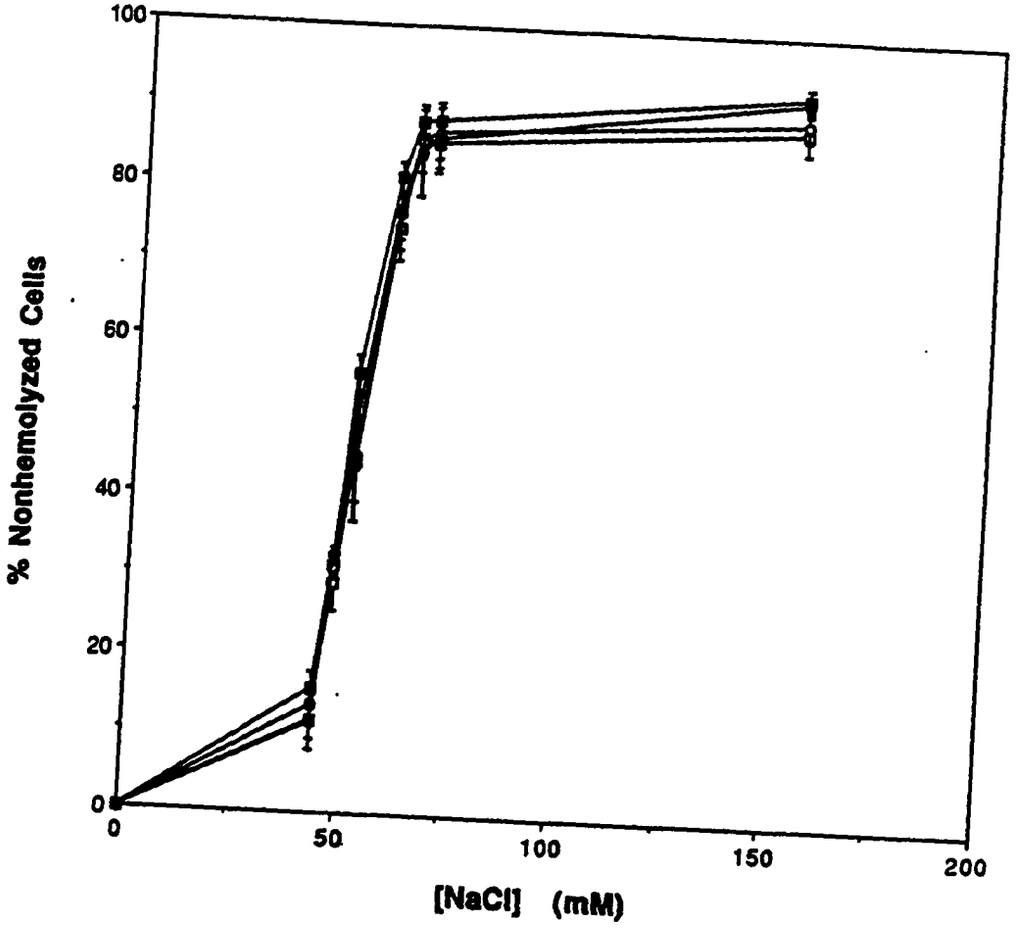

100
80
60
40
20
0
% Nonhemolyzed Cells
0
50
100
150
200
[NaCl] (mM)

Table 5. Osmotic fragility of chicken RBC's 1 day after heating.

Dilute NaCl solution (mM)	% Nonhemolyzed Cells	
	control*	heat-shocked†
44	0 ± 0	0.64 ± 0.64
48	1.44 ± 0.95	3.31 ± 2.17
52	7.63 ± 4.93	9.51 ± 6.14
60	8.30 ± 4.37	5.94 ± 3.86
64	13.39 ± 8.40	10.62 ± 9.33
68	12.04 ± 7.38	7.15 ± 6.93

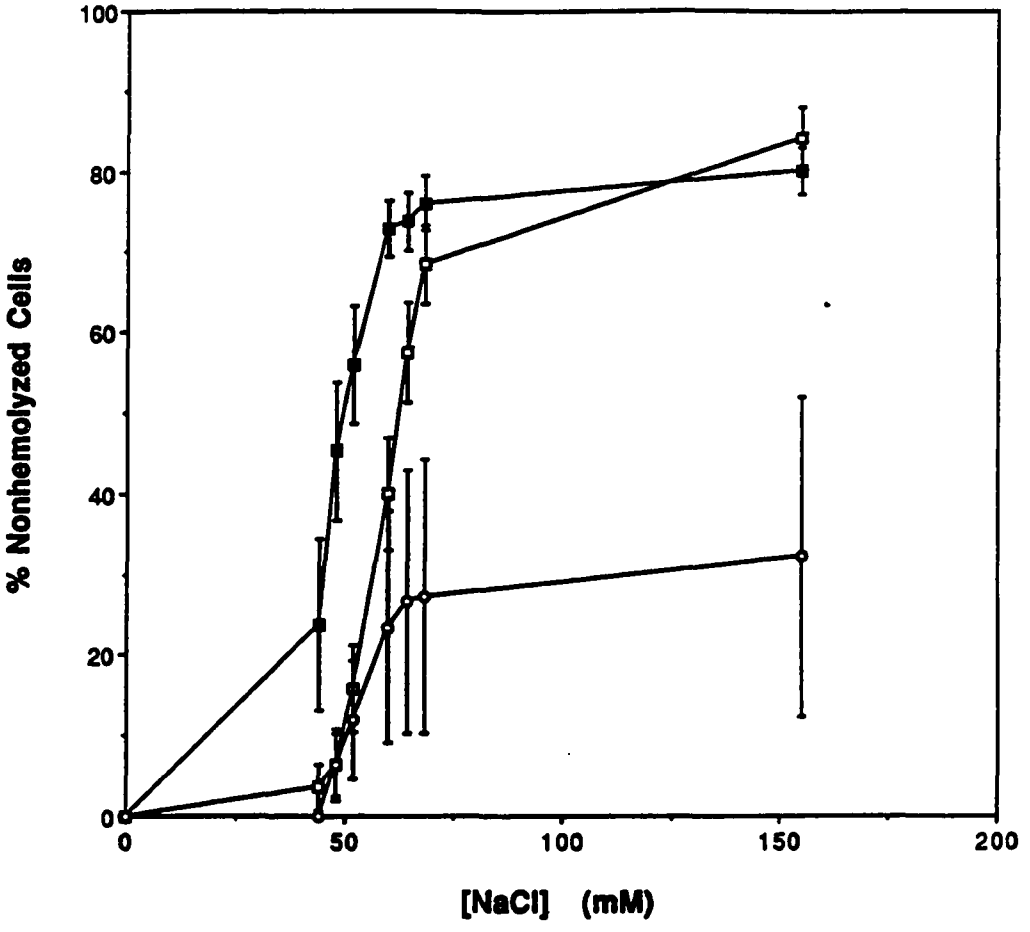
*control: 39.5 °C (3 hr) → 51.5 °C (30 min) → 39.5 °C (overnight incubation in isotonic-glucose solution) → osmotic challenge in dilute NaCl solution.

† heat-shocked: 51.5 °C (10 min) → 39.5 °C (3 hr) → 51.5 °C (30 min) → 39.5 °C (overnight incubation in isotonic-glucose solution) → osmotic challenge in dilute NaCl solution.

Each data point represents a mean of 4 experiments and for each experiment, duplicate samples were used.

$p > 0.10$

Figure 13. The effect of the heat shock treatment (51.5°C for 10 min) on the osmotic fragility of chicken RBC's. Osmotic fragility was tested immediately after the heat shock (open squares), 3 hr after the heat shock (closed squares) and 24 hr after the heat shock (open circles). Each data point represents a mean of 3 experiments in each of which duplicate samples were used. Student's t-test was performed to compare the osmotic fragility of heat-shocked cells immediately after heating and those that were incubated for up to 24 hr and $p < 0.05$.


100
80
60
40
20
0
% Nonhemolyzed Cells
0
50
100
150
200
[NaCl] (mM)

glucose medium with pH adjusted to 7.4, hemolysis 1 to 3 days after heating provided a reproducible measurement to score heat damage.

Several assays, each having some advantages and some disadvantages,.have been used to score erythrocyte damage:

(1) Ion leakage. Reports on changes in K^+ transport after hyperthermia are conflicting. An increase in active K^+ transport was reported when sheep erythrocytes were heated at 41°C (Joiner and Lauf, 1979); but hyperthermia of 15 min or greater at 43°C reduced the activity of (Na-K-Mg)-ATPase in bovine erythrocyte membrane (Grzelinska et al., 1982). In our study, heat-induced potassium leakage occurred within 20 min of heating at 51.5°C and in a dose-dependent manner. Interestingly, 24 hr after heating, the same amount of potassium was lost to the extracellular space regardless of heating duration. This was illustrated in Figure 4. Longer heating durations (≥ 100 min), however, caused greater amount of K^+ leakage immediately after heating than 24 hr after heating. No studies on the activity of (Na-K-Mg)-ATPase 24 hr after heating was available. It would be interesting to see if there is any return of activity by the pump hours after heating. Although all of our samples had similar amounts of K^+ leakage 24 hr after heating, they did not have similar hemolysis whether or not potassium was present in the suspending medium. Clearly K^+ leakage did not correlate with hemolysis. Ion leakage in proliferative cells also did not appear to correlate with cell survival (Borrelli et al., 1986a; Borrelli et al., 1986b).

Since K^+ leakage was an immediate process, I wanted to learn if thermotolerance could be observed promptly after heating. When K^+ leakage was used as an endpoint, thermotolerance was not found. Because the suspending medium used in these experiments contained no potassium, the lack of potassium in extracellular fluid was considered as a possible reason for this. Therefore, similar heat shock experiments were repeated and hemolysis was scored for 2 days in K^+-free solution. Under K^+-free conditions,

thermotolerance was still not observed using our hemolytic assay. This result made the potassium leakage experiments difficult to interpret.

(2) Osmotic fragility. Studies on the effect of heat on a variety of red blood cells showed that osmotic fragility was inversely proportional to temperature (Jacobs et al., 1936; Leyko and Jozwiak, 1987). This relationship was observed for temperatures below 45°C. At higher temperatures (45° to 52.6°C) osmotic fragility of human and dog erythrocytes increased with heating temperature (Ham et al., 1948). I also observed an inverse response between osmotic fragility and temperature in chicken RBC's when the temperature was below 40°C but at 51.5°C, osmotic fragility of chicken RBC's increased to the level equivalent to that of 22°C. Because of the complicated relationship between osmotic fragility and temperature, hyperthermic damage on erythrocytes measured by this assay would be difficult to interpret. Furthermore,there are some indications that osmotic fragility of heated RBC's changes with time (unpublished data), but all these studies measured osmotic fragility immediately after heating; therefore, any latent effect of heat was not recorded. It seems that it would be more logical to study the effects of heat on red blood cells by measuring hemolysis in isotonic solution for an extended period of time so that any latent effects of heat could be accounted for under more physiological conditions. Only rarely has hemolysis in isotonic solution been used to score the effects of lethal physical agents such as heat or ionizing radiation on RBC's for beyond 24 hr after treatment (Ducoff et al., 1989; Kondo et al., 1989).

(3) "Spontaneous" hemolysis in various solutions:

(a) Slightly hypotonic medium. Hemolysis in isotonic saline solution is a slow process. By incubating erythrocytes in slightly hypotonic saline solution, the process is hastened. This assay was used by Rusu and Baghdadi (1978) to study radiation-induced hemolysis in hen erythrocytes. In addition to measuring RBC damage under a non-

physiological condition, results of this method may be modified by altered osmotic fragility.

(b) Iso-osmotic glycerol solution. In this assay heat-induced hemolysis was measured under iso-osmotic conditions and the time required for 50% hemolysis was shown to be reduced by increasing temperature (Grzelinska et al., 1982). The authors stated that hemolysis in iso-osmotic glycerol solution occurred faster than in isotonic PBS solution, but the data for hemolysis in PBS solution were not given. Glycerol interacts with cell membranes and the presence of glycerol during treatment has been shown to protect against heat-induced cell deformities (Ho and Lin, 1991). Glycerol complicates studies of the effects of stressors on erythrocytes because of its free radical scavenging activity.

(c) Isotonic saline solution. T_{50} (time required for 50% hemolysis) has been used as an endpoint to score radiation damage on human erythrocytes (Brown, 1983). This assay was very convenient because samples could be read at anytime and the T_{50} value could simply be extrapolated. However, presentation of results as a single value ignores the detailed patterns of hemolysis.

In our assay, heat-induced hemolysis was measured under isotonic conditions, and followed for 72 hr in order to study any latent effects of heat. Heat-induced hemolysis did not occur immediately. This was demonstrated by the lack of hemolysis when cells were scored immediately after heating. However, when they were scored one to three days later, the extent of hemolysis was directly proportional to heating temperature and duration.

Heat Dose-response. The pattern of heat-induced hemolysis was similar for exposure to either 50.5° or 51.5°C, but the 51.5°C-heating resulted in greater amount of hemolysis at every corresponding heating durations. For the short heating durations ($\leq$ 80 min at 51.5°C), hemolysis proceeded slowly; it developed most rapidly between day 1 and 2, and complete hemolysis was not observed within 72 hr. Longer heating durations ($\geq$

120 min at 51.5°C) did cause complete hemolysis. Cells heated at 50.5°C exhibited similar hemolytic patterns and complete hemolysis was only achieved when they were heated for 180 min.

