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thesis entitled

THE EFFECTS OF A THIOL REDUCING AGENT
ON INDICATORS OF STORED RBC VIABILITY

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in Clinical Laboratory Sciences

Major professor

Date May 9, 1995

THE EFFECTS OF A THIOL REDUCING AGENT ON INDICATORS OF STORED RBC VIABILITY

by

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Clinical Laboratory Sciences
1995

ABSTRACT

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by

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Post-transfusion red blood cells (RBCs) are subject to the same selective removal process as RBCs *in vivo*. These processes involve deleterious effects known as storage lesions. One of these lesions is the presence of Heinz Bodies. Numerous lines of evidence show that storage lesions are caused by increases in oxidative events or decreases in thiol reducing equivalents. The effects of adding the reducing agent dithiothreitol (DTT) to Adsol stored RBCs, to reduce oxidative damage, were evaluated. The effects were followed by monitoring osmotic fragility and glucose consumption as well as the accumulation of lactate, methemoglobin and Heinz Body counts. The results indicate that the addition of 5 mM DTT can inhibit Heinz body formation but, at the cost of increasing cellular fragility.

ACKNOWLEDGMENTS

I would like to acknowledge Donna Duberg, MA, MS, MT(ASCP)SM for her scientific and moral support. I would also like to express extreme thanks to Carey Loveland, MT(ASCP) and Kathleen MacDonald, MT(ASCP) of Michigan State University's Clinical Center Laboratory as well as James Ivey, Laboratory Manager of St. Lawrence Hospital for their financial and technical support, which was vital in finalizing this project. I thank David Thorne, Phd for the opportunity to work on this project. Finally, I would like to thank the members of my guidance committee for their wisdom and support.

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INTRODUCTION

The elucidation of the circulatory system in 1628 allowed the successful transfusion of blood from person to person (1). The ability to type and store blood products provided safer access to this therapeutic procedure on demand (1). A factor that determines the effectiveness and availability of these products in a therapeutic regime is the quality of the product. Many variables have been defined as affecting the quality of these products, among these the content and composition of the preservative solution plays an important role (2). The current success of modern transfusion practices have been determined by improvements in the components of preservative solutions or the conditions under which they are used (2). This research will evaluate the addition of the thiol reducing agent, Dithiotreitol (DTT) to standard Adsol stored RBCs. It is believed that DTT can act as an additional RBC antioxidant and thus maintain stored RBC viability and as a result possible increase longevity.

In the last few decades situations have arisen where the availability of blood products have been low due in part to a limited shelf-life (3). This low availability therefore limits the benefits of transfusion therapy. Further, not all of the units designated for transfusion are transfused (4). Due to low demand, the remaining units are usually of the A, B or AB types which are antigen specific and are discarded because of the expiration of their shelf life (4). However, in this era of decreased allogenic blood donations, an enzymatic technique has been developed to transform these units to O cells, which can be transfused to anyone (4). Thus, the quality and longevity of these units are still important issues. Further, due to the fear of acquiring deadly diseases from an unsafe blood supply, many patients have turned to autologous units for transfusions (5). Autologous units are important in many patient management programs (8,9). Therefore, the quality and shelf-life of autologous units are now important issue as well (8,9).

In response to this need, attempts have been made to extend the shelf-life of stored blood (9). In the past, the demand for blood products due to decreased availability has been met by modifications in the current preservative solutions or storage conditions of the product. Successful attempts in this area have centered around modification of the storage medium to improve the biological or biochemical aspects of the product (10,11). These changes have presented with products which show improvements in cellular function, post-transfusion survival and extended shelf-life (10,11).

1. PRESERVATIVE SOLUTIONS

In 1828, the first successful transfusion was achieved (12). Researchers realizing the potential life saving benefits of this procedure, set about making it safer (13). In 1900, the hit and miss strategy of the original technique was refined with the discovery of the RBC blood group antigens (14). Anticoagulants solutions aided the transfusion process by allowing the storage of blood products for transfusion at a later date (15). Since the discovery of these solutions, a solution that would yield a better product under prolonged storage conditions has been a continuing goal.

In 1943, a solution of acid, citrate and dextrose (ACD) became the standard blood preservative solution (16). This solution provided dextrose as an energy substrate and the calcium chelating agent, sodium citrate, as the anticoagulant. The solution also contained citric acid to maintain a pH at 4^0C of 7.4. This pH was found to maintain RBC enzyme activities and thus RBC function. This solution allowed the preservation of RBCs for 21 days at 4^0C.

The addition of phosphates was the next major improvement in the composition of the preservative solution (17). This addition allowed better maintenance of the cellular adenosine 5'-triphosphate (ATP) content. The product, was now called citrate-phosphate-dextrose (CPD). This formulation yielded a more viable product but did little to improve the shelf-life of the product. The addition of a second energy source, adenine, further enhanced the ATP content and increased the shelf-life to 35 days (18,19). This preservative solution was called citrate-phosphate-dextrose-adenine (CPDA).

In 1978, Swedish scientists developed a storage technique that contained a secondary storage solution (11,20). This secondary storage solution was for use after the blood was drawn into a suitable primary anticoagulant solution, usually CPD. This procedure had a two-fold purpose. The first was to remove the buffy coat and thereby remove leukocytic enzymes which have been implicated as a possible cause of RBC hemolysis (11,20,21). The secondary solution, saline-adenine-glucose (SAG), was then used to store the leukocyte depleted RBCs in a saline diluent. This formulation was shown to improve the shelf-life to greater than 35 days (11,20). However, this formulation also resulted in an increased supernatant hemoglobin content and an increase in echinocytes, a concern in massive transfusion therapy. Normal cellular fragility and physiology were recovered after incubation in fresh frozen plasma and the RBCs were infused after a two-fold saline wash. The post transfusional viability was greater than 70% after 48 hours (11,20).

At the same time, in the United States (U.S.), a similar formulation was developed. This formulation was called Adsol (22). It contained roughly the same components as SAG (Appendix A), with extra glucose and added mannitol to control the hemolysis (11). This solution increased the shelf-life to 49 days, yielding post transfusional viability of greater than 75% after 24 hours (22). Adsol is, at present, the best liquid preservative solution used for cold storage in the U.S. (16,23,24).

Several attempts have been made to improve upon the Adsol solution (25,26). Researchers have attempted to use ion-exchange resins (27). It was found that the resins sterilized the product by removing bacteria and created a buffering effect which allowed increase energy utilization (27). However, this did not significantly improve the product quality or shelf-life (27). Other researchers have experimented with freezing and lyophilizing blood products (2,28,29). It was found that these techniques, although allowing for increased longevity with apparent viability, are tedious to perform and RBC recovery is low (2). This could be detrimental in an emergency situation.

The quality and longevity of stored RBCs has been defined by American Association of Blood Banks (AABB) standards (23) to be the maximum storage period in which at least 80% of the stored RBCs are recovered with a hematocrit of no greater than 80% and minimal supernatant hemoglobin (23), which allows at least 70% of the transfused RBC's to be present in circulation 24 hrs after transfusion (24). One unit of RBCs should only raise an adults hemoglobin 10 g/L and the hematocrit by 3% (24). These criteria were imposed due to a group of storage defects, which are collectively called, "storage lesions" (30).

Many aspects of the storage lesions, which define the quality and longevity of stored RBCs, have been linked to oxidation of intracellular constituents (30). As a result, several attempts have been made to improve the basic Adsol formulation by the addition of various oxidative and reductive compounds. Among these are malonate (26), oxalate (26), glutathione (32,33,34,35), and the antioxidant vitamins C (26,31,36,37) and E (36,37). Reduced glutathione (GSH) is not cell permeable and thus, has no effect on intracellular events (32). The compound oxalalate and the analog, malonate, had no effect and may be toxic (26). Vitamin C is water soluble but has no active mechanism to enter the cell (36,37). Therefore, vitamin C functions extracellularly with minimal passive intracellular activity (36,37). Vitamin E partitions into lipids and due to this hydrophobic nature, also has minimal intracellular activity (36,37). At this time, there are no studies investigating the addition of thiol reducing agents, to replace the ones lost during storage.

In attempting to understand the role of oxidation in RBCs, several oxidative agents have been tested. Among them are phenylhydrazine (38,39), dapsone (38), cumene hydroperoxide (40), damide (41), GSSG (32), ozone and peroxides (40,42). All have been found to produce some aspects of store lesions. They also cause disulfides and mixed disulfides which decrease the intracellular formation of GSH concentrations and increase RBC removal or destruction (38,39,40,41,42). The disulfide GSSG (oxidized glutathione) has been shown to inactivate enzymes by oxidizing their sulfhydryl (thiol) groups (32,33). It has been demonstrated that the addition of monothiol reducing agents to oxidatively stressed RBC and nonRBC systems reduces lysis due to oxidative damage (31,39).

2.THIOL\DISULFIDE INTERACTIONS

In vivo, several factors influence RBC survival (43). These factors limit the endogenous life span of the RBC to 120 days (30,43). On a cellular and biochemical basis, these factors include, morphological changes, modifications in membrane antigenicity, changes in membrane integrity and changes in the concentrations of RBC enzymes or modifications in their activities (44). These effects are regulated by metabolic processes which govern the production and utilization of energy substrates (26,43). The decreased production and utilization of energy substrates have been linked to changes in thiol containing, RBC membrane, proteins (45,46,47). The effects of which lead to modifications in RBC membrane structure and function which may preclude RBC senescence (48,49). These same effectors may play a large part in determining the viability of stored RBCs *in vitro* (50,51).