The amount of heat-induced hemolysis in isotonic-glucose medium immediately after heating has been shown to be negligible for 60 min at temperatures up to 49.0°C in human erythrocytes and heating for 60 min at 52.6°C was required to induce 47% hemolysis (Ham et al., 1948). Since human body temperature is lower than that of chicken, either much higher temperature or much longer heating duration would be necessary to achieve immediate hemolysis of chicken erythrocytes in isotonic solution. In the study by Ho and Lin (1991), heat-induced structural changes in human erythrocytes was compared to that of chicken erythrocytes. They reported that 6 min at 48.5°C resulted in about 50% abnormal (i.e., wavy edges and cell fragments) human erythrocytes, whereas 6 min at 56°C was required to induce similar levels of abnormality in chicken erythrocytes. According to Ham et al., (1948), early structural changes were not accompanied by detectable hemolysis. However, it is possible that if hemolysis was scored for some time after heating, the amount of structural abnormality would have corresponded to the amount of eventual hemolysis.

Development of Thermotolerance. Thermotolerance has been studied extensively in cultured mammalian cells as well as many other proliferating systems. Nevertheless, very little is known about the phenomenon in terminally-differentiated tissues. There is electrophoretic evidence that avian red cell preparations can synthesize heat shock proteins (HSP's) in response to a heat shock treatment (Banerji et al., 1984; Atkinson and Dean, 1985), but neither addressed the development of thermotolerance. Our report is the first on the induction of thermotolerance in avian red cells.

Chicken erythrocytes developed thermotolerance when prior heat shock of either 42.6°C or 43.1°C was given but the 42.6°C group was heat shocked for 0.5 to 2 hr and was followed immediately by a challenge of 40 min at 51.5°C; the highest level of thermotolerance was observed for heat shock durations of 60 to 90 min. The 43.1°C group was heat shocked for 15 min but was incubated for 0 to 2 hr at 35°C before the challenge of 40 min at 51.5°C was given. This resulted in lower levels of heat protection than the 42.6°C group. We have not attempted to determine the maximum level of heat protection obtainable in the chicken red cell preparation. It is clear, however, that the degree of thermotolerance is influenced by previous heat shock treatments. This is not surprising since the development of thermotolerance in other systems has been shown to be modified by the duration and temperature of the heat shock treatment (Boon-Niermeijer et al., 1986).

According to Banerji et al. (1984), chicken reticulocytes heat shocked at 43°C for 60 min produced the highest level of HSP 70 synthesis. Also, HSP 70 synthesis decreased when chicken reticulocytes were incubated for 120 min and its synthesis was virtually undetectable when the cells were incubated for 180 min. Thus, there is apparent correlation between the level of HSP 70 synthesis found by Banerji et al. (1984) and the thermotolerance observed in our study

The maximum level of heat protection produced by heat shock in our study ranged 12 to 22%. This is much lower than the levels reported in proliferative cells. It is possible that the thermotolerance observed here involved only the reticulocytes present in blood preparation. It is uncertain whether the genetic information of mature avian red blood cells is irreversibly repressed. Reports on HSP synthesis in avian red cells used reticulocyte-enriched preparations (Banerji et al., 1984; Dean and Atkinson, 1985). In addition, avian blood has been reported to contain 10 to 20% reticulocytes in its peripheral circulation

(Lucas and Jamroz, 1961). Reticulocyte counts in our study ranged from 9 to 12%. Therefore, the thermotolerance I observed might be attributed to reticulocytes.

The sensitivity of chicken RBC's to heat appeared to have seasonal variation. Cells collected in the summer were consistently more heat resistant than those collected in the winter and spring. Comparing Figures 7 and 9, percent of nonhemolyzed cells of non-heat-shocked controls in Figure 9 was much higher than that of Figure 7. Data points in Figure 7 were pooled from 4 experiments performed in the month of February, 1987 and data in Figure 9 were pooled from 3 experiments performed in the month of June, 1987. The seasonal variation in heat resistance might represent natural heat stress chickens experience during summer months. Since the cells were already heat shocked before they received the heat treatment, they appeared to be more resistant to heating. Therefore, the reproducibility of the heat shock experiments was restricted to the seasonal proximity during which the RBC's were collected.

For the protocols tested here potassium leakage was not a suitable endpoint to measure thermotolerance. Failure to develop thermotolerance as scored by hemolysis in K+-free solution suggest interesting possible role of potassium in developing thermotolerance but this has not been further pursued.

Heat-induced heat resistance could also be scored using osmotic fragility as the endpoint. The kinetics of thermotolerance was different for hemolysis and osmotic fragility. When hemolysis was used as the endpoint, thermotolerance developed slowly, and was maintained throughout the time course of observation. On the other hand, when osmotic fragility was used as the endpoint, heat-induced heat resistance was measurable immediately but it disappeared within 24 hours. Heat-induced heat resistance measured by osmotic fragility was probably unrelated to the type of thermotolerance that is believed to be associated with heat shock proteins, which has been shown to persist beyond 50 hours at

37°C (Li and Hahn, 1983). The initial heat shock might induce alterations in membrane configuration making the cells more resistant to osmosis, but as time progressed, the latent effect of heat manifested itself; hence, the cells became more sensitive to osmosis.

5. EFFECTS OF IONIZING RADIATION

The critical target of ionizing radiation is believed to be the DNA. Radionuclide experiments using ^{125}I-iododeoxyuridine and ^{125}I-concanavalin A showed that cells are much more sensitive to ^{125}I-iododeoxyuridine, which is associated with DNA, than ^{125}I-concanavalin A, which is associated with membranes (Warters et al., 1977). However, damage to cellular membranes can also lead to cell death. This mode of action is most likely through lipid peroxidation. Free radicals formed by radiolysis of water can oxidize polyunsaturated fatty acyl chains found in phospholipids, and the peroxidized lipids can cause damage to membrane-bound proteins. This leads to ion leakage, thus disturbing the delicate ionic balance of the cell and eventually killing the cell. Radiation has been shown to change a variety of ion permeabilities including Na^+, K^+, anions, spin-labelled substances, and substrates of glyceraldehyde 3-phosphate dehydrogenase (Suzuki and Akamatsu, 1980; Bartosz et al., 1978; Gwozdzinski et al., 1981; Kong et al., 1981).

In this chapter, the effects of ionizing radiation on chicken erythrocytes will be discussed.

5.1 Radiation Dose-response

Radiation dose-response of chicken RBC's was constructed by exposing cells to 0-100 Gy of radiation at a dose rate of approximately 10 Gy/min (See Figure 14). Hemolysis was not observed immediately after irradiation, but when hemolysis was scored one, two, three and four days following the irradiation, it occurred in a dose-dependent manner for doses greater than 50 Gy. For doses of 30 Gy or less, the amount of hemolysis remained similar to nonirradiated controls. The pattern of radiation-induced hemolysis for various doses is plotted in Figure 15. Chicken RBC's were exposed to 10.4, 31.2, 53.2, 74.5 or

Figure 14. Radiation-dose response of chicken RBC's. Hemolysis was scored immediately after irradiation (open squares), 1 day after irradiation (closed squares), 2 days after irradiation (open circles) and 3 days after irradiation (closed circles). Dose rate = 10.4 Gy/minute. Each data point represents a mean of 4 experiments and for each experiment, triplicate samples were used.

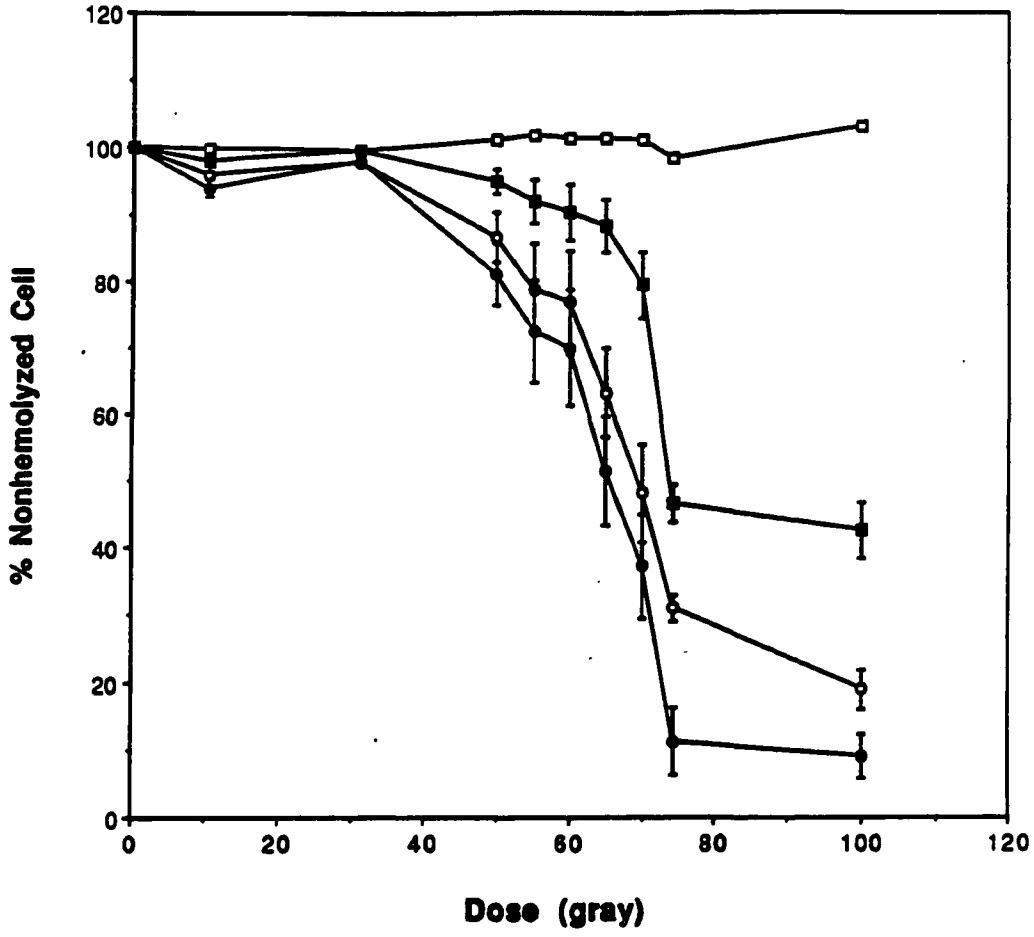
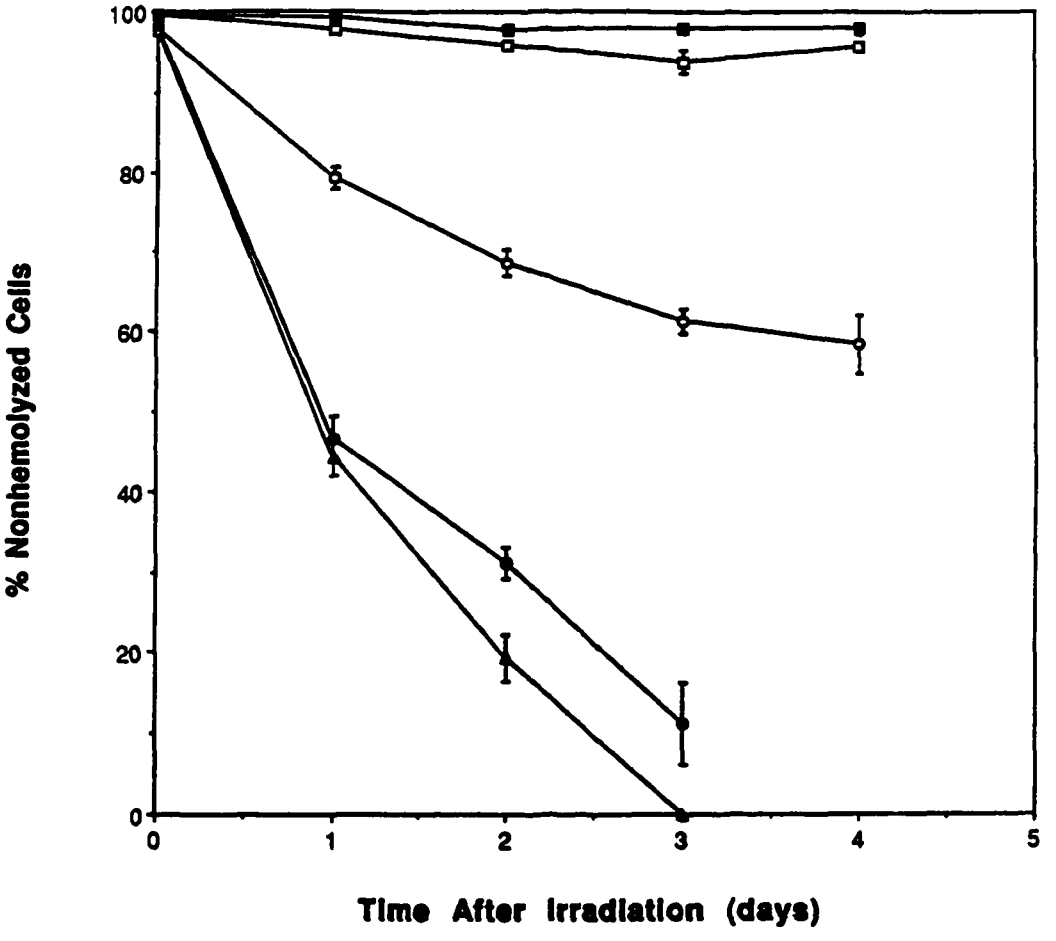

120
100
80
60
40
20
0
% Nonhemolyzed Cell
0
20
40
60
80
100
120
Dose (gray)

Figure 15. The pattern of radiation-induced hemolysis in chicken RBC's. 10.4 gray (open squares), 31.2 gray (closed squares), 53.2 gray (open circles), 74.5 gray (closed circles) and 106.4 gray (open triangles). Each data point represents a mean of 4 experiments and for each experiment, triplicate samples were used.