Thiols are sulphur containing moieties which are contained in most protein structures (52,53). They are usually in the form of cysteine or a modified form thereof (52,53). These compounds are referred to as thiols when in their reduced state and as disulfides when they have been oxidized. In aerobic systems, the oxidation of two thiols yields one disulfide and the reduction of one disulfide yields two thiols (52,53). Thiol/disulfide status is a major factor regulating protein structure and thus function (52,53). Changes in the oxidation or reduction of the thiol groups of some proteins are believed to regulate their function (52,53). In some proteins, the changes are permanent, in others, reversible. For example, the proteins, lysozyme and ribonuclease A, are activated only in their disulfide state. They are inactivated and lost when converted to their dithiol moieties. In contrast, the enzymes glucose-6-phosphate dehydrogenase and phosphofructokinase, can be switched between their active reduced thiol states and their inactive disulfide states. A major determinant of protein thiol/disulfide status is the availability of electron donors or acceptors with the appropriate redox potentials (52,53).

2.1 Thiol\Disufide Effects on RBC Structure

Research has shown a correlation between oxidative damage and cellular aging (45,46,48,54,55). It has been demonstrated that the levels of oxidatively modified constituents in circulating RBCs increases with age (44,45). This change is linked to a loss of protein structure and thereby function of RBC constituents (44,45,46,48,54). It has been reported that the concentrations of RBC enzymes remain constant during the life span of the RBCs, but their activity declines (45). In many instances this loss of activity has been associated with irreversible structural oxidative damage of the proteins (44,45,56).

The oxidation of the sulfhydryl groups within the structures of many proteins inhibits their normal cellular functions (43,34). Sulfhydryl modulation also destabilizes some protein-protein interactions (43,34,57). This finding is notable in the role of the hemoglobin molecule, which appears to have a stabilizing effect on the membrane protein spectrin's dimer-dimer associations (57,58). Further, it was shown that hemoglobin degradation products can have a destabilizing effect on spectrin dimers (57,58). Spectrin is the major constituent of the red cell's skeletal structure (57), therefore, destabilization of this protein can lead to decreased structural integrity and lysis (57-59).

2.2 RBC Thiol/Disulfides

The main non-protein thiol/disulfide compound in RBCs is glutathione (GSH, GSSG)(60). GSH is maintained in high levels in the red cell. This high concentration is maintained by a rapid rate of turnover that is responsive to oxidative stress (34). This turnover is governed by two primary factors. They are the rate of GSH de novo synthesis and the rate of GSH regeneration due to the reduction of oxidized glutathione (GSSG)(34,61).

The synthesis of GSH is a two step process. First, L-glutamate and L-cysteine are combined by L-glutamylcysteine synthetase to form L-glutamylcysteine. L-glutamylcysteine and glycine are then combined to form GSH (34,60). Both of these enzymatic reactions utilizes ATP (34).

The reduction of the disulfide GSSG, may be accomplished by two pathways. The majority of GSSG is reduced by the action of glutathione reductase and the NADPH generated from the HMP shunt (34,35). Methemoglobin reductase, which uses the NADH generated from the glycolytic pathway, can also catalyze the same reaction, although, less efficiently (34,43). Both the reducing equivalents of NADPH and NADH as well as the levels of GSH in the RBC are dependent on the amount of glucose consumed by the glycolytic pathway (34). Also noteworthy is that GSH can not leave the intracellular space, however, at high concentrations, GSSG can (34,35). The release of GSSG appears energy dependent (34). This may be an important factor because it limits the concentration of GSSG available for the regeneration of GSH inside the RBCs.

In one study, methemoglobin was chemically induced causing a decrease in the concentration of normal hemoglobin (35). The glycolytic pathway was blocked to avoid spontaneous reversion mediated by methemoglobin reductase (35). As the concentration of GSH declined, the concentration of normal hemoglobin increased (35). This indicates that GSH is capable of reducing methemoglobin without the aid of methemoglobin reductase and without glycolysis, GSH is not replenished. In addition, within fresh cells where normal hemoglobin was predominant, GSH concentrations were lower than those of older cells which are prone to oxidative damage (34). This is evidence that GSH concentrations are inducible under oxidative challenge. Further evidence that GSH is a powerful antioxidant was shown when the use of exogenous GSH proved effective in reducing or preventing injury to cellular systems under extracellular oxidative stress (35). These experiments show: 1) GSH is capable of acting alone to directly reduce methemoglobin; 2) concentrations of GSH are inducible under oxidative stress; and 3) GSH is a potent antioxidant in cellular systems.

3. RBC ENERGY METABOLISM

3.1 Adenosine 5'-triphosphate (ATP)

In RBCs, as in all cell types, ATP is the primary source of free energy (43,62). Experimental depletion of ATP in RBC's, most notably, results in loss of normal morphology and other aspects relating to structural integrity (43,62). These defects are due to the diminution of a wide variety of critical ATP dependent enzymes (43). In general, no distinguishable cellular changes have been noted with cellular ATP depletion of 50% or less (43). Decreases in ATP levels and posttransfusional survival of RBC's have been described (30,50). However, the exact mechanism for the removal of these ATP depleted cells has not been completely elucidated (50). It appears that multiple signals or triggers may exist secondary to ATP depletion, such as changes in membrane proteins (56), or changes in membrane integrity (46) leading to the possible production of senescent antigens (58).

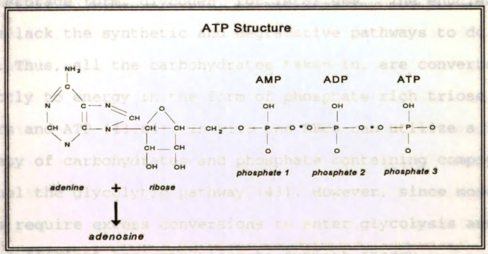

Figure 1. Chemical makeup of adenosine 5'- triphosphate

3.2 RBC ATP Synthesis

ATP is composed of adenosine and three high energy phosphate bonds. The compound adenosine is composed of adenine and ribose (Figure 1). In nucleated cells, the production of ATP can be achieved in several ways. However, since the mature red blood cell lacks both a nucleus and mitochondria (43,63,64), it can not access the pathways of gluconeogenesis and fatty acid utilization for energy. Thus, the RBCs must rely on existing carbohydrates as their primary energy source (43,63). This means that the RBC is dependent on the anaerobic Embden-Meyerhof glycolytic pathway, the Hexose-monophosphate shunt and the purine salvage pathways for the maintenance of ATP. These pathways convert carbohydrates, especially glucose and its derivatives, to ATP.

In nucleated cells, excess carbohydrates are converted to a storage form, glycogen, for later use. The enucleated RBC's lack the synthetic and degradative pathways to do this. Thus, all the carbohydrates taken in, are converted directly to energy in the form of phosphate rich triose sugars and ATP (43,64). *In vivo* the RBCs can utilize a variety of carbohydrates and phosphate containing compounds to fuel the glycolytic pathway (43). However, since most of these require excess conversions to enter glycolysis and are in insufficient concentrations to support energy requirements, quantitatively, glucose is the primary source of RBC energy (62,63). For this reason glucose is added to the preservative media of stored RBC's at an optimal concentration (60-80 mM) to ensure the proper maintenance of ATP concentrations (65).

Glucose utilization via the glycolytic pathway involves multiple enzymatic steps. Significant among these is the action of the enzymes hexose kinase and phosphofructokinase (43). Hexose kinase converts glucose to a form useable for glycolysis and the HMP shunt (43,66). Phosphofructokinase is the rate limiting enzyme of glycolysis. The primary regulator of glycolysis appears to be a negative feedback of the ATP concentration (43,66).

3.3 Hexose Monophosphate Pathway (HMP shunt)

The HMP shunt is an alternate branch of the glycolytic pathway which has several functions in nucleated cells (43). However, the role of this pathway in enucleated RBCs is primarily to generate reducing equivalents of NADPH (34,47,63). NADPH is an essential cofactor for rapid enzymatic reduction of disulfide form of glutathione (GSSG) (34,47,63). Glutathione has been linked to cellular oxidative protection. Further, data shows that the amount of glucose drawn from the glycolytic pathway and shuttled though the HMP shunt is regulated by the concentration of NADP+ present for reduction (34). Therefore, the amount of glucose drawn into the HMP shunt is directly related to the rate of reduction of GSSG, which is ultimately related to the oxidation rate of GSH (34,35,43).

The conversion of 6-phosphogluconate (6PG) to the pivotal compound ribulose-5-phosphate (RU5P) is another important step in this pathway and also requires the cofactor NADP+ (34). RU5P provides a pathway for purine salvage compounds generated from adenosine to reenter the HMP shunt and ultimately glycolysis (34,47,63). Thus, the HMP shunt is of great importance to the red cells because it maintains adequate concentrations of reducing equivalents and supplements energy production.

The importance of the HMP shunt has been further demonstrated by studies of glucose-6-phosphate dehydrogenase (G6PD), one of the most studied and best known enzymes in the RBC (34,47,63). This enzyme regulates the first committed step of the HMP shunt. RBCs with low G6PD activity or no G6PD activity at all, exhibit decreased concentrations of GSH. Under oxidative stress, these RBCs express abnormal morphologies and increased numbers of Heinz bodies (38,54). RBCs with this trait are prone to lysis and present with a shorter average life-span than do normal RBCs (38,54).

The rate of glucose utilization by the HMP shunt and the level of G6PD activity are directly linked to the concentration of NADP+ present and the reduction rate of GSSH. The NADP+ concentration yields an equimolar concentration of NADPH. NADPH functions to maintain an adequate concentration of GSH by reducing an equimolar concentration of GSSG. GSH protects the cell from oxidative damage which allows the cells to maintain normal morphology and cellular functions.

3.4 Purine ATP Production

Adenine is linked to ATP production through several biochemical pathways (67). It can be incorporated into adenosine 5'-monophosphate (AMP) via the purine salvage pathway. In this pathway, the compound phospho-ribosylpyrophosphate (PRPP) combines with adenine to generate AMP (63). AMP can then be salvaged by combining AMP and ATP to yield two molecules of ADP. ADP can be easily converted to ATP in the presence of free phosphate and the enzyme adenylate-phosphokinase (63,67).

In a related biochemical pathway, AMP can be deaminated to adenosine and further converted to inosine then ribose-1-phosphate, a key intermediate (63). This compound may follow several biochemical pathways (63). In particular, the conversion to ribose-5-phosphate allows entrance into the HMP shunt. Further metabolism of this compound allows it to enter directly into glycolysis as a triose phosphate and ultimately produce 1 molecule of ATP (63,67).

The final product of adenine utilization *in vitro* is hypoxanthine (67). *In vivo*, hypoxanthine can be further oxidized by xanthine oxidase to form uric acid. The red cell lacks xanthine oxidase, which is found only in the liver and small intestinal mucosa (67). The end result is that there is no mechanism for the removal of hypoxanthine in the red cell. Therefore, as adenine is utilized during *in vitro* storage, hypoxanthine accumulates. The rate of hypoxanthine accumulation should therefore be equal to the rate of adenine depletion (43,47,67). The build up of hypoxanthine may play an important role in the enzymatic recycling of the compound PRPP (43,47,67). Hypoxanthine plus PRPP is converted to inosinic acid (IMP)(63,67). IMP can then be converted inosine (63,67). Inosine regenerates hypoxanthine and yields ribose-1-phosphate (63,67). The compound ribose-1-phosphate can be converted to ribose-5-phosphate by the enzyme phosphoribosylmutase (63,67). Ribose-5-phosphate from this pathway, and to a smaller extent, from the hexose monophosphate shunt, can couple with ATP to regenerate PRPP (Figure 2)(63,67). As stated above PRPP can go on to generate more ATP. This reaction is regulated by feedback inhibition of IMP, GMP, and AMP and is catalyzed by the enzyme phospho-ribosyl-pyrophosphokinase (PPK)(63,67).

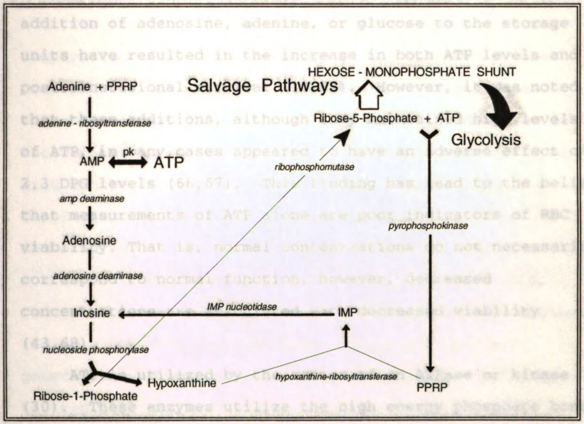

Figure 2. Pathways of purine salvage

3.5 RBC Utilization of ATP

Numerous reports have correlated the ATP concentrations of stored RBCs to posttransfusional survival (50). Evidence for the relationship of ATP and red cell viability was found when the red cells of individuals, who expressed abnormally high levels of red cell ATP, where found to have high storage capacity (10). Further studies involving the addition of adenosine, adenine, or glucose to the storage units have resulted in the increase in both ATP levels and posttransfusional survival (10,67). However, it was noted that these additions, although they maintained high levels of ATP, in many cases appeared to have an adverse effect on 2,3 DPG levels (66,67). This finding has lead to the belief that measurements of ATP alone are poor indicators of RBC viability. That is, normal concentrations do not necessarily correspond to normal function, however, decreased concentrations are associated with decreased viability (43,68).

ATP is utilized by the action of an ATPase or kinase (30). These enzymes utilize the high energy phosphate bonds to drive a variety of ATP dependent reactions (43). One of the most important ATP-dependent reactions is the regulation of the intracellular Na^{+2} and K^{+2} (30). Within RBCs, Na^{+2} is maintained in low concentrations and K^{+2} in high concentrations by the actions of a Na^{+2}/K^{+2} ATPase pump (30). Since this ATPase pump is linked to ATP utilization, it is also dependent on glycolysis (28,43). It has been observed that decreased regulation of these electrolytes (Na^{+2}, K^{+2}) due to decreased ATP concentrations is associated with increased cellular fragility (43). It has been noted that decreased regulation can also occur due to oxidation of the thiol groups contained in the proteins of this pump (52,53).

4. RBC ANTIOXIDANT ABILITIES

The function of the red cell is to mediate tissue respiration (68). This function is accomplished by the transport of oxygen to and the removal of carbon dioxide from the tissues (69). The role of oxygen transporter is facilitated by the red cell protein, hemoglobin (69). At high concentrations oxygen (O_2) binds to hemoglobin displacing (CO_2) and has a negative allosteric effect on 2,3 DPG binding (66,69). During respiration and some disease states, O_2 dissociates from oxyhemoglobin a super oxide radical $O_2^{\cdot}$ which generates peroxides and dangerous hydroxal radicals ($OH^{\cdot}$)(70,71). To counter this free radical generation and any potential oxidative damage due to them, the red cell employs several reducing agents (34,61,71). These agents include, NADH, NADPH, superoxide dismutase and reduced glutathione (GSH) (35,38,43,61). All of these are linked to the HMP shunt (35,38,43,61). These reducing agents are known as antioxidants (43,61). This is important in the red cell because evidence suggest that the oxidative product of hemoglobin, methemoglobin, is a possible precursor to membrane damaging precipitates (30,34,63). Antioxidants function by directly donating reducing equivalents (electrons) in the form of hydrogen or indirectly by the use of enzymes, to reactive oxygen species (superoxides), converting them to peroxides and water (34,35,38,43). Superoxide dismutase is an enzyme which catalyzes the consumption of superoxides in the presence of a hydrogen donor (NADPH or NADH), and free metals, to form peroxide, hydroxides and free oxygen (34,61) (Figure 3). The hydroxides generated by this reaction can then be converted to water by a second equivalent of hydrogen (34,35,38,43).

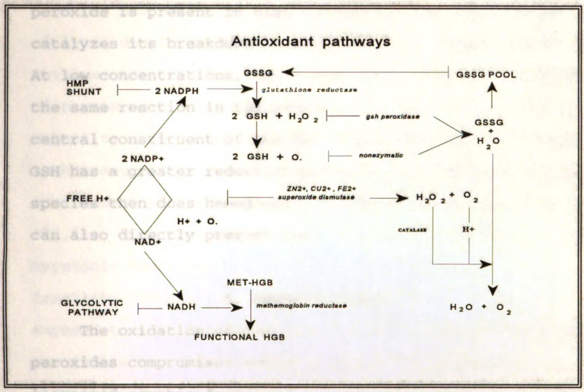

Figure 3. Antioxidant Pathways of the RBC.

The peroxides generated from both mechanism can be converted to water and free oxygen in one of two ways. First, the red cell is a rich source of the enzyme catalase (47). When peroxide is present in high concentrations, this enzyme catalyzes its breakdown to water and free oxygen (34,47,61). At low concentrations, the enzyme GSH-peroxidase catalyzes the same reaction in the presence of GSH (34,61). GSH is a central constituent of the RBC antioxidant system (34,61). GSH has a greater reduction potential for reactive oxygen species then does hemoglobin or membrane proteins, thus it can also directly prevent their oxidation. (34,61).

5. OSMOTIC FRAGILITY

The oxidation of membrane lipids by free radicals and peroxides compromises membrane integrity leading to increased osmotic fragility and potassium leakage (34,61). The loss of K^+ is important to the RBC, because the RBC intracellular volume is controlled by sodium and potassium regulation (34). When the RBC loses this ability, there is an efflux of intracellular potassium and an influx of extracellular sodium. This causes the RBC to swell. However, when the potassium efflux is less than the sodium influx, the RBC shrinks (34,61). In both cases the cell eventually expires. With RBC enlarlargement, lysis occurs when the elasticity of the cell membrane reaches a critical point. These are commonly observed as macrocytic cells. In situations where the RBC shrinks, the leaky RBCs lose membrane fragments and present as spherocytes. Spherocyctes, due to their reduced surface to volume ratio, lyse easily when hypotonically challenged (34,61).

The evaluation of the membrane can be made by analysis of lysis due to hypotonic shock (72,73). This analysis involves the testing of a population of RBCs in a gradient of physiologic saline, typically ranging from 0.0 (distilled water) to 0.9% NaCL (isotonic saline). RBCs that have lost membrane elasticity are more fragile in a hypotonic environment and are said to posses increased fragility (72,73). RBCs that can expand, are less fragile in a hypotonic environment, and are said to posses decreased fragility. Therefore, following a population of RBCs, exposed to a series of increasing osmotic fluids, over time, can indirectly indicate the relative changes in membrane elasticity due to oxidative damage to lipids and other membrane constituents.

6. RBC HEMOGLOBIN

6.1 Hemoglobin and Methemoglobin

The structure of hemoglobin is a complex one involving two pairs of globin chains, one pair is alpha globin and the other pair is either gamma or beta globin (74). The structure of the globin chain is determined by its amino acid sequence and, as with most globins, contains at least one sulfhydryl group (34,57,74). The sulfhydryl groups are necessary to maintain the molecule's structure and its function as an O_2 and CO_2 transporter (34,57). Each of these sequences enfolds a complex heme group which contains a molecule of iron (Fe^{+2}) at its center (71,74). This molecule of iron binds loosely to one molecule of oxygen. The binding of oxygen to the heme group changes the configuration of the globin chain such that the hemoglobin molecule itself is changed. The resulting hemoglobin molecule, with an oxygen molecule bound to all four heme groups is termed oxyhemoglobin and appears redder than deoxyhemoglobin (74).