100
80
60
40
20
0
0
1
2
3
4
5
% Nonhemolyzed Cells
Time After Irradiation (days)

106.4 Gy of radiation and were followed for up to four days after irradiation. The 10.4 and 31.2 Gy samples were virtually indistinguishable from nonirradiated controls. Four days after irradiation they contained 95.6% and 97.8% nonhemolyzed cells, respectively. The 53.2 Gy sample hemolyzed fairly quickly in the first 24 hours i.e., approximately 50% of maximum amount of hemolysis was reached by the end of 24 hours, but after the first 24 hours, hemolysis occurred slowly; very little additional hemolysis was observed between day 3 (61.2% nonhemolyzed cells) and day 4 (58.3% nonhemolyzed cells). The 74.5 and 106.4 Gy samples hemolyzed quite rapidly in the first day and complete hemolysis was observed in four and three days, respectively. Radiation-dose response curves are replotted on a probit graph in order to obtain the LD_{50} of the system (See Figure 16). The $LD_{50/2\ day}$ estimated from the probit graph was 68.2 Gy.

5.2 Split-dose Fractionation

Sparing Effect Of Dose Fractionation. Split-dose experiments were designed to study the sparing effect of dose fractionation (sdf) in chicken erythrocytes. Using radiation-induced hemolysis as the endpoint, sdf was observed in chicken RBC's (See Figure 17). The survival of fractionated samples (total dose of 100 Gy) was similar to a single dose of 60-65 Gy (values are derived from comparing Figure 17 to Figure 14). This represented a reduction of 35-40 Gy. Having observed this, several aspects of sdf in chicken RBC's were further investigated, which included the kinetics of split-dose effect, the effect of multifractionation and the effect of incubation temperature.

Kinetics Of Split-dose Repair. In split-dose experiments, the effect of a single radiation dose was compared to a similar total dose but fractionated into two doses. By varying the time between the two fractions, the kinetics of split-dose repair was characterized.

Figure 16. Survival of chicken RBC's 2 days after irradiation plotted on probit graph. Each data point represents a mean of 4 experiments and for each experiment, triplicate samples were used.

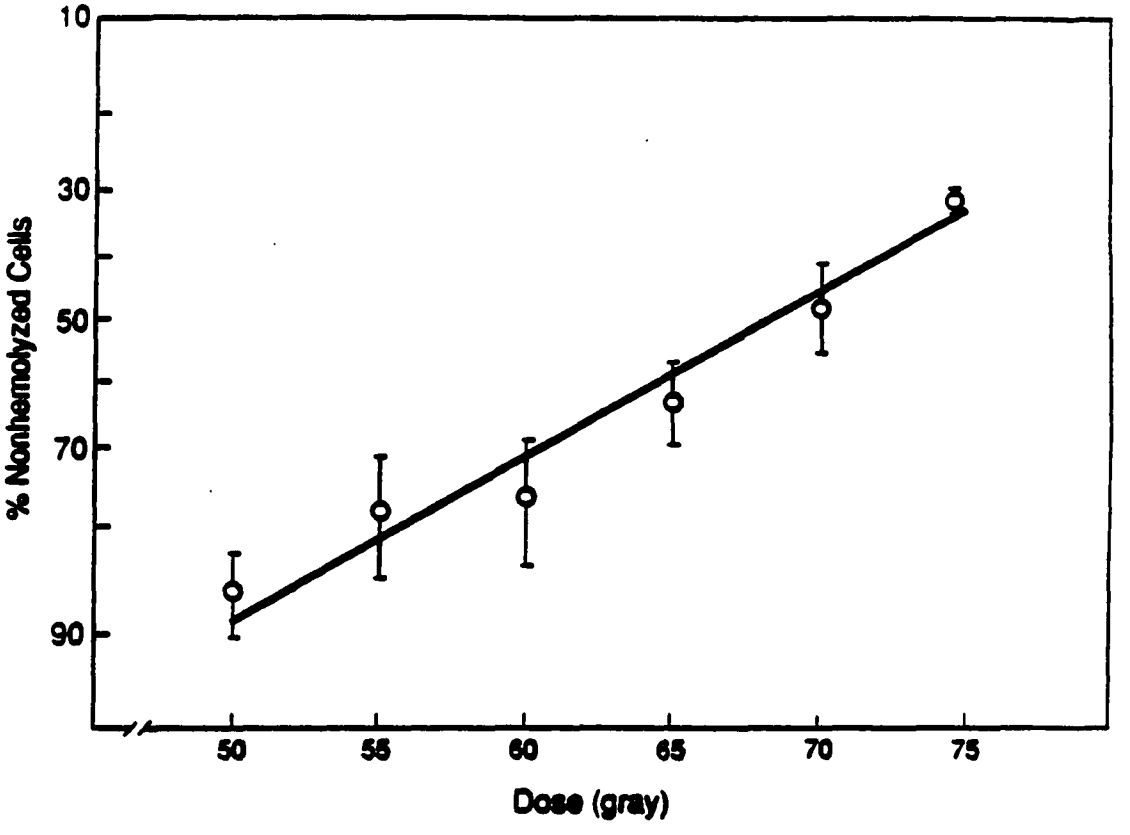
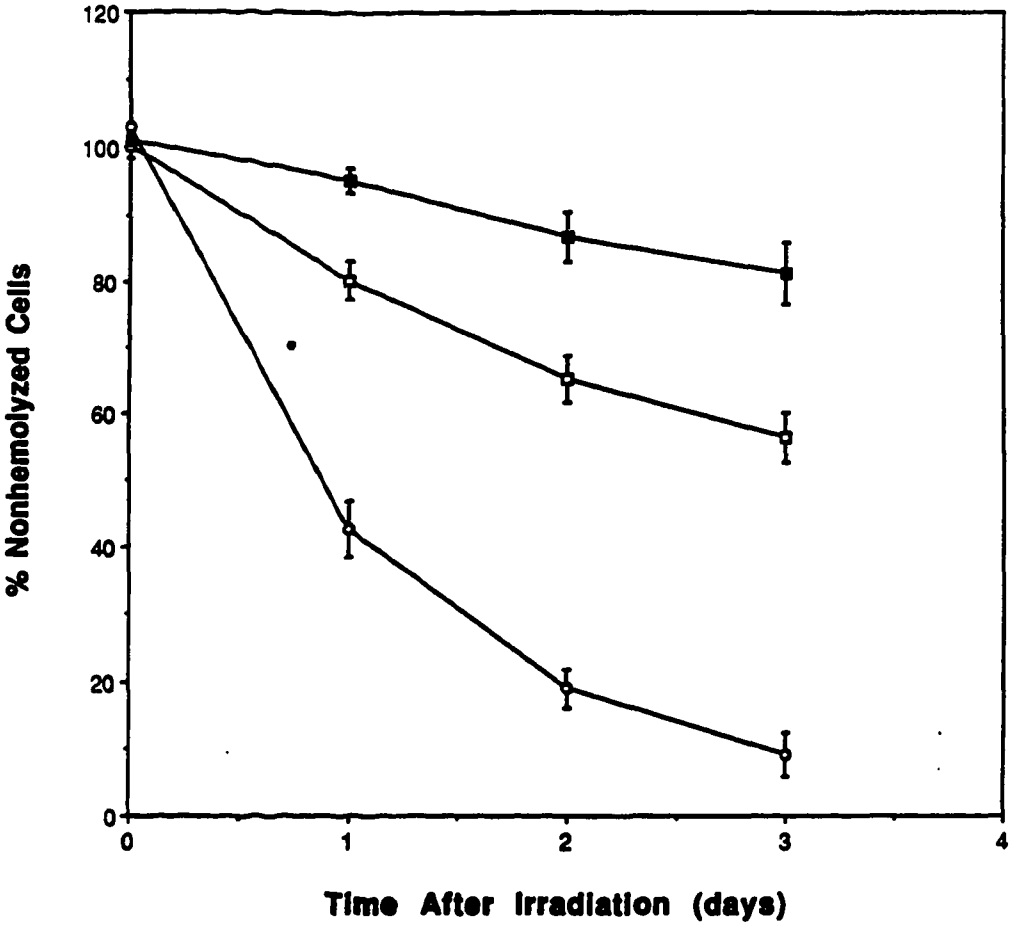

10
30
50
70
90
% Nonhemolyzed Cells
50
55
60
65
70
75
Dose (gray)

Figure 17. The effect of split-dose fractionation on chicken RBC's. Cells received a total dose of 100 Gy given in either 2 equal doses or as one single dose. The interfractionation time was 1 hour at 35°C. Some cells received a single dose of 50 Gy for comparison purposes. Fractionated samples (open squares), 100 Gy (open circles) and 50 Gy (closed squares). Each data point represents a mean of 4 to 5 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare the fractionated treatment with the nonfractionated treatment and $p < 0.001$.


120
100
80
60
40
20
0
% Nonhemolyzed Cells
0
1
2
3
4
Time After Irradiation (days)

In the kinetics experiments, chicken RBC's received a total dose of 100 Gy given in two equal fractions. The incubation time between the two fractions varied from 0 to 4 hours, and the incubation temperature was 35°C. No sdf was detected when incubation was less than 20 minutes, but when the time between the two fractions was lengthened to 30 minutes or longer, recovery from radiation damage was evident (See Figure 18). The $t_{1/2}$ of maximum sparing effect calculated from Figure 18 was 26.8 minutes.

Effect Of Multifractionation On Chicken Erythrocytes. In the multifractionation experiments, a total dose of 100 Gy was delivered in four equal fractions and in between irradiations, cells are kept at 35°C for 60 minutes. Multifractionation produced more dose sparing than bifractionation. The survival of bifractionated samples was similar to a single exposure of 60-65 Gy, whereas the survival of multifractionated samples was similar to a single dose of 50-55 Gy (See Figure 19). This represented a dose reduction of approximately 16% between multifractionation and bifractionation treatment.

Effect Of Incubation Temperature On sdf. To test the effect of temperature on sdf, cells were incubated at either 22°C or 35°C for the 1 hour between the two exposures. A total dose of 100 Gy was used. Figure 20 illustrates that sdf in chicken RBC's was influenced by temperature. Incubation at 22°C for 1 hour was not as effective as a similar incubation at 35°C in sparing radiation damage to the cells. A longer incubation time at 22°C might be required to obtain the same survival level achieved by 1 hour at 35°C. It was also possible that at higher temperatures [than 35°C] the half time for maximum sparing effect will be less than 26.8 minutes.

5.3 Discussion

Radiation-induced hemolysis (0 to 100 Gy) in isotonic condition did not occur immediately following irradiation. Time was required for significant hemolysis to result.

Figure 18. The kinetics of recovery in chicken RBC's. Cells received a total dose of 100 Gy given in 2 equal fractions. The interfractionation time varied from 0 to 4 hours, and during this time the cells were placed in a 35°C incubator. Scored immediately after irradiation (open squares), 1 day after irradiation (closed squares), 2 days after irradiation (open circles) and 3 days after irradiation (closed circles). Each data point represents a mean of 3 to 5 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare the fractionated treatment with the nonfractionated treatment and for samples with interfractionation time of greater than 30 minutes, $p < 0.001$.