During normal respiration, a small percentage of oxygen releases from the ferrous iron of hemoglobin as free radicals. This results in the iron being oxidized to a ferric state (70,74). This leaves the hemoglobin molecule as a nonfunctional protein, called methemoglobin. The conversion back to the ferrous state and a functional protein is normally mediated by the activity of methemoglobin reductase (43,63,74). Methemoglobin reductase enzymatically reduces methemoglobin back to oxyhemoglobin (34,43,47,76). It does this by combining the reducing equivalents of NADH with the oxidized iron, thereby reducing the metal back to its ferrous state (Figure 4)(71,74). The reducing equivalents in this pathway are generated from the glycolytic pathway (34,38,74). The equivalents can also come from GSH, which can act directly to reduce methemoglobin or as a cofactor for methemoglobin reductase (34,35,61). The reducing equivalents for these reactions are generated from the HMP shunt. Thus, the concentration of methemoglobin is dependent on NADH, NADPH and GSH concentrations, and ultimately, on the amount of glucose utilization.

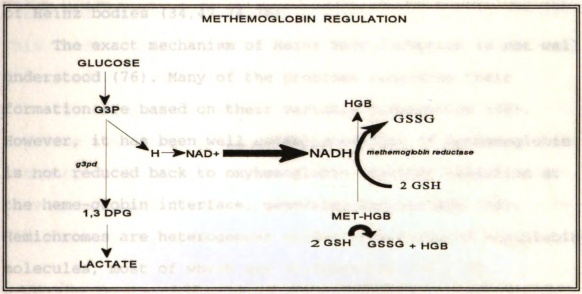

Figure 4. The generation of methemoglobin reducing equivalents.

6.2 Heinz Bodies

Methemoglobin has been strongly implicated as a precursor for Heinz body formation (38,45). In the absence of the reducing equivalents NADH, NADPH and GSH, as seen in low glucose or enzyme deficient situations, methemoglobin concentrations increase (38,45). Methemoglobin can then be further oxidized to intermediate forms called hemichromes (28,59,60). Data suggest that it is here that the reducing compound, GSH, is vital as an antioxidant (71,73,75). Convincing evidence concerning the relationship between GSH and hemoglobin oxidation is described in G6PD and GSH reductase deficiencies, both of which display low GSH concentrations. It has been observed that under these conditions, the RBCs are prone to exhibit abnormally high concentrations of methemoglobin as well as increased numbers of Heinz bodies (34,47,74,76).

The exact mechanism of Heinz Body formation is not well understood (76). Many of the problems regarding their formation are based on their variable composition (38). However, it has been well established that if methemoglobin is not reduced back to oxyhemoglobin, further oxidation at the heme-globin interface, generates hemichromes (38). Hemichromes are heterogenous oxidatively modified hemoglobin molecules, most of which are irreversible (38). If hemichromes are subjected to further oxidation, Heinz Bodies are formed (38,63).

Heinz Body formation appears to primarily involve oxidation of the globin proteins of the hemoglobin molecule (30,63,71). This oxidation occurs in susceptible amino acid side chains (77). These side chains possess four sulfhydryl groups which are oxidized in the process of Heinz Body formation (77). At this point, oxidation causes the three dimensional structure of the molecule to become unstable (77). Subsequent structural changes cause the proteins to precipitate and aggregate, forming Heinz Bodies (77).

Newly formed Heinz Bodies exist in the cystol of RBCs (51,78,79). However, microscopic and biochemical evidence indicates that the majority are membrane associated

(51,78,79). Further, this attachment requires another oxidative event. This oxidative event provides a point of attachment by oxidizing the membrane cytoskeletal protein sulfhydryls (78,79). Membrane bound Heinz Bodies cause localized decreases in the RBC membrane flexibility (78,79). This leads to sequestration and lysis in capillary beds and the spleen (78,79).

The antioxidant, GSH counters this formation by reducing some forms of hemichromes back to methemoglobin and methemoglobins back to oxyhemoglobin (34,61). GSH may also prevent membrane attachment by reducing oxidative damage to the membrane cytoskeleton (34,61).

7. RBC RHEOLOGY

7.1 Mechanical Removal

The spleen is a regulatory organ that maintains a healthy red cell population by removing cells that have a change in shape, contain inclusions, lack deformability or possess some other factor associated with RBC aging. As the flow of blood passes through the spleen, red cells are passed through narrow slit pores (56,63). These pores are created by rows of endothelial cells in the sinus vessels of the spleen (56). Cells with fluid membranes flow though easily, those with inclusions, rigid membranes or antigenic triggers can not pass through and are sequestered. Most of the *in vivo* lysis is believed to occur as the spleen attempts to remove the inflexible portions of the membrane through a process termed pitting (56,63). Pitting removes a portion of the membrane containing rigid defects and reduces the cells surface area in the process (30,43). The reduction of cell surface area leads to the formation of irregular cell shapes such as echinocytes and spherocytes which further affect the flexibility of the cells membrane (30,43,46). Changes in membrane flexibility are known as rheologic properties. These properties cause the RBCs to be sequestered in the capillary beds of the spleen and\or removed by luekocytic phagocytosis (30,43,46). This process is also operative in pathological conditions such as sickle cell disease (30,43,46).

7.2 Immunological Removal

Other factors appear to be involved in marking the RBC for removal. It has been found that the concentration of Heinz bodies in circulating RBCs are increased after splenectomy while the RBC turnover is not altered (54). This supports the existence of another mechanism of RBC removal (54,58). Since the formation of Heinz bodies involves the membrane, the process of their formation may also lead to changes in the antigenicity of the RBCs (58). This antigenic change appears to be due to the peroxidation of lipids, a reduction in intracellular enzyme concentrations and oxidative damage to membrane bound receptors and proteins (44,58,68). These changes are due, in part, to the oxidation of sulfhydryl groups within membrane proteins and enzymes. (34,54,56). These changes cause the RBCs to be recognized as nonself or foreign and stimulate the production of naturally occurring autoantibodies which mediate complement lysis and luekocytic phagocytosis (34).

Further evidence has shown that the sialic acid content of old RBCs is 10-15% less than that of younger cells (34,56). Sialic acid is the major building block of RBC membrane proteins, a primary determinant of cellular antigenicity. It has also been shown that sialidase treated cells are recognized by the spleen as well as the liver (34,56). Further data shows that desialyated red cells were removed from the circulation of rabbits within a day, while ATP depleted cells took up to 3 days for complete removal (34,56). This suggests that ATP may not be directly related to cellular removal. However, declining concentrations of ATP initiates a cascade of events which results in a series of changes including changes in antigenicity (34,56,58). Thus, the existence of a pathway, starting with ATP depletion and ending with a change in red cell antigenicity, triggering cell removal, appears possible.

Recent studies have identified a 62,000 MW glycoprotein, on the surface of old and oxidatively damaged RBCs which has been termed the senescent cell antigen (56,59). It has been concluded that, since the RBC lacks a nucleus, it can not synthesize proteins, therefore this antigen must be a modified form of one that already exist

(58). Further, an IgG autoantibody has been identified which is specific for this modified antigen and initiates phagocytosis (58). Therefore, it appears that the removal of senescent RBCs can be accomplished by the spleen and\or by the immune system to maintain the RBC turnover rate of 120 days (54,58).

7.3 Oxidation and Reduction

The role of oxidation in living cells has been well documented. Oxidation serves to transform dietary materials to cellular constituents, some of which are used for energy (43). Oxidation also appears to play a significant role in host cellular protection against intracellular parasites and viruses (42,80,81). Studies also show that oxidation is a factor in various pathological conditions as well as the normal aging process (39,42). The role of oxidation is notable in RBCs, which contain several oxidatively sensitive systems which are constantly exposed to reactive oxygen species. Therefore, RBCs have developed systems to reduce, remove or trap damaging oxygen species (34,61). When the capacity of this antioxidant system is overloaded, the RBC is compromised and lysis occurs (42,71).

8. SUMMARY

It appears that multiple factors determine the viability of post-transfusion RBCs (38,56,61,74,81). In particular, factors associated with oxidative alterations of protein structure and function (56,59). Previous work has identified the benefits of maintaining reducing equivalents in individual systems (35). Important to this theme is the overall thiol\disulfide status (52). This appear to be a major factor controlling cellular functions. Within RBCs, endogenous GSH is the most effective thiol reducing substance (60,61). However, as described previously, GSH is not membrane permeable (35). Therefore, adding exogenous GSH to stored RBCs would be ineffective in countering intracellular oxidation (34,35). However, if a nontoxic, cell permeable, thiol specific reducing agent can be identified and added to the stored cells, intracellular antioxidant ability of the RBCs may be maintained.

A promising compound is the compound 2,3-dihydroxy-1,4-dithiolbutane. This compound exists in two isoforms, dithiothreitol (DTT) and dithioerythritol (DTE), collectively called Cleland's reagents (82). Cleland's reagents are similar in function and reduction potential to GSH (82,83). As a result they are relatively specific for the reduction of disulfides and other related oxidative species. Cleland's reagents form stable irreversible cyclic disulfide structures when oxidized (52,82,83). Due to their amphoteric nature, these compounds are membrane permeable and water soluble (82). Research with this compound has demonstrated that oxidized RBCs incubated in DTT show loss of auto-IgG binding (84). In contrast, research with the oxidant compound, damide, demonstrated increased auto-IgG recognition and binding (41,84).

Preliminary research in this lab has shown favorable results with DTT. In experiments in glass bottles, this compound expressed a 3.0 fold decrease in Heinz body positive RBCs over controls (Thorne, D., et al, unpublished). The effectiveness of similar compounds to reduce oxidative stress has been well established (10,26,31,55). Most notable is the effects of ascorbic acid (vitamin C) and α-tocopherol (vitamin E). Ascorbic acid is an effective extracellular antioxidant. However, it is limited due to the fact that it has no mechanism for cellular entry (36,37). Thus, it has no effect on intracellular oxidative events. Likewise, α-tocopherol, is limited in intracellular activity due to an excessive hydrophobic nature (36,37). However, it too, is an excellent extracellular antioxidant which targets the cell membrane (36,37). Although it may be usefull in preventing Heinz Body-membrane associations, it is limited in the deterrence of Heinz Body formation (36,37). Thus, the effects of DTT on reducing intracellular oxidation in stored RBCs appears better than those seen with ascorbic acid, further research in this area is warranted.

The goal of this research was to evaluate if there is any improvement to the Adsol storage solution by adding DTT, a thiol specific protective agent. Knowing the relationship between RBC energy metabolism and Heinz body formation, further studies were conducted to determine if DTT decreases

Heinz body formation by increasing the rate of glucose utilization with subsequent production of ATP and reducing equivalents. To accomplish this, the rate of glucose depletion was monitored. Since the final product of glucose utilization is lactate, lactate determinations was used to estimate the total rate of energy production due to glycolysis. The assumption that methemoglobin is the oxidative substrate for Heinz Body formation was evaluated by monitoring the methemoglobin content and performing Heinz Body Counts. Further, the integrity of the RBC structure was analyzed by the standard osmotic fragility test.

MATERIALS AND METHODS

1. EXPERIMENTAL DESIGN

The sample size (N) for this experiment was determined by an independent source to be a minimum of 5 (N = 5) (Appendix D). An N of 6 was chosen. Blood was drawn from randomly chosen individuals to acquire a pool of three males and three females. Specimens were then alphabetized and coded 1 through 6, respectively. Following a matched paired design, each specimen was then split into two parts, one for control and the other for a treatment group. To each control tube, 15.4 ml of adsol was added. To each treatment tube, 15.4 ml of Adsol containg 2.2 g of DTT was added. The specimens were coded 1 through 12, such that all controls were odd numbered and all treatment samples were even numbered, for example, #1= control of #2= treatment. Each sample was aliquoted into individual specimens of 3 ml each and placed in 150 ml or 300 ml standard transfer bags (Fenwal, a division of Baxter) and labled appropriatly. The complete experiment contained 120 bags, 20 bags per specimen (control = 10 and treatment = 10). This allowed individual samples of the specimens to be available for weekly analysis over a 10 week period. All specimens were stored at 4^0C (23,24).

Monday through Friday, the samples were visually inspected for gross contamination and each bag was rotated 10 to 15 times to mix the contents. All procedures were performed aseptically and specimen sterility was validated by gram stain and on weeks 5 and 10, all 12 samples were submitted to an independent laboratory for culturing (Appendix C). On a weekly basis specimens were removed from storage and the following assays were performed:

1. Heinz body count
2. Methemoglobin
3. Glucose
4. Lactate
5. Osmotic fragility
6. Gram stain

2. HEINZ BODY COUNTS

Heinz body counts were conducted by a method similar to one previously described by Beutler (86). To 3 drops of well mixed specimen, 2 drops of 2% w\v crystal violet (certified, Sigma, St. Louis, MO.) was added and mixed by inversion. This suspension was allowed to stand at room temperature for 7-9 min. A smear was made from the stained sample and allowed to air dry. The prepared smears were observed under oil immersion. Heinz Bodies appeared as dark intracellular membrane associated particulates (86). A total of 1000 RBCs were counted per smear. The percent Heinz Body count was calculated using the following formula.

Calculation:

$$\frac{\text{\# of Heinz body positive cells}}{\text{\# of cells counted}} \text{ X } 100 = \text{\% Heinz bodies}$$

3. METHEMOGLOBIN

Methemoglobin content was determined by differential spectrophotometry. This procedure was described by Tietz (87) and is based on the work of van Kampen (88). This procedure is based on the fact that methemoglobin gives a spectral peak at 630 nm which disappears as methemoglobin is converted to cyanmethemoglobin in the presence of cyanide. The resulting decrease in absorbance at 630 nm is proportional to the methemoglobin content. The content of methemoglobin, expressed as a percentage of the total hemoglobin fraction, yields the percent methemoglobin content.

PROCEDURE:

To 3.9 ml of distilled water add 0.1 ml of well mixed adsol stored RBCs and vortex to mix. To this mixture add 4.0 ml of 0.15 M potassium phosphate buffer, pH 6.6 and vortex to mix. Place 3.0 ml of this mixture in a fresh tube and label it C2. To a second tube add another 3.0 ml and label C3. To a third tube add 1.5 ml of distilled water and 1.5 ml 0.15 M phosphate buffer, pH 6.6 and label this tube blank or C1. Set a spectrophotometer (Gilford Stasar III) to 630 nm and zero with the C1 blank. Measure the absorbance of C2 at 630 nm and call this 2a. Add 100 uls of potassium ferricyanide solution (2.0 g\10 ml dh_2o) to cuvet C3. Mix by inversion and let stand 2 minutes. Measure the absorbance of C3 at 630 nm and call this 3a. Add 100 uls potassium cyanide solution (0.5 g\10 ml dh_2o)to all tubes. Mix by inversion and let sit 5 minutes. Set spectrophotometer to 630 nm and zero with C1. Read absorbance of C2 and call this 2b. Read absorbance of C3 and call this 3b. Calculate percent methemoglobin from the following formula and plot verses time.

Calculation:

$$\frac{2a - 2b}{3a - 3b} \times 100 = \%\ \text{Methemoglobin}$$

4. GLUCOSE

This method employed a coupled hexose kinase method as described by Bonder and Mead (89). In this procedure glucose is converted to glucose-6-phosphate (G6P) by the enzyme, hexose kinase (HK). The glucose-6-phosphate produced is then converted to 6-phospogluconate by the action of glucose-6-phosphate dehydrogenase (G6PDH). This reaction reduces the cofactor NAD^+ to NADH. The increase in NADH is directly proportional to the glucose concentration. The NADH concentration is monitored spectrophotometrically at a wavelength of 340 nm. This method does not differentiate between glucose and glucose-6-phosphate.

glucose + ATP |------- HK --------> G-6-P + ADP

G-6-P + NAD |----- G-6-PDH -----> 6-PG + NADH

With this method, turbid, icteric and extremely hemolyzed samples may yield falsely elevated results. To correct for this, a sample blank was used as recommended. The blank was prepared the same way the sample was, except isotonic saline or deionized water was used in place of the reagent. The absorbance of the blank was subtracted from the absorbance of the sample before final calculations were made.

PROCEDURE:

Place 0.2 ml of well mixed Adsol RBCs in to an eppendorf tube and spin at 15,600 x g for 5 minutes. Remove supernatant immediately after centrifugation and place in a fresh eppendorf tube. Add an equal volume of distilled water and vortex to mix. Place samples in refrigerator until needed. From the hexose kinase kit (Sigma, 16-UV, St. Louis, MO.), prepare the glucose (HK) reagent by removing 1 bottle and adding 50 ml distilled water. Swirl to mix and let stand 5-10 minutes. Using 10x15 tubes, lable 2 each for blank, standard and samples. To each tube add 1.5 ml of glucose (HK) reagent. To the blank, pipet 0.01 ml of distilled water to each tube. To the control tubes, pipet 0.01 ml of 300 mg/dl standard (Sigma, 16-11). To the sample tubes, pipet 0.01 ml of the appropriate sample to each tube. To control for excessive hemolysis a sample blank was added for each sample. This was made by adding 1.5 ml distilled water to a fresh tube and addind 0.01 ml of the sample. Mix each tube by slow vortex or inversion. Place rack of tubes in a 37°C water bath for 10 minutes. Set Gilford spectrophotometer to 340 nm and zero with distilled water. Read and record the absorbance of each tube. Calculate the glucose content of each sample using the following procedure.

Calculations:

1. Add the absorbances of the blanks and divide by 2. Subtract this value from each of the other absorbances.
2. Combine all the standard samples and determine the mean and standard deveations. Do the same for the test samples. Using the means of the standard and test samples, calulate the absorbance of each sample from the following formula.

Calculation:

$$\frac{\text{absorbance of sample}}{\text{absorbance of STD}} \times 300 \times 2 = \text{glucose concentration}$$

5. LACTATE

This enzymatic method is based on one described by Gloster and Harris (90). In this method, lactate is converted to pyruvate by the action of lactate dehydrogenase (LD). This reaction reduces the cofactor NAD^+ to NADH. The increase in NADH is directly proportional to the lactate concentration. Since this reaction is in equilibrium, hydrazine is added to trap the formed pyruvate and force the consumption of lactate. Therefore, the increase in absorbance measured at 340 nm, is proportional to the total concentration of lactate present. The presence of excess α- and β-keto and hydroxy acids can interfere with this assay by presenting as substrate for lactate dehydrogenase (90). These substrates, however, are not present in significant quantities in body fluid tested for lactate under normal circumstances (90).

Pyruvate + NADH <----- LD -----> Lactate + NAD^+

PROCEDURE:

To eppendorf tubes, add 0.4 ml ice cold 10% trichloacetic acid. To these tubes, add 0.2 ml well mixed adsol stored RBCs and lable apppropriately. Vortex vigorously for 0.5-1 minute and place in 4^0C refrigerator for 0.5-1 hour. Spin tubes at 15,600 x g for 10 minutes and remove supernatant. To a fresh tubes, add 0.35 ml distilled water. Lable appropriately and add 0.05 ml of the respective supernatant and vortex.

Remove enough lactate reagent vials to test samples in duplicate, 1 vial does 2 tests. These vials contain 10 mg of NAD each. Reconstitute by adding the following to each vial: 2 ml of glycine buffer (provided), 4 ml of distillerd water and 0.1 ml of lactate dehydrogenase (Sigma, 826-6). Gently invert 11-15 times, combine into a single flask or beaker and swirl to mix. Using 10x15 tubes, pipet 2.9 ml into tubes for blank, control and samples. To the blank tubes add 0.1

ml 10% trichloroacetic acid. To control tubes, add 0.1 ml of Sigma metabolite control (S-3005). To sample tubes, add 0.1 ml of the respective samples. Mix all tubes by gentle vortex or inversion and incubate in 37^{0}C waterbath for 15 minutes. Set spectrophotometer to 340 nm, zero with the blanks, read and record absorbances. Calculate the lactate content of the samples with the following protocol.