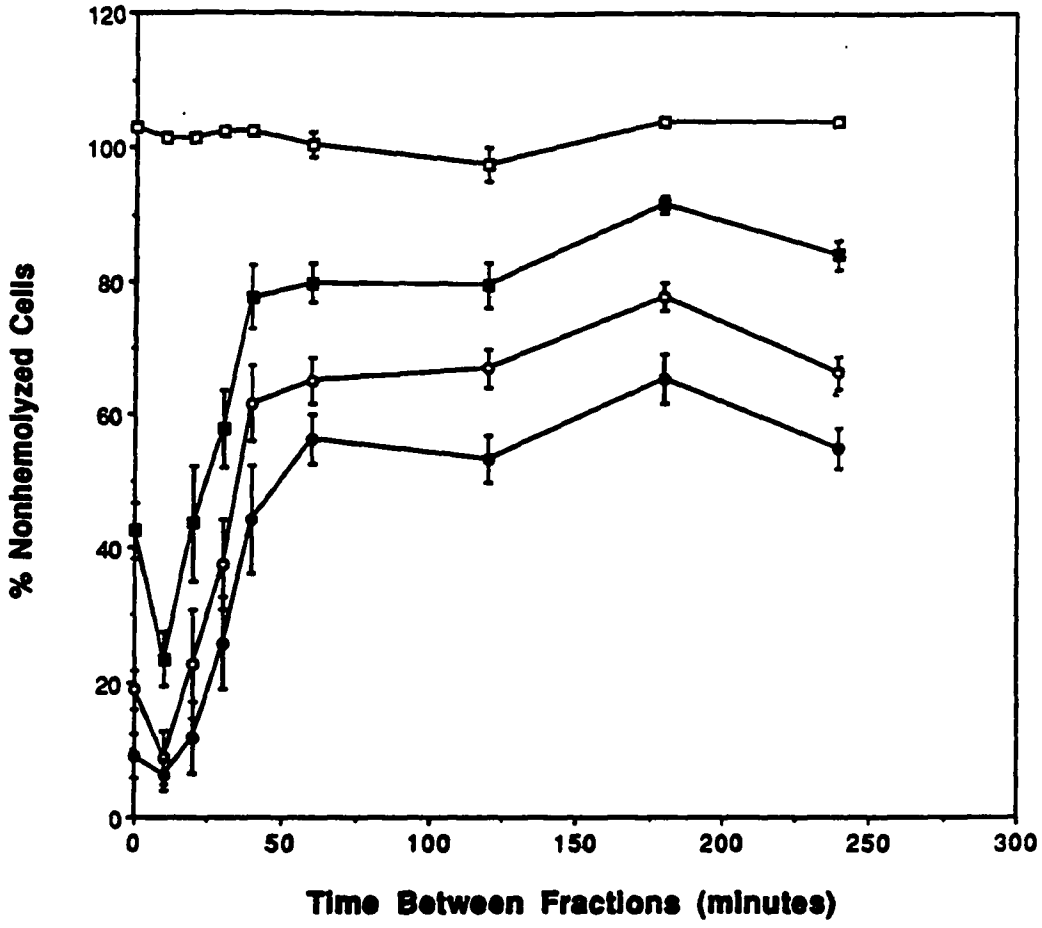

120
100
80
60
40
20
0
% Nonhemolyzed Cells
0
50
100
150
200
250
300
Time Between Fractions (minutes)

Figure 19. The effect of multifractionation. Chicken RBC's received a total dose of 100 Gy given in either 4 equal fractions or as a single dose. For the fractionated experiments, cells were incubated for 1 hour at 35°C between fractions. Some cells also received a single dose of 25 Gy for comparison purposes. Multifractionated samples (open squares), 100 Gy (open circles) and 25 Gy (closed squares). Each data point represents a mean of 5 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare the fractionated treatment with the nonfractionated treatment and $p < 0.001$.

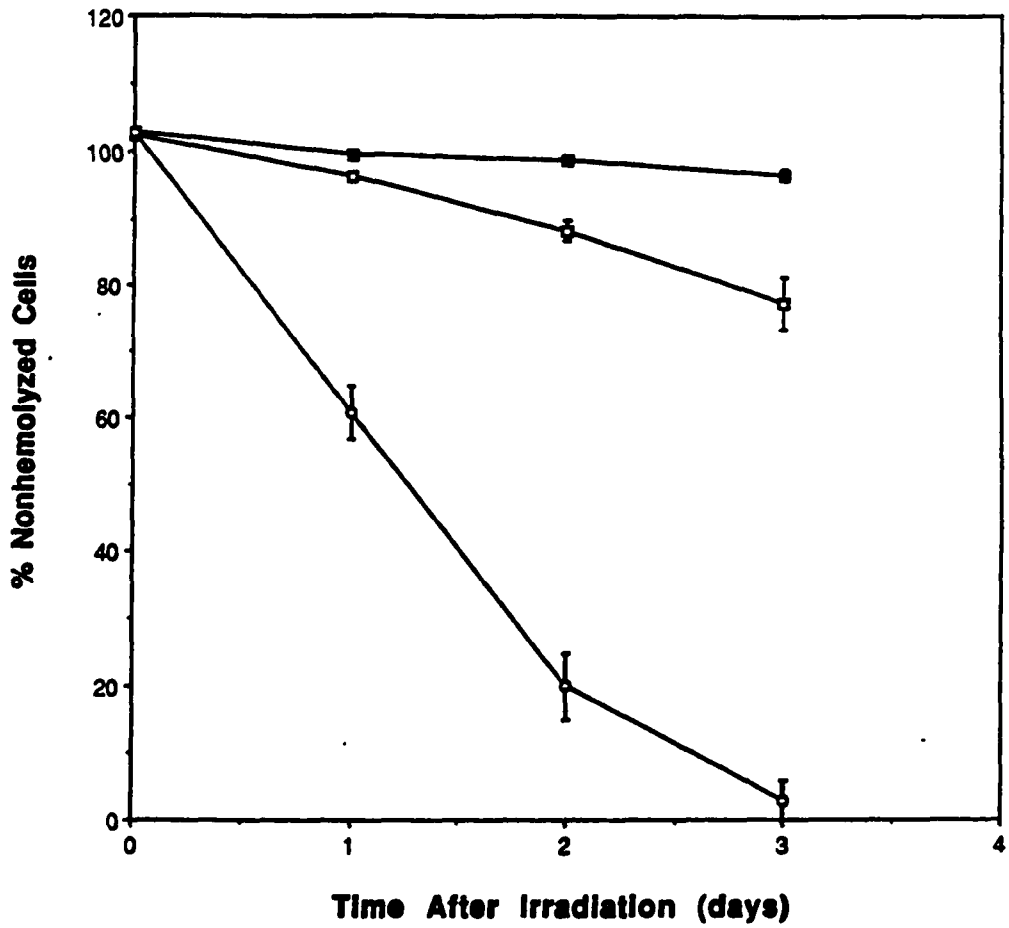
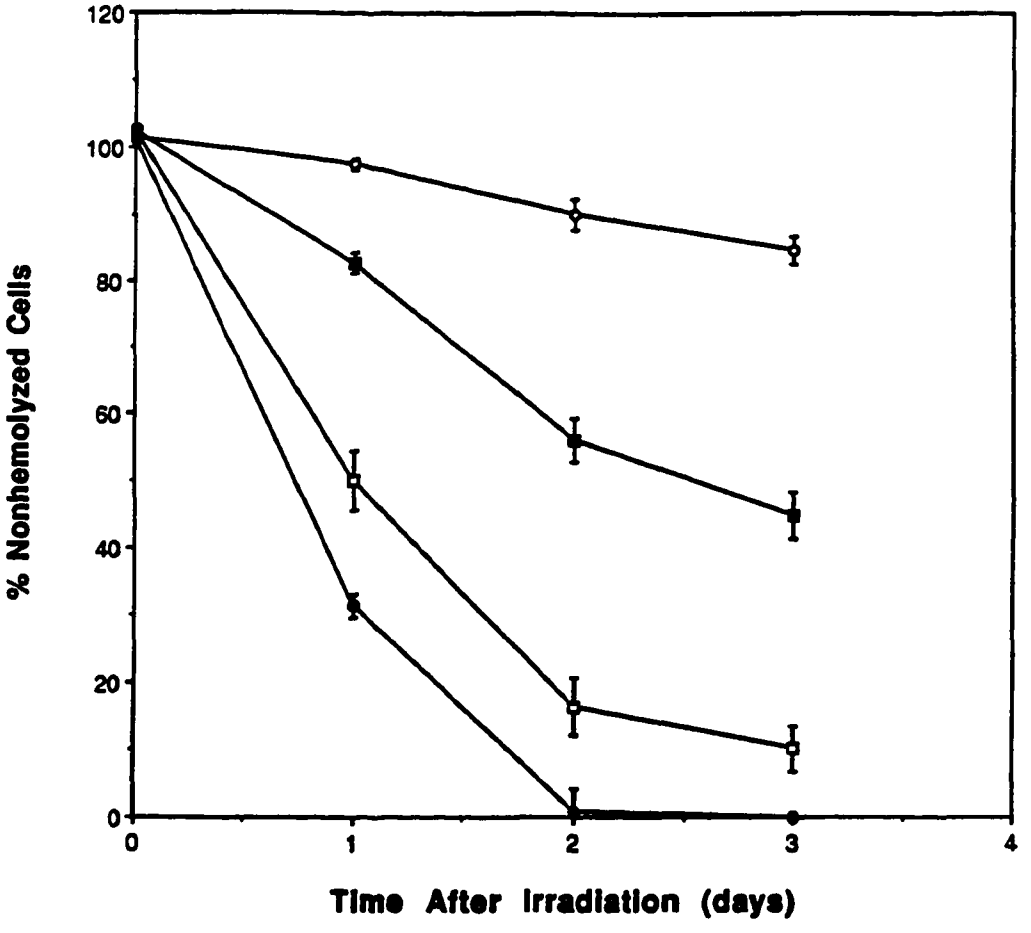

120
100
80
60
40
20
0
% Nonhemolyzed Cells
0
1
2
3
4
Time After Irradiation (days)

Figure 20. The effect of temperature on repair. Chicken RBC's received a total dose of 100 Gy in either 2 equal fractions or as a single exposure. For the splt-dose experiments, cells were held for 1 hour at either 22°C or 35°C between the two fractions. Some cells received a single dose of 50 Gy for comparison purposes. Cells held at 22°C (open squares), cells held at 35°C (closed squares), 100 Gy (closed circles) and 50 Gy (open circles). Each data point represents a mean of 4 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare the fractionated treatment with the nonfractionated treatment and $p < 0.001$.


120
100
80
60
40
20
0
0
1
2
3
4
% Nonhemolyzed Cells
Time After Irradiation (days)

When hemolysis was scored one, two, three and four days after irradiation, chicken RBC's hemolyzed in a dose-dependent manner with a threshold of at least 31.2 Gy. Below this threshold, irradiated cells were indistinguishable from controls.

Radiation-induced hemolysis has been studied since the early 1900's (Henri and Mayer, 1904; Holthusen, 1923). Back then, a latent period was found between radiation exposure and the subsequent hemolysis. Proposal for the mechanism of radiation-induced hemolysis focused mainly on electrolyte imbalance (Sutherland et al., 1967; Kollmann et al., 1969). These investigators proposed that radiation oxidizes sulphydryl groups in the erythrocyte membrane leading to potassium loss, sodium accumulation, cell swelling and hemolysis. While osmotic hemolysis was a consequence of ion imbalance in RBC's (Dacie and Lewis, 1984), it could not suffice as the sole explanation for radiation-induced hemolysis. For example, twenty hours after exposure to 40 kR, human erythrocytes lost more than 70% of their intracellular potassium but less than 10% hemolysis resulted (Kollmann et al., 1969). Also, dinitrophenol has been shown to cause sodium accumulation in hen RBC's, but it protected against radiation-induced hemolysis (Rusu and Baghdadi, 1978). Therefore, ion imbalance alone could not account for radiation-induced hemolysis.

Another hypothesis for radiation-induced hemolysis was the formation of free radicals. Free radicals formed during radiolysis of water can cause lipid peroxidation, hydrolysis of phospholipid head groups, lipid-lipid crosslinks, disulfide bridge formation, amino acid residue damage in membrane proteins, and lipid-protein crosslinks (Leyko and Bartosz, 1986). These changes in membrane structures also affect the cytoskeleton (Vranska et al., 1985). The combined effects of free radicals on erythrocyte membrane and cytoskeleton contribute to the eventual leak of hemoglobin out of the cells. Convincing evidence to support the free radical hypothesis included free radical scavengers, which

reduced the amount of radiation-induced hemolysis (Bartosz and Leyko, 1981; Miller and Raleigh, 1983), and antioxidants such as vitamin E and reduced glutathione, which have been shown to protect human erythrocytes against radiation-induced hemolysis (Brown, 1983; Kollmann et al., 1969).

Recovery from radiation damage has been investigated in mammalian cells (Elkind and Sutton, 1959), green algae (Jacobson, 1957; Davies, 1966), protozoa (Calkins, 1965), and insects (Yang and Ducoff, 1971). All these cell systems have a complicated recovery kinetics from radiation due to cell cycling. By manipulating the incubation temperature, the effect of the cell cycle could be minimized (Elkind et al., 1965). However, terminally-differentiated cells such as avian red blood cells would be more suitable for studying sdf without cell cycle interference since they are noncyclic cells.

From my split-dose studies, chicken RBC's were shown to recover from radiation damage. The amount of hemolysis observed in the bifractionated samples was similar to a single dose of 60-65 Gy. The recovery was even greater when cells received the multifractionated treatment (100 Gy split into 4 fractions), in which an additional dose reduction of 10 Gy was observed. Recovery from radiation-induced hemolysis in chicken erythrocytes was also influenced by temperature. When the cells are kept at 22°C rather then 35°C in between fractions, less recovery was observed. The survival of fractionated samples kept at 22°C for 1 hour was only a slight improvement from samples that received the total irradiation in a single dose.

From the kinetics studies, the half time for maximum recovery at 35°C in chicken RBC's was approximately 30 minutes. This was comparable to the value obtained from other post-mitotic systems which has a half time for maximal repair ranging from 25-50 minutes at 22°C (Ducoff et al., 1984). It was also comparable to the fast component of split-dose repair in plateau-phase CHO cells which has a mean time of 67.7 minutes

(Nelson et al., 1990). However, the avian erythrocyte system did not exhibit the immediate repair process seen in insect and some plateau-phase mammalian cells (Ducoff et al., 1984; Malcolm and Little, 1979). In chicken RBC's no repair was detectable when the interfractionation time was less than 20 minutes whereas in the insect and some plateau-phase mammalian cell systems recovery was clearly visible within intervals as short as a minute. For the very short interfraction time (e.g., 10 minutes) there was more hemolysis present than the nonfractionated samples. The reason for this was unclear but by 2-3 days after irradiation the additional hemolysis seen in the 10-20 minute samples was statistically insignificant from the nonfractionated samples.

There is continued controversy about the genetic activity of avian RBC's. Avian RBC's are nucleated although the function of the nucleus is unknown. Mature hen erythrocytes have been shown to synthesize mRNA but the mRNA is not believed to translocate to the cytoplasm and therefore not translated (Zentgraf et al., 1975). Mature avian erythrocytes also do not carry out semi-conservative DNA replication (Williams, 1972). Despite the controversy, our study clearly demonstrated that avian RBC's can repair radiation damage.

Although the mechanism for radiation repair in avian RBC's is unclear, I would like to provide a few speculations. Ionizing radiation induces free radical formation via radiolysis of water. Free radicals can cause damage to either lipids or membrane-bound proteins which can lead to cell rupture. One possible mechanism for repair of radiation damage in chicken RBC's is radical scavenging. Erythrocytes contain enzymes such as superoxide dismutase, glutathione peroxidase and catalase which can protect cells from radiation by scavenging superoxide anions, hydroxyl radicals and hydrogen peroxide (Witting, 1980; Cross et al., 1987). Antioxidants such as α-tocopherol and ascorbic acid exist in cells and can also scavenge free radicals (Machlin and Bendich, 1987). These

molecules can reduce the amount of free radicals present in cell membrane while the cells are incubating after the first radiation dose. Consequently, less free radicals will be present in the fractionated samples than in the nonfractionated samples. The fact that radiation repair is affected by incubation temperature is a good indication that it is a enzymatic process. However, this hypothesis has a weakness in that radical scavenge process should occur fairly rapid, but no recovery is observed for the short fractionation interval in the chicken RBC's.

Proteins are reported to play a role in protecting against radiation-induced membrane damage (Verma and Rastogi, 1990). Using protein-inserted liposomal preparations, they demonstrated that protein binding with lipids can inhibit the formation of lipid peroxides. Since erythrocytes contain various proteins, it is possible that these proteins can protect erythrocytes against radiation-induced hemolysis. Proteins may interact with the lipid component of the membrane during the first irradiation dose and inhibit radiation-induced peroxidation. While during the incubation, the proteins can return to their original state, and when the second dose of radiation was delivered, there will be proteins available to counter the effect of radiation. Hence, the total amount of hemolysis is reduced.

There is evidence for repair of radiation-induced damage in intact human erythrocytes (Sutherland and Pihl, 1968). By incubating the irradiated human erythrocytes in presence of glucose at 37°C for 1 hour, the cells recover about 60% of the sulphydryl groups they lost on irradiation. The recovery is only observed in intact erythrocytes not in isolated ghosts. Also, recovery does not occur without the presence of glucose or at 4°C. Given this and the two above hypothesis, sdf should be present in mammalian erythrocytes as well. We have preliminary data indicating that rat RBC's do not exhibit split-dose repair. From the existing literature (Sutherland et al., 1967; Kollmann et al., 1969; Kondo et al., 1989) and our own experiments, mammalian erythrocytes appear to be much more

radioresistant than chicken RBC's. Doses in excess of 400 Gy are often used to induce hemolysis in mammalian RBC's and 24 hours after irradiation usually less than 10% hemolysis results. On the other hand, 100 Gy is sufficient to induce more than 50% hemolysis in chicken RBC's 24 hours after irradiation. It is possible that the presence of the nuclear components in the chicken RBC's render them more sensitive to radiation but able carry out split-dose repair.

6. EFFECTS OF HYPERTHERMIA AND RADIATION

Hyperthermia in conjunction with ionizing radiation is currently recognized as an effective method of treating human cancers. The combination of hyperthermia and radiation results in more cell killing than either alone. This has been demonstrated in a variety of biological systems (Li et al., 1976; Lai and Ducoff, 1977; reviewed by Overgaard, 1984). Several hypotheses have been proposed to explain the synergism between heat and radiation. First, cells have different heat and radiation sensitivities during the various stages of the cell cycle and their sensitivity reciprocates each other. Second, cells exposed to chronic hypoxia are quite radioresistant but more heat sensitive. A tumor mass has a necrotic core which is more radioresistant. Hyperthermia in conjunction with radiotherapy can increase tumor killing by eliminating tumor cells in the necrotic core. Third, heat decreases the repair capability of cells resulting in more radiation damage and thus, more cell death. To determine whether the synergism between heat and radiation is due to heat potentiation of radiation damage or radiation potentiation of heat damage, the kinetics of organism or cell death induced by different treatment sequences (i.e. preirradiation heating versus postirradiation heating) have been examined (Lai and Ducoff, 1977; Hofer, 1987). Their data suggested that enhanced killing by the combination of heat and radiation is attributed heat potentiation of radiation rather than radiation potentiation of heat.

In this chapter, the synergistic effect of heat and radiation on the induction of hemolysis in chicken RBC's will be examined.

6.1 Treatment Sequence

Chicken RBC's received 60 Gy and 40 minutes at 51.5°C for the combined treatment. Heating was done immediately before or after the irradiation. No immediate hemolysis was observed for the cells that received the preirradiation heating sequence, but

immediate hemolysis was observed for the cells that received the postirradiation heating sequence. The results are plotted in Figure 21. Hemolysis was then periodically scored for the next three days. Throughout the course of observation, postirradiation heating samples contained more hemolyzed cells than the preirradiation heating group although the amount of additional hemolysis at 24 and 48 hours is similar for postirradiation heating as for the other heating protocols. The pattern of hemolysis of preirradiation heating was quite similar to the samples that had received the heat treatment only (40 minutes at 51.5°C), while samples treated with radiation only (60 Gy) had fairly low levels of hemolysis as expected.

To further examine the interaction of heat and radiation, chicken RBC's were exposed to either 30 Gy or 60 Gy split into two equal fractions, and then followed by 40 minutes of heating at 51.5°C (See Figure 22). After a split radiation exposure (two equal fractions), no immediate hemolysis was observed. The split dose samples did hemolyze slightly more than the 30 Gy samples at 24 hour, but at 48 and 72 hours, the difference was not significant. Of all the treatments I examined, only the single dose of 60 Gy followed by heating sequence resulted in immediate hemolysis. All the other treatments had similar hemolytic patterns

6.2 Kinetics Of Recovery From Radiation-induced Heat Sensitivity

The ordinate used in Figure 23 was different from the previous graphs because the data required to construct this figure were obtained in a 3-month span. Due to the seasonal variation of heat response of chicken RBC's [already discussed in a previous chapter], the hemolysis values differed significantly among experiments. In order to preserve the general trend of the experiments, values were normalized by plotting the ratio (N/N_0), where N equals the percent of nonhemolyzed cells of a sample and N_0 equals the

Figure 21. The effect of radiation and heat on chicken RBC's. Chicken RBC's received 60 Gy and 40 minute of heating at 51.5°C. Cells were also given the heat and radiation treatment alone for comparison purposes. Preirradiation heating (open circles), postirradiation heating (closed circles), heat alone (open squares) and radiation alone (closed squares). Each data point represents a mean of 4 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare postirradiation heating treatment with either heat or radiation alone treatment and $p < 0.001$.

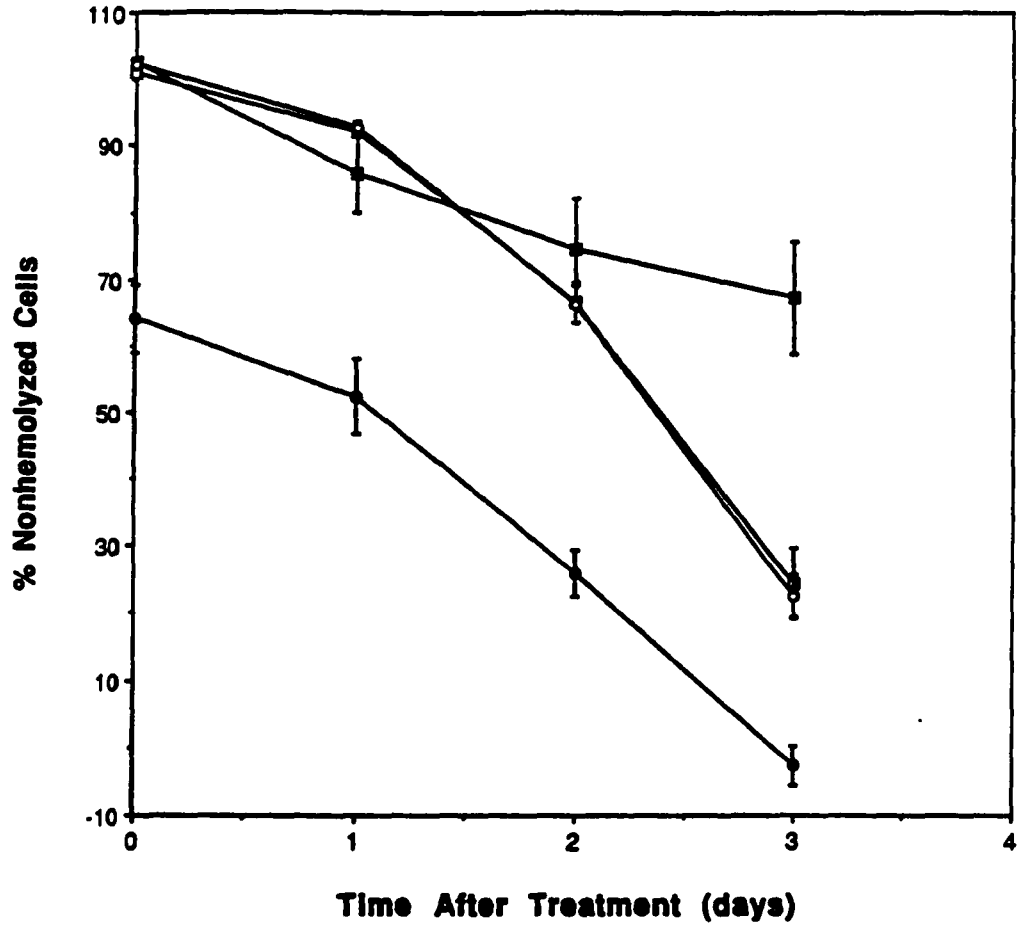

110
90
70
50
30
10
-10
0
1
2
3
4
% Nonhemolyzed Cells
Time After Treatment (days)

Figure 22. The effect of split-dose fractionation on the response of chicken RBC's to the combined treatment of heat and radiation. Cells received a total dose of 60 Gy split into two equal fractions. Immediately following the second radiation dose, cells were heated for 40 minutes at 51.5°C. The effect of a smaller radiation dose (30 Gy) preceeding the same heat treatment was also examined. Split dose followed by heating (open circles), 30 Gy followed by heating (closed squares) and 60 Gy followed by heating (open squares). Each data point represents a mean of 4 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare split-dose treatment with either heat or radiation alone treatment and $p < 0.001$