Calculations:

1. Calculate the mean and standard deviation for the control and test samples.
2. Calculate the mean of the control. Since this was not diluted it is 3x the posted value of 21 mg/dl so concentration of control = 63 mg/dl
3. Calculate and plot mg/dl lactate per week by multipling the mean absorbance by (8 x 130.5 mg/dl).

6. OSMOTIC FRAGILITY

RBC's in hypotonic solution will take up water by osmosis (72,91). This increase in intracellular water causes the cell volume to increase. At a critical volume, either due to the membrane elasticity or integrity, the cell will lyse. During lysis, hemoglobin is released and can be monitored spectrophotometrically at 540 nm. A graph of absorbance versus percent isotonic saline concentrations yields a sigmoid curve (72,91). This method is described by Beutler (72). This method was verified using healthy volunteers and 1 day old Adsol stored RBCs acquired from the

American Red Cross (Lansing, MI.), both of which produced results within the published normal range (Figure 1)(72).

PROCEDURE:

A stock solution of phosphate buffer saline (PH 7.4) was prepared containing 18.0 g sodium chloride, 2.73 g sodium phosphate (dibasic) and 0.374 g sodium phosphate (monobasic). Label sterile bottles or flask as follows: 0.0, 0.25, 0.30, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, and 0.90 for a blank. Multiply each label by 10 and add that amount of milliliters to its corresponding container. Bring the volume of each container up to 100 ml. For each sample label a tube for each solution with the exception of the 0.90 solution. Add 1.98 ml of the corresponding solution to each tube. Pipet 20 uls of well mixed packed cells to each tube and mix well. Let tubes sit for 1 hour at room temperture. Set Gilford spectrophotometer to 540 nM and blank with the 0.90 solution. Centrifuge tubes for 5 minutes at 833 x g (Sorvall T6000). Read and record absorbances. Calculate the percent hemolysis from the formula below.

Calculation:

$$\frac{\text{abs sample}}{\text{abs tube 0}} \times 100 = \%\ \text{HEMOLYSIS}$$

7. STERILITY

7.1 Gram Stain

A smear was prepared weekly for each stored sample. They were allowed to air dry and stained following a standard Gram stain technique (92). Each smear was visually inspected to determine the relative sterility of the product.

1. Stain with crystal violet for 1 minute (2.0 g dissolved in 20.0 ml 95% etoh + 0.8 g ammonium oxalate dissolved in 80.0 ml dh_20). Rinse with distilled water.
2. Add iodine for 1 minute (1.0 g iodine and 2.0 g potassium iodine dissolved in 300 ml dh_2o). Rinse with distilled water.
3. Destain with acetone/alcohol for 30 seconds (30 ml acetone dissolved in 70.0 ml 95% etoh). Rinse with distilled water.
4. Counter stain with safranin for 1 minute (0.25 g safranin dissolved in 10 ml 95% etoh bring to 100.0 ml with dh_2o). Rinse with distilled water.
5. Allow to air dry and view under oil immersion.

7.2 Culture

Definitive sterility of each of the 6 samples was determined by cultures performed on the week 5 and 10 samples by an independent laboratory (Appendix C).

8. COMPOUNDS, CHEMICALS AND EQUIPMENT

All reagents were from Sigma (St. Louis, MO.), Aldrich (Milwaukee, WI) or Michigan State University (M.S.U.) Stores (M.S.U., East Lansing, MI). The Adsol solution and the transfer bags used were acquired from the American Red Cross (Lansing, MI) and Fenwal Laboratory (A division of BAXTER). All equipment was supplied by Michigan State University Medical Technology program (N. Kedzie, M.S.U., East Lansing, MI).

9. STATISTICS

The determination of sample size was performed by an independent statistical consultant (Appendix D). Statistical differences within data sets were determined using the matched paired T-test (paired sample method). For outliers, values which showed a large difference from the group mean, the following formula was applied (93). The t_o value was acquired from the student's t distribution.

$$t_o = \frac{X - X_m}{SD}$$

The generated data was analyzed comparing lines of best fit which were determined by least squares analysis of linear and nonlinear functions. Lines of best fit, the corresponding graphics and their statistical analysis were produced using the Slide Write plus 2.1 program (94).

RESULTS

1. EXPERIMENTAL DESIGN

This research was conducted as outline in the methods section. The data obtained from these methods is listed below.

2. HEINZ BODY COUNTS

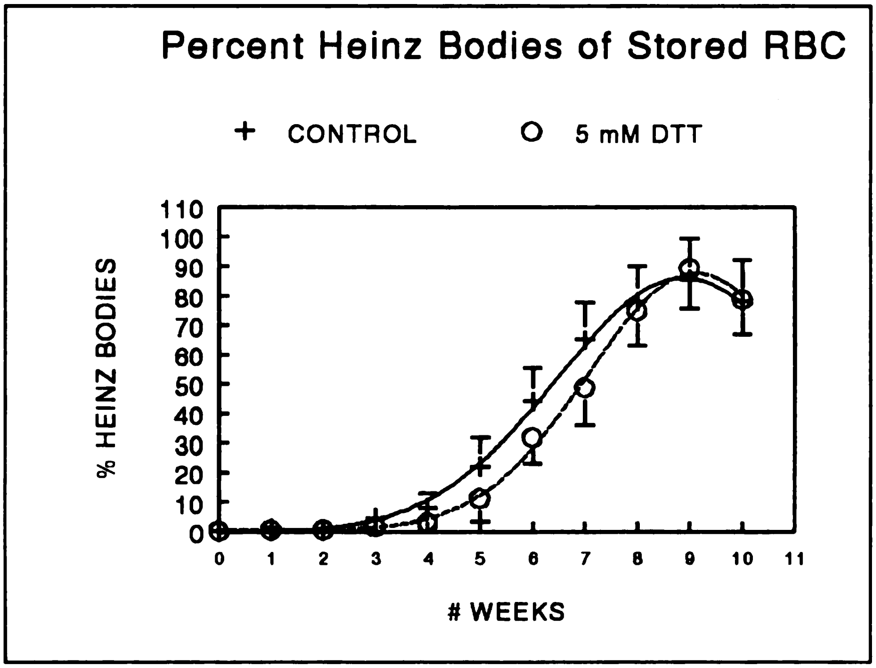

Figure 5. Percent Heinz Bodies in Control and 5 mM DTT Treated RBCs Over Time (N=5 +/- SD). Percent positive Heinz bodies was obtained by microscopic evaluation. A line of best fit predicts 32.5% positive in the control group at week 6.

Figure 5 compares the relative content of Heinz Bodies in control and in 5 mM DTT treated RBCs. From week 0 to week 3, no statistical difference between control and treated cells was observed. At week 4 there was a notable rise in percent Heinz Bodies in both the control and treatment groups. This data shows that the mean Heinz Body content of the control group is approximately two times that of the treatment group at this time. Using the matched paired T-test (p<0.05), statistical differences were found to be significant at weeks 5 through 7.

3. METHEMOGLOBIN

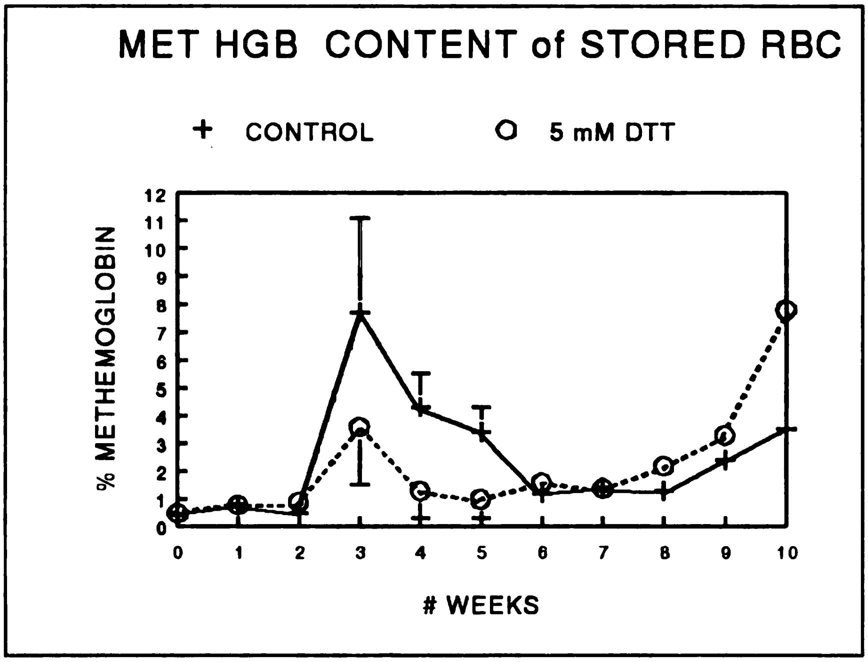

Figure 6. Comparison of Methemoglobin Content in Control and 5 mM DTT Treated RBCs Over Time (N=6 +/- SD). Significant differences in methemoglobin content was noted at weeks 3, 4, and 5 (p<0.05) using the paired T-test.

The methemoglobin (met-hgb) assay was performed as a single test set (i.e. no duplicates). The mean values for the control and 5 mM DTT treated RBCs were then used to generate the graph in figure 6. During weeks 0 though 2, no statistical difference was observed between treatment and control groups establishing a baseline at 0.8%. At week 3, the met-hgb content of both the control and treatment groups rose sharply. At this time the met-hgb content of the control rose to 7.7% ($^{+/-}$3.4%) which is 9.6 times the baseline of 0.8% set at weeks 0,1 and 2, by both the control and treatment groups. At week 3 the met-hgb content of the 5mM DTT treated group rose to 3.6% ($^{+/-}$2.1) which is 2.14 times less than that of the control group. At weeks 6 and 7, another baseline of approximately 1.4% was established for both control and DTT treated groups. At week 8, the met-hgb content of the treatment group rose over that of the control. This trend continued to the end of the experiment. The graphs of the control and treatment groups were generated by plotting weekly mean values (N=6 $^{+/-}$SD). Statistical differences, at a 95% confidence interval ($p<0.05$), using the matched paired T-test were established at weeks 3, 4 and 5.