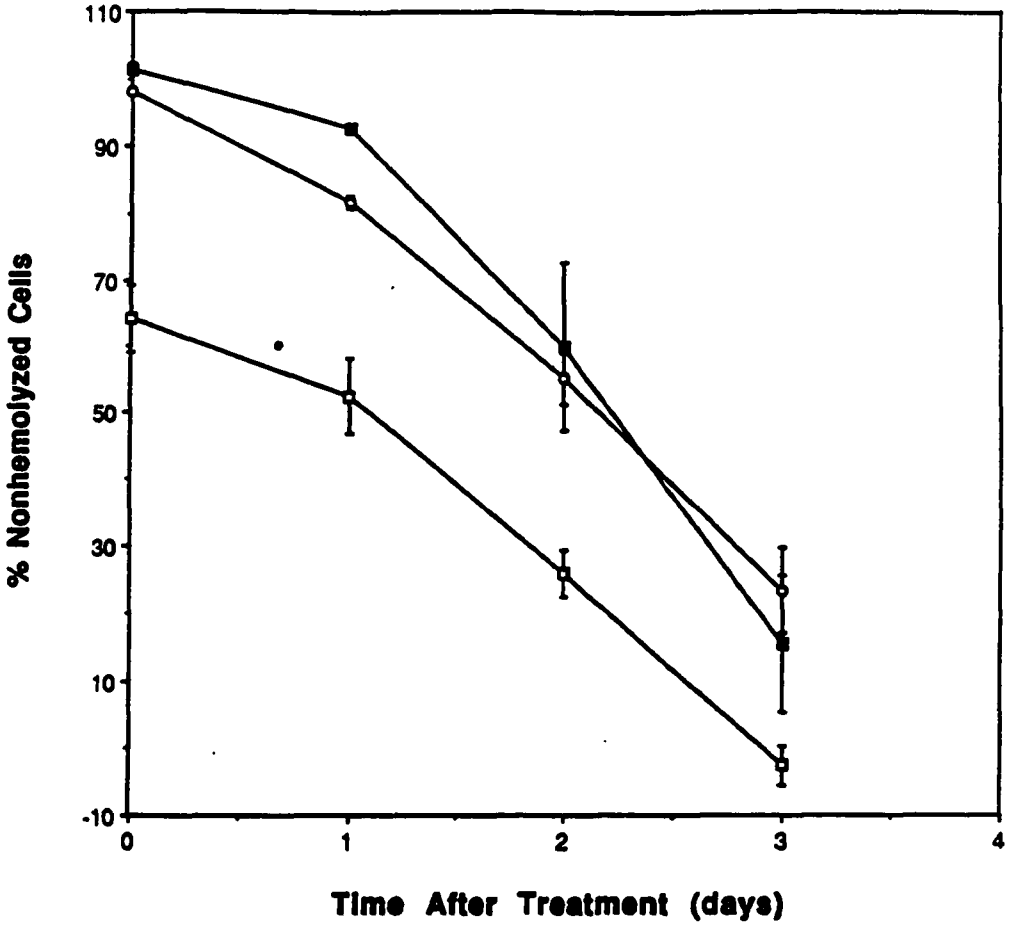
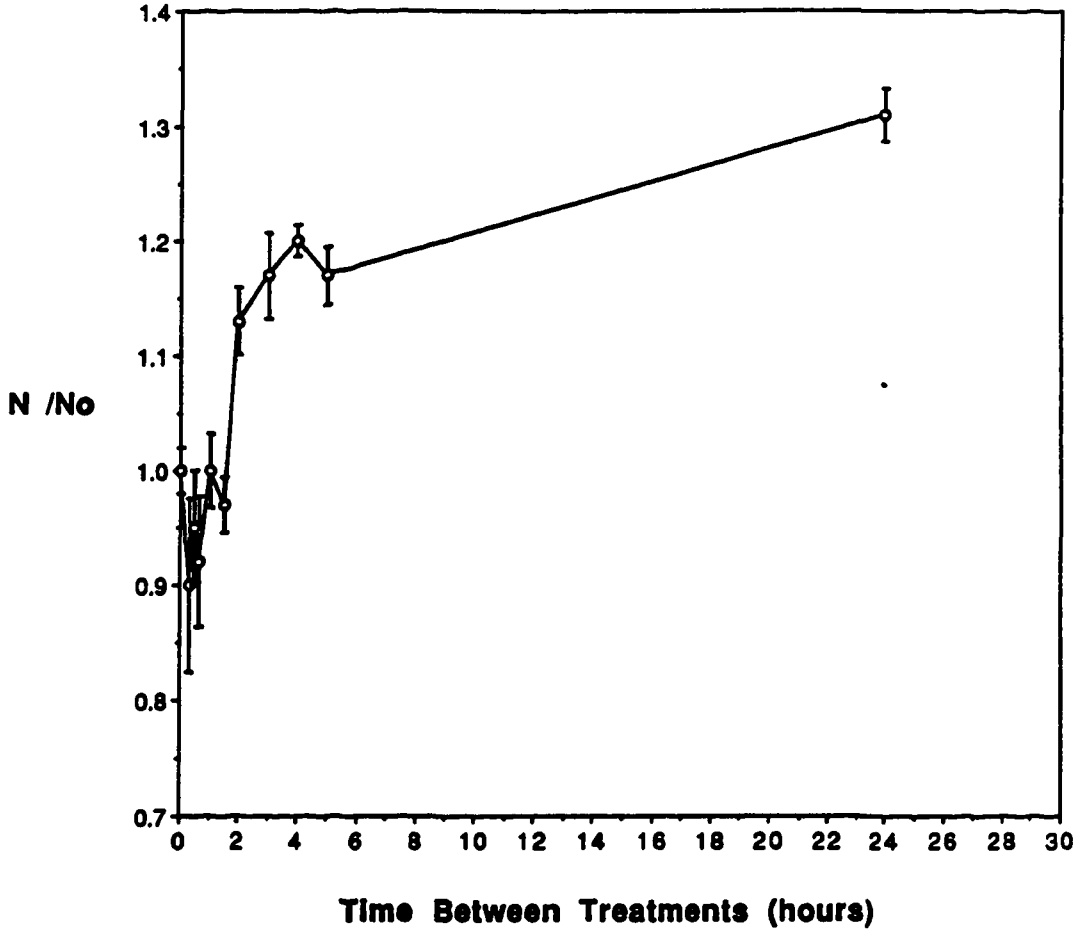

110
90
70
50
30
10
-10
0
1
2
3
4
% Nonhemolyzed Cells
Time After Treatment (days)

Figure 23. The kinetics of recovery from radiation-induced heat sensitivity in chicken RBC's. Irradiated cells were incubated at 35°C for 0 to 24 hours before receiving the heat treatment. The ordinate is plotted as (N/N_0), where N equals the percent of nonhemolyzed cells of the sample, and N_0 equals the percent of nonhemolyzed cells in the samples without intertreatment incubation at the time of treatment completeion. The data were collected immediately after treatment. Each data point represents a mean of 6 experiments and for each experiment, triplicate samples were used. Student's t-test was performed to compare irradiated cells that were incubated before heating with those that received no intertreatment incubation and for intervals greater than 2 hours, $p < 0.05$.


1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
N /No
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time Between Treatments (hours)

percent of nonhemolyzed cells in samples without intertreatment incubation at the time of treatment completion.

Irradiated (60 Gy) chicken RBC's were incubated at 35°C for 0 to 24 hours before receiving the heat treatment (51.5°C for 40 minutes). When incubation was less than 2 hours, immediate hemolysis was observed and the amount of hemolysis was similar to the samples that received no incubation between treatments. However, when irradiated cells were incubated for 2 or more hours, less prompt hemolysis was observed (See Figure 23).

6.3 Discussion

The combination of heat and radiation achieves more cell killing than either agent alone, but there is no consensus on which treatment sequence is more lethal to cells (Boone et al., 1976; Li et al., 1976; Raaphorst et al., 1988). It appears that the efficacy of the treatment sequence may be dependent on the individual cell system. In chicken RBC's, my data clearly indicated that postirradiation heating was more effective than preirradiation heating in causing cell lysis. In fact, preirradiation heating did not result in more hemolysis than cells that received hyperthermia alone. Since radiation by itself does not produce much hemolysis, it is not clear whether the amount of hemolysis produced by preirradiation heating is simply the addition of heat and radiation. Nevertheless, the data presented in this chapter imply that the interaction of heat and radiation is synergistic when postirradiation heating is given.

Hemolysis induced by the combination of radiation and heat appeared to be affected by the amount of radiation damage the cells acquired prior to the heating. This was demonstrated either by giving the cells less radiation (30 Gy instead of 60 Gy) or by splitting the total dose (60 Gy) into two equal fractions. In either case no hemolysis occurred promptly and the absolute amount of hemolysis was less than the postirradiation

heating group (60 Gy followed by 40 minutes at 51.5°C) throughout the course of observation. Interestingly the amount of additional hemolysis at 24 and 48 hours was similar for all heating protocols regardless of the prior [radiation] treatment.

The kinetics of recovery from radiation-induced heat sensitivity was performed to determine whether the interaction of radiation and hyperthermia was affected by the temporal proximity of these physical insults. When heat was applied within 90 minutes following irradiation, hemolysis occurred promptly and the degree of hemolysis was similar to the samples that received heating immediately after irradiation. However, when heating was done 2 to 24 hours after irradiation, prompt hemolysis occurred but in a less significant degree.

The synergism between heat and ionizing radiation could theoretically be either heat potentiation of radiation damage or radiation potentiation of heat damage. Study by Hofer (1987) showed that cell death after heating was fast and virtually complete within 2 days but cell death after irradiation was slow and delayed for at least 3 days. Postirradiation heating (6 Gy followed by 15 minutes at 44°C) caused greater cell killing than radiation alone. However, the enhanced cell killing was manifested only in the delayed component of cell death; the early component was unaffected by even massive doses of radiation (≥ 300 Gy). These data suggested that the synergism between heat and radiation was heat potentiation of radiation killing rather than radiation potentiation of heat killing. Studies performed using *Tribolium confusium* yielded similar results (Lai and Ducoff, 1977).

The same type of argument would be difficult to make in chicken RBC's because the kinetics of heat- and radiation-induced hemolysis were not distinct. In my study, postirradiation heating was the only protocol that induced immediate hemolysis. The level of hemolysis caused by preirradiation heating was similar to the heat-only group.

Thermal enhancement of radiation cell killing has often been attributed to decreased repair capacity (i.e., heat-induced chromatin condensation making it inaccessible to repair enzymes). The chromatin of the avian RBC's is already very condensed; therefore, the absence of additional effect of heat before radiation is not so surprising.

Although synergism between heat and ionizing radiation is well recognized, the mechanism(s) behind the different levels of cell killing induced by changing the treatment sequence is not. In the chicken RBC's system, synergism is observed only in the postirradiation heating treatment. In the following paragraphs, I will speculate on a few possibilities:

(1) When heat is applied, some protein denaturation will take place. As long as irreversible change does not occur, when conditions returned to normal, proteins will renature. Since the proper protein folding is built into the primary amino acid sequence, it is not unreasonable to assume that the renatured protein is restored to its original structure and function. When cells are irradiated first and then immediately exposed to heat , free radicals generated by radiation will not be scavenged efficiently because proteins that normally scavenge free radicals are under attack by heat and have lost their proper 3-dimensional structure to scavenge free radicals. However, when cells are heated prior to irradiation, some heat-induced protein structural changes can be restored during irradiation. This minimizes the radiation damage. Hence, greater amounts of hemolysis result from the post-irradiation heating treatment.

(2) Hemoglobin usually has high affinity for ferrous ions, but if it becomes denatured by heat, the ferrous ions are released and can provide a catalyst for converting hydrogen peroxide to hydroxyl radicals via Fenton reaction. While it is true that most free iron molecules are in the form of ferric, superoxide anions, which are produced by irradiation, enhance the reduction of ferric ion to ferrous (Cotran et al., 1989). The

differences observed in preirradiation versus postirradiation heating could be a result of more ferrous ion being available for the generation of hydroxyl radicals.

(3) Membrane-associated proteins can also be affected by heat. Study by Verma and Rastogi (1990) indicated proteins interact with the lipid component of the membrane and reduce lipid peroxidation. When protein structures are changed by heat, they cannot interact properly with lipids to prevent peroxidation. Preirradiation heating, in a sense, allows a greater chance for proteins to recover to their original state and, therefore, greater protection for radiation damage.

The above speculations are based on the idea that ionizing radiation creates a transient perturbation in chicken RBC's, which is exacerbated by the presence of heat, leading to greater and prompt hemolysis.

7. CONCLUSION

Avian erythrocytes are terminally-differentiated cells. Unlike mammalian erythrocytes, they are nucleated and are shaped like flying saucers rather than biconcaved. They have limited transcriptional and translational activity. The main purpose of this thesis is to characterized the effects of heat and/or ionizing radiation on avian erythrocytes and to develop a possible model for terminally-differentiated tissues.

(1) Since colony formation assay was inappropriate for measuring survival of chicken erythrocytes, a hemolytic assay was developed to score the effects of heat and/or radiation. After testing different isotonic solutions, buffering conditions and pH adjustments, an isotonic-glucose medium containing 140 mM NaCl, 10 mM KCl, 10 mM glucose, 1.5 mM $MgCl_2$, and 10 mM HEPES buffer (pH adjusted to 7.4) was used. Under this condition, hemolysis was a reproducible measurement.

(2) Chicken erythrocytes were subjected to either 50.5°C or 51.5°C for 0 to 180 minutes. Hemolysis was scored at 0, 24, 48 and 72 hours after heating. Heat-induced hemolysis occurred in a dose-dependent manner, but not promptly after heating. One to two days was required for the development of significant hemolysis. When hemolysis became apparent, its magnitude increased with heating duration and with temperature.

(3) Two other endpoints were used to score heat damage, namely potassium leakage and osmotic fragility. With these two indices, the effects of heating was immediately apparent. Heat-induced (51.5°C) K^+ leakage was directly proportional to heating duration (0 to 120 minutes). When cells were returned to incubation temperature (35°C), K^+ continues to leak, but the amount of post-heating leakage within 40 minutes was less than active continuous heating at 51.5°C.

Osmotic fragility of chicken RBC's was inversely related to temperature when they were exposed to temperatures less than 40°C. At 51.5°C osmotic fragility returns to the level exhibited by 22°C.

(4) Thermotolerance was induced using two different heat shock treatments. The two heat shock treatments were: 42.6°C for 0 to 2 hours and immediately followed by a heat challenge at 51.5°C for 40 minutes, and 43.1°C for 15 minutes, followed by 0 to 2 hours of incubation at 35°C and than heat challenged at 51.5°C for 40 minutes. Both treatments successfully induced thermotolerance but the levels of heat resistance achieved by them were different. Heat-shocked samples maintained a higher level of nonhemolyzed cells than non-heat-shocked controls for at least 3 days regardless of which heat shock temperature was given.

Using osmotic fragility as an index, thermotolerance was also observed. Cells that received a heat shock prior to heat challenge could withstand higher osmotic force. The increase in osmotic resistance was not stable. It disappeared overnight. Since the stability of thermotolerance scored by spontaneous hemolysis and by osmotic hemolysis were not similar, they might represent different phenomena. Thermotolerance observed using spontaneous hemolysis might be associated with heat shock proteins, which has been reported to last for about 72 hours, whereas heat resistance measured by osmotic hemolysis might be temporary membrane alterations. No thermotolerance was recorded with K^+ leakage as an endpoint.

(5) Chicken RBC's received 0 to 100 Gy of radiation at dose rate of 10 Gy/minute. Radiation-induced hemolysis also occurred in a dose-dependent manner but not immediately after irradiation. Hemolysis became apparent at 24 hours after treatment. Threshold for radiation dose response was observed. At doses below 40 Gy, hemolysis in irradiated samples was indistinguishable from nonirradiated controls.

(6) Split-dose experiments showed that chicken RBC's were able to repair radiation damage. The half time for maximum recovery was about 30 minutes at 35°C. Recovery from γ radiation was affected by the interfraction temperature. More cells recovered from radiation damage when they were incubated at 35°C rather than at 22°C for 60 minutes. Since repair was influenced by temperature, an enzymatic repair process was suspected.

(7) The combination of hyperthermia and ionizing radiation produced more hemolysis than heat or radiation alone. Different treatment sequence resulted in different level of hemolysis. Postirradiation heating was the only protocol that resulted in prompt hemolysis. If RBC's were allowed to recover for 2 to 24 hours from radiation before receiving heat treatment, some reduction in hemolysis was observed. The hemolytic kinetics of preirradiation heating was very similar to the one exhibited by the heat-only sample. Because of this, it would be difficult to determine whether allowing cells to recover from heat damage [before irradiation] would decrease hemolysis. From the limited data that I have (data not shown), this did not occur. By allowing time to elapse before irradiating heat-treated cells, the amount of hemolysis actually increased slightly and no immediate hemolysis was recorded. Definitive synergistic effect of the combined treatment was observed for postirradiation heating sequence only.

Most studies on the effects of heat and/or radiation are performed on proliferating cells, when in reality many tissue organs do not normally go into mitosis. They contain either stable or nondividing cells. The data presented in this thesis demonstrated that the response of terminally-differentiated cells to physical insults such as heat and radiation is similar to proliferative cells.

REFERENCES

Alper, T. (1979) Cellular Radiobiology. Cambridge University Press, Cambridge.

Altschuler, M. and Mascarenhas, J. P. (1982) Heat shock proteins and effects of heat shock in plants. Plant Mol. Biol. 39: 35-77.

Altschuler, M. and Mascarenhas, J. P. (1985) Transcription and translation of heat shock and normal proteins in seedlings and developing seeds of soybean exposed to a gradual temperature increase. Plant Mol. Biol. 5: 291-297.

Anderson, R. L. and Parker, R. (1982) Analysis of membrane lipid composition of mammalian cells during development of thermotolerance. Int. J. Radiat. Biol. 42: 57-69.

Anderson, R. L., Shiu, E. S., Fisher, G. A. and Hahn, G. M. (1988) DNA damage does not appear to be a trigger for thermotolerance in mammalian cells. Int. J. Radiat. Biol. 54: 285-298.

Atkinson, B. G. and Dean, R. L. (1985) The effects of stress on the gene expression of amphibian, avian and mammalian blood cells. In "Changes in Eukaryotic Gene Expression in Response to Environmental Stress" (B. G. Atkinson and D. H. Walden, Eds.,): 159-181. Academic Press, Inc., Orlando.

Banerji, S. S., Therdorakis, N. G. and Morimoto, R. I. (1984) Heat shock-induced translational control of HSP 70 and globin synthesis in chicken reticulocytes. Mol. Cell. Biol. 4: 2437-2448.

Bardwell, J. C. and Craig, E. A. (1984) Major heat shock gene of *Drosophila* and *Escherichia coli* inducible dnak gene are homologous. Proc. Natl. Acad. Sci. USA 81: 848-852.

Bartosz, G. and Leyko, W. (1981) Radioprotection of bovine erythrocytes to haemolysis. Int. J. Radiat. Biol. 39: 39-46.

Bartosz, G., Sieron, Z. and Leyko, W. (1978) Effect of gamma-irradiation on the non-electrolyte and anion permeability of erythrocytes. Studia Biophys. 68: 163-169.

Ben-Hur, E., Elkind, M. M. and Bronk, B. V. (1974) Thermally enhanced radioresponse of cultures Chinese hamster cells: inhibition of repair of sublethal damage and enhancement of lethal damage. Radiat. Res. 58: 38-51.

Boone, M. L. M., Leith, J. T. and Gerner, E. W. (1976) The interaction of hyperthermia and radiation (X-ray or accelerated helium ion) in tissue culture cells. Radiat. Res. 67: 517 [abstract].

Boon-Niermeijer, E. K., Tuyl, M. and Van De Scheur, H. (1986) Evidence for two states of thermotolerance. Int. J. Hyperthermia 2(1): 93-105.

Borrelli, M. J., Carlini, W. G., Ransom, B. R. and Dewey, W. C. (1986b) Ion-sensitive microelectrode measurements of free intracellular chloride and potassium concentration in hyperthermic-treated neuroblastoma cells. J. Cell. Physiol. 129: 175-184.

Borrelli, M. J., Wong, R. S. L. and Dewey, W. C. (1986a) A direct correlation between hyperthermia induced membrane blebbing and survival in synchronous G1 CHO cells. J. Cell. Physiol.126: 181-190.

Brown, M. A. (1983) Resistance of human erythrocytes containing elevated levels of vitamin E to radiation-induced hemolysis. Radiat. Res. 95: 303-316.

Burdon, R. H. (1982) The human heat-shock proteins: their induction and possible intracellular functions. In "Heat Shock: From Bacteria to Man" (M. J. Schlesinger, M. Ashburner and A. Tissieres, Eds.): 283-288. Cold Spring Harbor Lab. Cold Spring Harbor, New York.

Calkins, J. (1965) A study of the time course of recovery of *Paramecium aurelia* from the lethal effects of X-rays. Radiat. Res. 26: 124-131.

Cotran, R. S., Kumar, V. and Robbins, S. L. (1989) Robbins pathologic basis of disease. W. B. Saunders Co., Philadelphia.

Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M. and Harman, D. (1987) Oxygen radicals and human disease. Ann. Internal Med. 107: 526-545.

Dacie, J. V. and Lewis, S. M. (1984) Practical Haematology. Churchill Livingstone, New York.

Dalrymple, G. V., Sanders, J. L. and Baker, M. L. (1967) Dinitrophenol decreases the radiation sensitivity of L cells. Nature (London) 216: 708-709.

Davies, D. R. (1966) Patterns and rates of recovery in synchronous populations of algal cells exposed to gamma radiation. Radiat. Res. 29: 222-235.

Dean, R. L. and Atkinson, B. G. (1985) Synthesis of heat shock proteins in quail red blood cells following brief, physiologically relevant increase in whole body hyperthermia. Comp. Biochem. Physiol. 8B: 185-191.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. and Schekman, R. (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature 332: 800-805.

Dewey, W. C. (1989) The search for critical cellular targets damaged by heat. Radiat. Res. 120: 191-204.

Dewey, W. C., Hopwood, L. E., Sapareto, S. A. and Gerwick, L. E. (1977) Cellular response to combinations of hyperthermia and radiation. Radiology 123: 463-474.

Dewey, W. C., Stone, L. E., Miller, H. H. and Giblak, R. E. (1971b) Radiosensitization with 5-bromodeoxyuridine of Chinese hamster cells X-irradiated during different phases of the cell cycle. Radiat. Res. 47: 672-688.

Dewey, W. C., Westra, A., Miller, H. H. and Nagasawa, H. (1971a) Heat-induced lethality and chromosomal damage in synchronized Chinese hamster cells treated with 5-bromodeoxyuridine. Int. J. Radiat. Biol. 20: 505-520.

Ducoff, H. S., Jayaraman, S. and Blumenthal, L. K. (1984) Repair studies utilizing a fly-pupariation model of postmitotic cell response. Br. J. Cancer 49: 249-252.

Ducoff, H. S., Lee S. W. and Kozma, T. G. (1989) Heat-induced hemolysis and thermotolerance: enucleate vs nucleated red cell. Proceedings of 5th International Symposium in Hyperthermic Oncology, Kyoto, Vol II: 359-361.

Elkind, M. M. and Sutton, H. (1959) X-ray damage and recovery in mammalian cells in culture. Nature (London) 184: 1293-1295.

Elkind, M. M., Sutton-Gilbert, H., Moses, W. B., Alesico, T. and Swain, R. W. (1965) Radiation response of mammalian cells in culture. V. Temperature dependence of the repair of x-ray damage in surviving cells (Aerobic and hypoxic). Radiat. Res. 25: 359-376.

Elkind, M. M. and Whitmore, G. F. (1967) The Radiobiology of Cultured Mammalian Cells. Gordon and Breach, New York.

Fricke, H. and Hart, E. J. (1965) In "Radiation Dosimetry, Vol. II. Instrumentation" (F. C. Attix, W. C. Roesch and E. Tochilin, Eds.): 167-239.

Gerner, E. W. And Schneider,.M. J. (1975) Induced thermal resistance in HeLa cells. Nature 256: 500-502.

Grzelinska, E., Bartosz, G., Leyko, W. and Chapman, I. V. (1982) Effect of hyperthermia and ionizing radiation on the erythrocyte membrane. Int. J. Radiat. Biol. 42: 45-55.

Gwozdzinski, K., Bartosz, G. and Leyko, W. (1981) Effect of gamma radiation on the transport of spin-labeled compounds across the erythrocyte membrane. Radiat. Environ. Biophys. 19: 275-285.

Hall, E. J. (1988) Radiobiology for the Radiobiologist. J. B. Lippincott Company, Philadelphia.

Hallberg, R. L. (1986) No heat shock protein synthesis is required for induced thermostabilization of translational machinery. Mol. Cell. Biol. 6: 2267-2270.

Ham, T. H., Shen, S. C., Fleming, E. M. and Castle, W. B. (1948) Studies on the destruction of red blood cells. IV. Thermal injury: action of heat in causing spheroidicity, osmotic and mechanical fragilities and hemolysis of erythrocytes; observations on the mechanisms of destruction of such erythrocytes in dogs and in a patient with fatal thermal burn. Blood 3: 373-403.

Harris, J. R., Murthy, A. K. and Belli, J. A. (1976) The effect of hyperthermia on the repair of radiation damage in plateau phase cells. Radiology 119: 227-229.

Heikkila, J. J., Kloc, M., Bury, J., Schultz, G. A. and Browder, L. W. (1985) Acquisition of heat-shock response and thermotolerance during early development of *Xenopus* larvis. Dev. Biol. 6: 663-673.

Henle, K. J. and Leeper, D. B. (1979) Effects of hyperthermia (45°) on macromolecular synthesis in Chinese hamster ovary cells. Cancer Res. 39: 2665-2674.

Henri, V. and Mayer, A. (1904) Action des radiations du radium sur les colloids l'hemoglobin, les ferments, et les globules rouges. Compt. Rend. 138: 521-524.

Ho, K. C. and Lin, P. S. (1991) Response of erythrocytes to heat in the presence of D_2O, glycerol and anisotonic saline. Radiat. Res. 125: 20-27.

Hofer, K. G. (1987) Heat potentiation of radiation damage versus radiation potentiation of heat damage. Radiat. Res. 110: 450-457.

Holthusen, H. (1923) Blutveranderungen durch Roetgenstrahlen und deren Sensibilisierung. Strahlentherapie 14: 561-570.

Jacobs, M. H., Glassman, H. N. and Parpart, A. K. (1936) Osmotic properties of the erythrocyte. VIII. on the nature of the influence of temperature on osmotic hemolysis. J. Cell. Comp. Physiol. 8: 403-417.

Jacobson, B. (1957) Evidence for recovery from X-ray damage in *Chlamdyomonas*. Radiat. Res. 7: 394-406.

Joiner, C. F. and Lauf, P. K. (1979) Temperature dependence of active K^+ transport in cation dimorphic sheep erythrocytes. Biochim et Bioph. Acta. 522: 540-545.

Jozwiak, Z and Palecz, D. (1988) Influence of heat treatment on osmotic fragility of carp erythrocytes. Int. J. Radiat. Biol. 54: 299-303.

Kassis, A. I., Sastry, K. S. R. and Adelstein, S. J. (1985) Intracellular localisation of Auger electron emitters: Biophysical dosimetry. Radiat. Prot. Dosim. 13: 233-236.

Kelley, P. M. and Schlesinger, M. J. (1978) The effects of amino acid analogues and heat shock on gene expression in chicken embryo fibroblast. Cell 15: 1277-1286.

Kollmann, G. K., Shapiro, B. and Martin, D. (1969) The mechanism of radiation hemolysis in human erythrocytes. Radiat. Res. 37: 551-566.