4. GLUCOSE

Figure 7 compares the glucose content of control and the 5 mM DTT treated RBCs over a 9 week period. The validity of the assay was questionable at week 10, thus this point was ignored. From these curves, week 0 and week 4 show no statistical difference by the matched paired method with a 95% confidence ($p<0.05$). The other time points however, show the treatment group consuming more glucose than the controls. At week 10, the validity of the assay was in question due to inconsistent results, this data was not included in the graph.

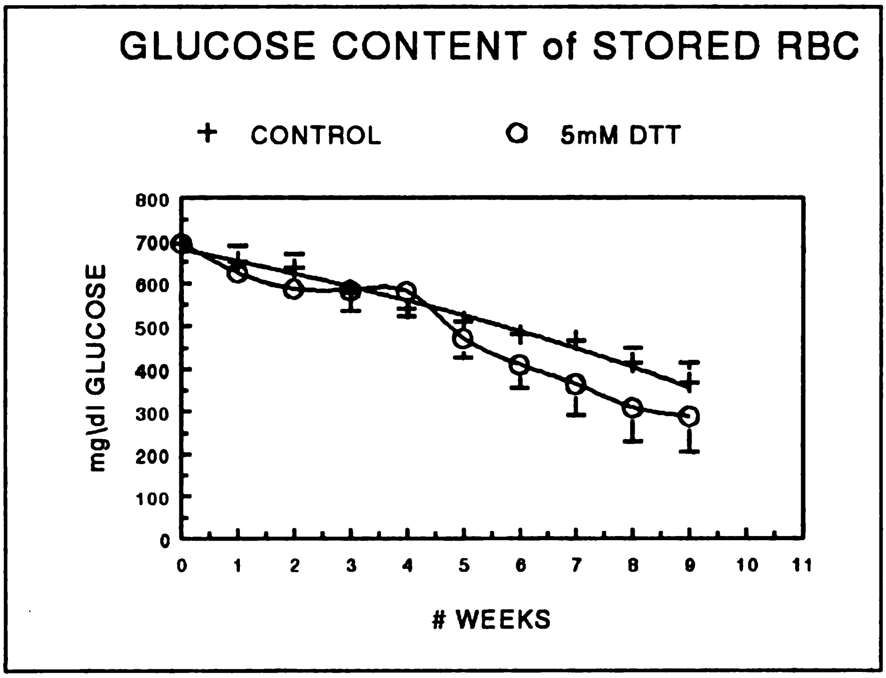

Figure 7. Comparison of Glucose Content of Control and 5 mM DTT Treated RBCs Over Time (N=6 +/- SD). No significant difference in glucose content was found between DTT groups and controls on weeks 0 and 4.

5. LACTATE

Figure 8 compares the lactate content of control and 5 mM DTT treated RBCs. At week 10, the validity of the assay was in question due to inconsistent results, this data was not included in the graph. All other points after week 4 were significantly different using the matched paired T-test with the treated RBCs producing less lactate than the control.

6. OSMOTIC FRAGILITY

6.1 Method Evaluation

This method was verified using healthy volunteers and 1 day old Adsol stored RBCs acquired from the American Red Cross, (Lansing, MI). Both produced results within the published normal range (72) and are graphically depicted in figure 9 .

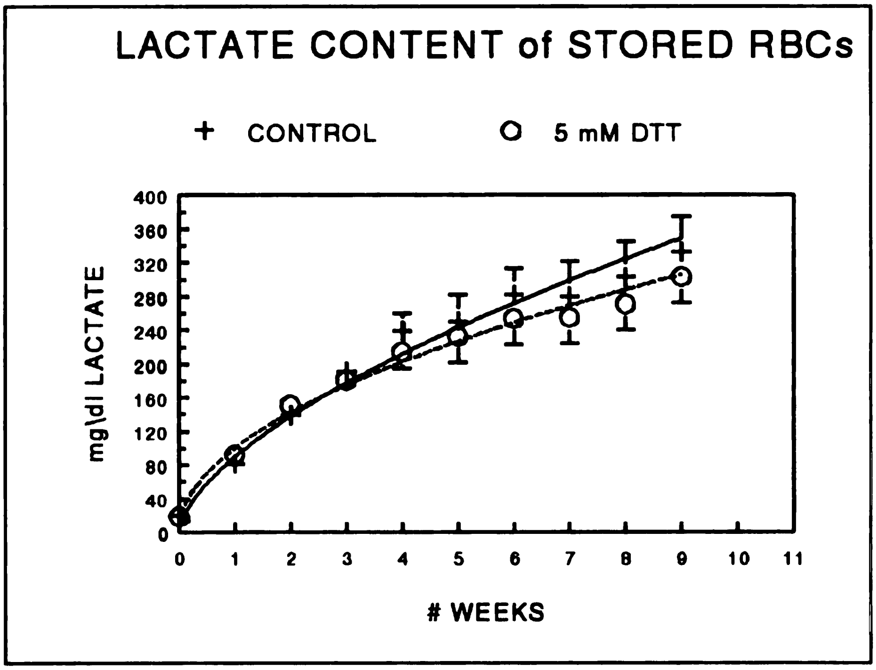

Figure 8. Comparison of Lactate control and 5 mM DTT Treated RBCs Over Time (N=6 $^{+/-}$ SD). The observed differences between the control and treatment groups after week 4 were statistically significant. Statistical significance was determined by the matched paired T-test (p=0.05).

6.2 Data

Figure 10 is a graph of osmotic fragility of untreated RBCs over a 10 week period. From this graph, two things can be observed. The first is a gradual shift to the right as the RBCs age. This is consistent with increasing cellular fragility. The second is a steady rise in basal hemolysis in isotonic saline (0.8%). The increase in basal hemolysis was termed "nonstress hemolysis", and serves as an indirect estimate of the degree of hemolysis present in this stress free isotonic environment.

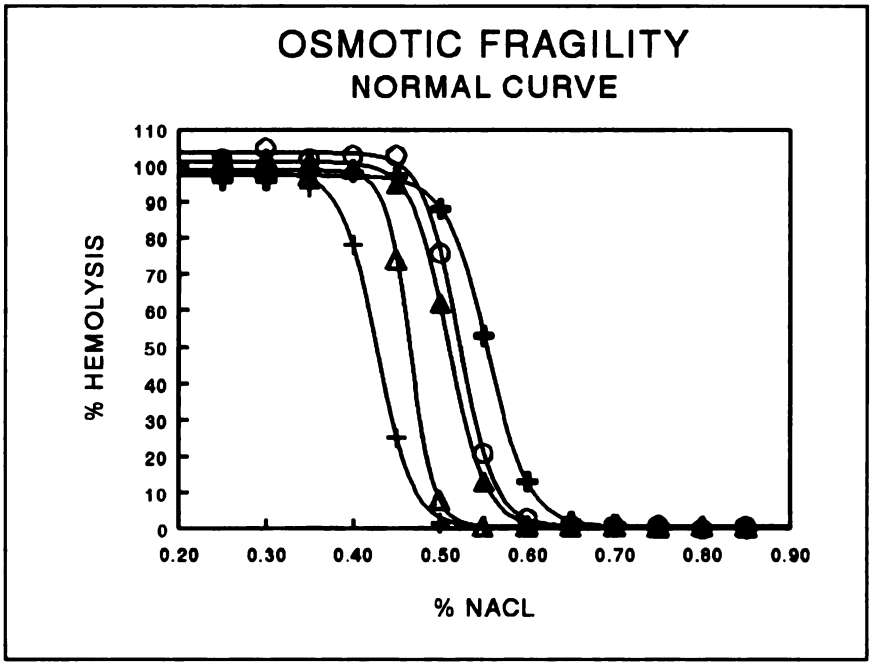

Figure 9. Normal Osmotic Fragility Curve. Curves Show Two Students (pt1=+ and pt2=△) and Three Donor Units (53=○, 89= ✚ and 98=▲). 1.0 = Isotonic Saline.

Figure 11 is a graph of the 5 mM DTT treated RBCs over a 10 week period. A shift to the right and a rise in the basal hemolysis was also noted. These changes are more pronounced in the treated RBCs than in the control RBCs. The treated RBC nonstress hemolysis is at least 1.7 fold that of the control group at all time points except week 0. These graphs were generated weekly from mean values (N=6 +/- SD).