Kondo, T., Fukushima, Y., Kon, H. and Riesz, P. (1989) Effect of sheer stress and free radicals induced by ultrasound on erythrocytes. Arch. Biochem. Biophys. 269(2): 381-389.

Kong, S., Davison, A. J. and Bland, J. (1981) Action of gamma-radiation on resealed erythrocyte ghosts. A comparison with intact erythrocytes and a study of the effects of oxygen. Int. J. Radiat. Biol. 40: 19-29.

Konings, A. W. and Ruifrok, A. C. C. (1985) Role of membrane lipid and membrane fluidity in thermosensitivity and thermotolerance of mammalian cells. Radiat. Res. 102: 86-98.

Lai, P.-K. and Ducoff, H. S. (1977) Kinetics of interaction of hyperthermia and ionizing radiation in *Tribolium confusium*. Radiat. Res. 72: 296-307.

Lakchaura, B. and Clark, J. B. (1971) Recovery from X-ray and nitrogen mustard inactivation of Escherichia coli by 2, 4-dinitrophenol treatment. Radiat. Res. 42: 320-330.

Landry, J., Bernier, D., Chretien, P., Nicole, L. M., Tanguay, R. M. and Marceau, N. (1982) Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res. 42: 2457-2461.

Lepock, J. R. (1982) Involvement of membranes in cellular responses to hyperthermia. Radiat. Res. 92: 433-438.

Leyko, W. and Bartosz, G. (1986) Membrane effects of ionizing radiation and hyperthermia. Int. J. Radiat. Biol. 49: 743-770.

Leyko, W. and Jozwiak, Z. (1987) Heat-induced heat resistance in mammalian erythrocytes. Proceedings of the 8th International Congress of Radiation Research, Edinburgh: F22-20P.

Li, G. C. (1985). Elevated level of 70,000 dalton heat shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. J. Cell. Physiol. 115: 116-122.

Li, G. C. (1983) Induction of thermotolerance and enhanced heat shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. J. Cell. Physiol. 115: 116-122.

Li, G. C., Evans, R.G. and Hahn, G. M. (1976) Modification and inhibition of repair of potentially lethal X-ray damage by hyperthermia. Radiat. Res. 67: 491-501.

Li, G. C. and Hahn, G. M. (1983) Induction and decay of thermotolerance: influence of environmental temperature. Proceedings of 7th International Congress of Radiat. Res.: D33-34.

Li, G. C. and Laszlo, A. (1985) Amino acid analogs while inducing heat shock proteins sensitize CHO cells to thermal damage. J. Cell. Physiol. 122: 91-97.

Lin, C-Y., Roberts, J. K. and Key, J. L. (1984) Acquisition of thermotolerance in soybean seedlings. Plant Physiol. 74: 152-160.

Lindquist, S. (1980) Translational efficiency of heat-induced messages in *Drosophila melanogaster* cells. J. Mol. Biol. 137: 151-158.

Little, J. B. and Williams, J. R. (1976) Enhancement of survival of irradiated plateau phase cells by dinitrophenol: Effect of dose-rate and cell strain. Radiat. Res. 66: 90-99.

Lucas, A. M. and Jamroz, C. (1961) Atlas of Avian Hematology. U.S. Deptartment of Agriculture.

Machlin, L. J. and Bendich, A. (1987) Free radicals tissue damage: protective role of antioxidant nutrient. FASEB J. 1: 441-445.

Malcolm, A. W. and Little, J. B. (1979) Rapid recovery in plateau-phase mammalian cell. Radiat. Res. 80: 38-48.

Massicotte-Nolan, P., Glofcheski, D. J., Kruuv, J. and Lepock, J. R. (1981) Relationship between hyperthermic cell killing and protein denaturation by alcohols. Radiat. Res. 87: 284-299.

McAlister, L. and Finkelstein, D. B. (1980) Heat shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. 93: 819-824.

Miller, G. G. and Raleigh, J. A. (1983) Action of some hydroxyl radical scavengers on radiation-induced hemolysis. Int. J. Radiat. Biol. 43: 411-419.

Mivechi, N. F. and Li, G. C. (1985) Thermotolerance and profile of protein synthesis in murine bone marrow cells after heat shock. Cancer Res. 45: 3843-3849.

Mizzen, L. A. and Welch, W. J. (1988) Characterization of the thermotolerant cell. I. effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. J. Cell Biol. 106: 1105-1115.

Munro, S. and Pelham, H. (1985) What turns on the heat shock gene? Nature 317: 477-478.

Murthy, A. K., Harris, J. R. and Belli, J. A. (1977) Hyperthermia and radiation response of plateau cells: potentiation and radiation damage repair. Radiat. Res. 70: 241-247.

Nelson, J. M., Braby, L. A., Metting, N. F. and Roesch, W. C. (1990) Multiple components of split-dose repair in plateau-phase mammalian cells: a new challenge for phenomenological modelers. Radiat. Res. 121: 154-160.

Ohtsuka, K., Tanabe, K., Nakamura, H. and Sato, C. (1986) Possible cytoskeletal association of 69,000- and 68,000-dalton heat shock proteins and structural relations among heat shock proteins in murine mastocytoma cells. Radiat. Res. 108: 34-42.

Overgaard, J., eds. (1984) Proceedings of the Fourth International Symposium on Hyperthermic Oncology, Vol. I. Taylor and Francis, London.

Overgaard, J. and Nielson, O. S. (1983) The importance of thermotolerance for clinical treatment with hyperthermia. Radiother. Oncol. 1: 167-178.

Overgaard, K. and Overgaard, J. (1972) Investigation on the possibility of a thermic tumor therapy II. Action of combined heat-roentgen treatment on a transplanted mouse mammary carcinoma. Eur. J. Cancer 8: 573-575.

Parker, C. S. and Topol, J. (1984a) A *Drosophila* RNA polymerase II transcription factor contains a promotor-region-specific DNA binding activity. Cell 36: 357-367.

Parker, C. S. and Topol, J. (1984b) A *Drosophila* polymerase II transcription factor binds to the regulatory site of an hsp 70 gene. Cell 37: 273-283.

Quastler, H. (1956) The nature of intestinal radiation death. Radiat. Res. 4: 303-320.

Raaphorst, G. P., Azzam, E. I. and Feeley, M. (1988) Potentially lethal radiation damage repair and its inhibition by hyperthermia in normal hamster cells, mouse cells, and transformed mouse cells. Radiat. Res. 113: 171-182.

Raaphorst, G. P., Freeman, M. L. and Dewey, W. C. (1979) Radiosensitivity and recovery from radiation damage in cultured CHO cells exposed to hyperthermia at 42.5 or 45.5°C. Radiat. Res. 79: 390-402.

Riabowol, K. T., Mizzen, L. A. and Welch, W. J. (1988) Heat shock is lethal to fibroblasts microinjected with antibodies against hsp 70. Science 242: 433-436.

Ritossa, F. (1962) A new puffing pattern induced by temperature shock and DNP in *Drosophila*. Experientia 18: 571-572.

Roti Roti, J. L. and Laszlo, A. (1988) The effects of hyperthermia on cellular macromolecules. In "Hyperthermia and Oncology" (M. Urano and E. Douple, eds.), Vol. 1: 13-56. VSP BV, The netherlands.

Rusu, M. and Baghdadi, M. (1978) Protection by dinitrophenol against radiation-induced hemolysis in hen erythrocytes. Radiat. Res. 76: 198-205.

Schlesinger, M. S., Ashburner, M. and Tissieres, A., Eds. (1982a) Heat Shock from Bacteria to Man, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schlesinger, M. S., Kelley, P. M., Aliporti, G. and Malfer, C. (1982b) Properties of three major chicken heat shock proteins and their antibodies. In "Heat Shock: From Bacteria To Man" (M. J. Schlesinger, M. Ashburner and A. Tissieres, Eds.,): 243-250. Cold Spring Harbor Lab, Cold Spring Harbor, New York.

Spitz, D. R., Dewey, W. C. and Li, G. C. (1987) Hydrogen peroxide or heat shock induces resistance to hydrogen peroxide in Chinese hamster fibroblast. J. Cell. Physiol. 131: 364-373.

Sutherland, R. M. and Pihl, A. (1968) Repair of radiation damage to erythrocyte membranes: the reduction of radiation-induced disulfide groups. Radiat. Res. 34: 300-314.

Sutherland, R. M., Stannard, J. N. and Weed, R. I. (1967) Involvement of sulphydryl groups in radiation damage to the human erythrocyte membrane. Int. J. Radiat. Biol. 12: 551-564.

Suzuki, S. and Akamatsu, Y. (1980) Increase of radiation damage to potassium-ion permeability in *E. coli* cells with decrease in membrane fluidity. Int. J. Radiat. Biol. 37: 475-482.

Tissieres, A., Mitchell, H. K. and Tracy, U. M. (1974) Protein synthesis in salivary glands of *Drosophila melanogaster:* Relation to chromosome puffs. J. Mol. Biol. 85: 380-398.

Tomasovic, S. P. and Koval, T. M. (1985) Relationship between cell survival and heat-stress protein synthesis in a *Drosophila* cell line. Int. J. Radiat. Biol. 48: 635-650.

Vandecasserie, C., Schnek, A. G. and Leonis, J. (1971) Oxygen-affinity studies of avian hemoglobin chicken and pigeon. Eur. J. Biochem. 24: 284-287.

Verma, S. P. and Rastogi, A. (1990) Role of proteins in protection against radiation-induced damage in membranes. Radiat. Res. 122: 130-136.

Vranska, Ts. G., Pantev, T. P., Ryzhof, N. I. and Fedorenko, B. S. (1985) Electrophoretic mobility of erythrocytes exposed to accelerated helium nuclei or to γ-rays, as modified by adeturone. Int. J. Radiat. Biol. 47: 285-290.

Warters, R. L. and Brizgys, L. M. (1987) Apurinic site induction in the DNA of cells heated at hyperthermic temperatures. J. Cell Physiol. 133: 144-150.

Warters, R. L., Hofer, K. G., Harris, C. R. and Smith, J. M. (1977) Radionuclide toxicity in cultured mammalian cells: Elucidation of the primary site of radiation damage. Curr. Top. Radiat. Res. Q. 12: 389-407.

Welch, W. J. and Suhan, J. P. (1986) Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell Biol. 103: 2035-2052.

Williams, A. F. (1972) DNA synthesis in purified populations of avian erythroid cells. J. Cell. Sci. 10: 27-46.

Witting, L. A. (1980) Vitamin E and lipid antioxidants in free-radical-initiated reactions. In "Free Radicals In Biology, Vol. IV" (W. A. Pryor, Eds.): 295-319. Academic Press, Inc., New York.

Yamanori, T. and Yura, T. (1980) Temperature-induced synthesis of specific proteins in *Escherichia coli*: Evidence for transcriptional control. J. Bacteriol. 134: 1133-1140.

Yang, T. C. H. and Ducoff, H. S. (1971) Recovery studies of X-irradiated *Tribolium castaneum* ("flour beetle") larvae. Radiat. Res. 46: 290-300.

Yatvin, M. B. (1977) The influence of membrane composition and procaine on hyperthermic death of cells. Int. J. Radiat. Biol. 32: 513-521.

Yatvin, M. B., Gipp, J. J., Werts, M. T. and Simone, G. (1984) Membrane aspects of radiation biology. In "Oxygen Radicals in Chemistry and Biology" (W. Bors, M. Saran and D. Tiat, Eds.): 563-579, de Gruyter, New York.

Zentgraf, H., Scheer, U. and Franke, W. W. (1975) Characterization and localization of the RNA synthesized in mature avian erythrocytes. Exp. Cell Res. 96: 81-95.

VITA

Susan Wei Shenn Lee was born April 17, 1964 in Taipei, Taiwan. She immigrated to United States of America in 1975 with her family. After graduating from West Torrance High School in Torrance, California, she attended University of Illinois at Urbana-Champaign, where she majored in biophysics. She was awarded the Bachelor of Science degree in August, 1985. She continued her graduate study in the Department of Physiology and Biophysics at the University of Illinois at Urbana-Champaign. In May, 1987 she received the degree of Master of Science. As a graduate student at University of Illinois, she was a Teaching Assistant for Introductory Human Physiology, Membrane Biophysics and Medical Physiology. In addition, she was a recipient of an NCI predoctoral traineeship in Radiation Oncology. Graduate research was conducted under the direction of Dr. Howard S. Ducoff on the effects of hyperthermia and ionizing radiation on terminally-differentiated tissues. After completing the requirements for the Ph.D., she will continue to work on the requirements for the M.D. degree which will take 2 more years to complete.

PUBLICATIONS

Erdos, G., Shinohara, K., Chen, H.-Q., Lee, S., Gillott, M. and Buetow, D. E. (1987) Chloroplast development and regulation of LHCP-GENE expression in greening cultured soybean cells. In "Progress in Photosynthesis Research, Vol. IV." (J. Biggins, eds.): 539-542. Martinus Nijhoff Publishers, Dordrecht.

Ducoff, H. S., Lee, S. W. and Kozma, T. G. (1989) Heat-induced hemolysis and thermotolerance: enucleate vs nucleated red cells. Proceedings of 5th International Symposium in Hyperthermic Oncology, Kyoto, Vol. II: 359-361.