Figures 12 and 13 compare the percent hemolysis seen in hypotonic solution (0.6% = 205 mOSM/L, calculated) to a calculated isotonic solution (0.8% = 275 mOSM/L, calculated). Figure 12 demonstrates the selection of a population of RBCs by hypotonic stress, which are less fragile than their counterparts at the time the assay was performed. This hemolysis due to hypotonic stress was termed "stress hemolysis". The weekly analysis revealed an overall population of cells, which on a weekly basis, were more fragile than the preceding weeks. The treated cells under hypotonic stress, presented with a weekly percent hemolysis which was at least 1.6 times the control. At week 10 the hemolysis of the treated cells was 100%. Complete

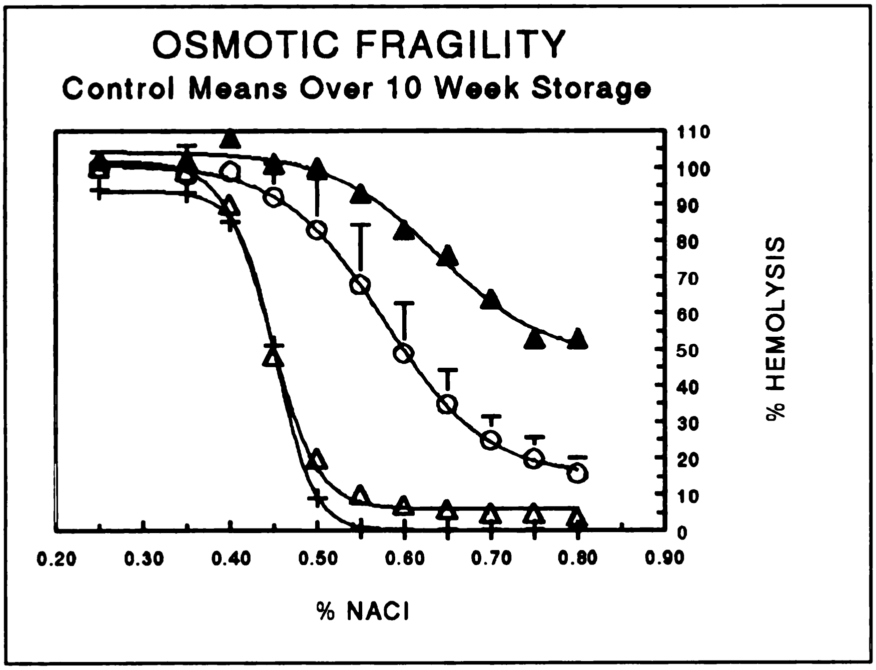

Figure 10. Changes in Osmotic Fragility of Adsol Stored RBCs Over Time. Mean values (N=6 +/- SD) were used to generate curves at indicated times. The degree of variance at week 6 was similar that noted at other time points (week 0 = + , week 2 = △ , week 4 = ○ , week 10 = ▲). Selected data points were omitted to make the graph legible.

lysis was verified by visual inspection in which no visual cell button was observed after centrifugation. In contrast, the control at week 10 showed 83% hemolysis. This indicates that 17% of the control population remains at this time.

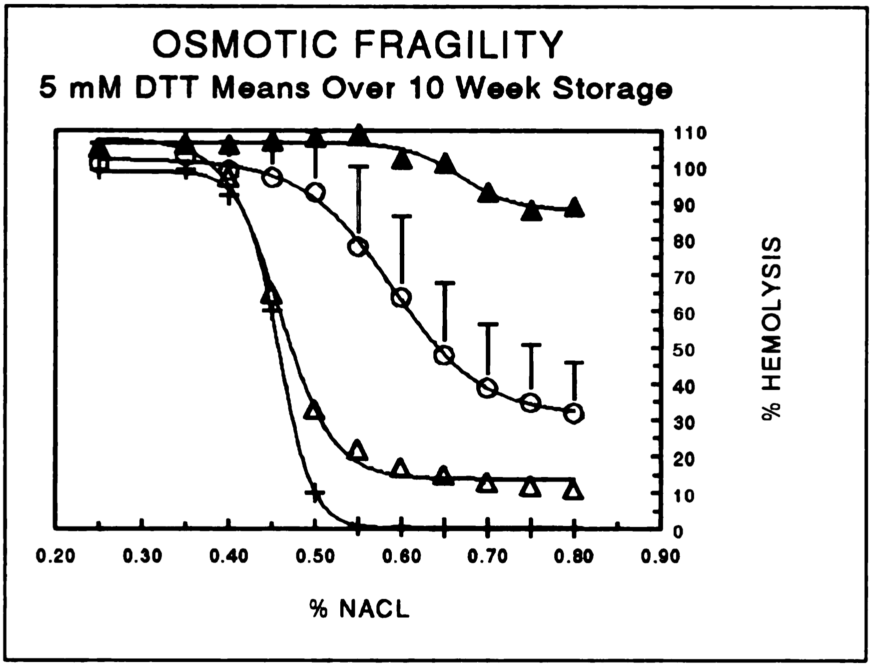

Figure 11. Changes in Osmotic Fragility Curves of 5 mM DTT Treated RBCs Over Time (N=6 +/-SD). Mean values were used to generate curves at the indicated times. The degree of variance at week 6 was similar to that noted at other time points (week 0 = + , week 2 = ▵ , week 6 = ○ , week 10 = ▴). Selected data points were omitted to make the graph legible.

Likewise figure 13 demonstrates the selection of a population of RBCs by nonstress means. This is the natural lysis due to aging in an isotonic medium. At week 10 the hemolysis of the treated RBCs was 87%, indicating that 13% of the treated RBCs remained unlyzed. In contrast the control, at this time, only showed 53% hemolysis, indicating that 47% of the control RBCs remained after being suspended in an isotonic phosphate buffered saline solution, pH 7.4 (PBS). The hemolysis seen in both figures was due to the osmolality of the solutions. The distinction of stress and nonstress hemolysis is important because one is due to normal lysing as the RBCs aged (nonstressed, Figure 13). The other was due to an added exogenous stress factor (stressed, Figure 12). This information gives added insight about the behavior of the RBCs in the environment of the storage bags.

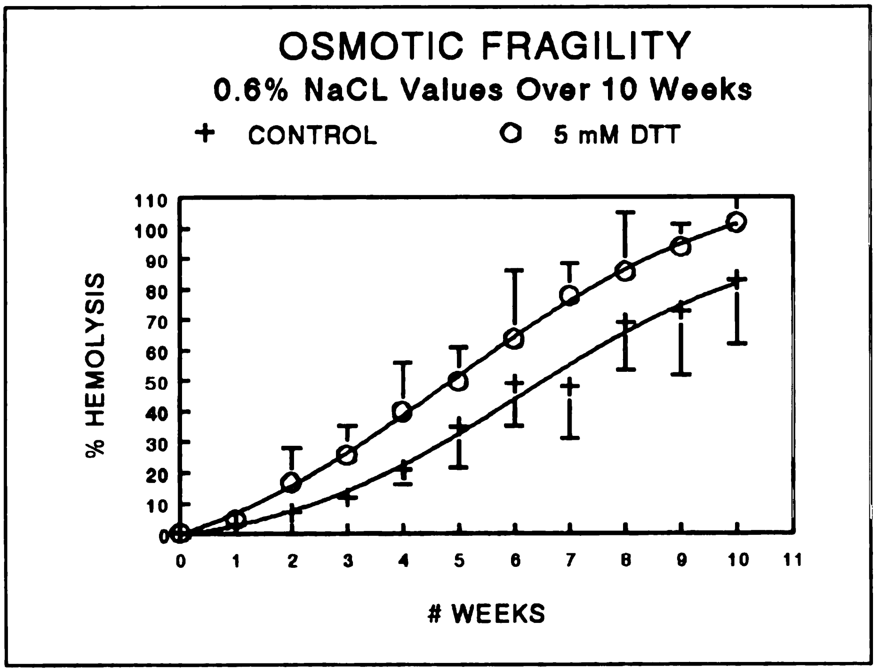

Figure 12. Graph of Percent Hemolysis in 0.6% NaCL vs Time. The 0.6% NaCL solution corresponds to a calculated osmolality of 205 mOSM/L. Data (N=6 +/- SD) suggest 5mM DTT treated RBCs are more fragile (susceptible to lysis) compared to controls.

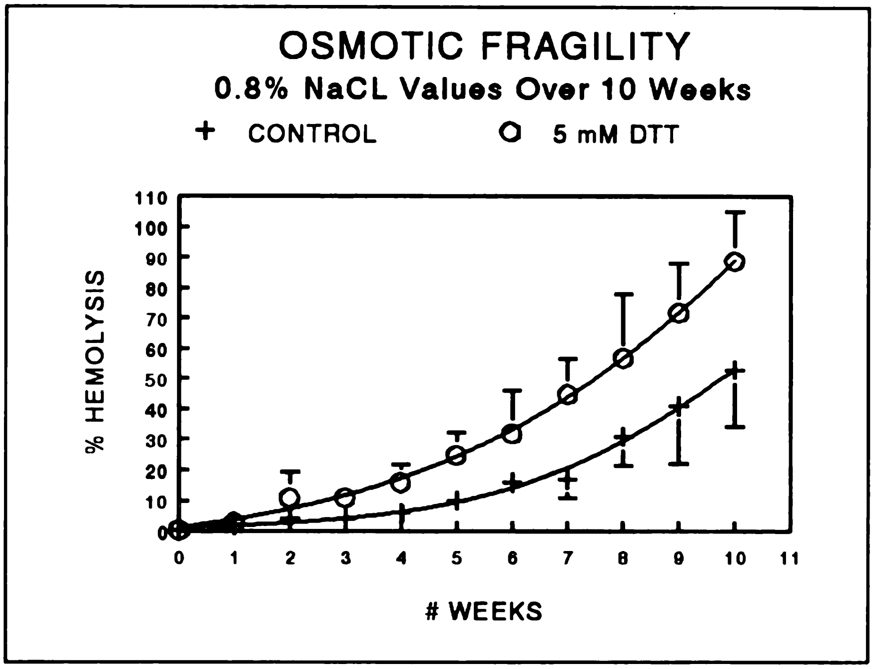

Figure 13. Graph of Percent Hemolysis in 0.8% NaCL vs Time. The 0.8% solution corresponds to a calculated osmolality of 275 mOSM/L. Data (N=6 +/- SD) suggest 5 mM DTT treated RBCs are still more fragile (susceptible to lysis) compared to controls.

7. STERILITY

7.1 Gram stain

All gram stains were negative until week 9. At week 9, specimen 1 (a control) was positive with an organism which presented as a poorly staining bacillus. At week 10, the concentration of this bacillus increased drastically and may be the cause of the aberrant results observed at week 10.

7.2 Culture

The first culture taken at week 5 was negative for all specimens. However, at week 10, specimens 1,5 and 11 were positive. The organism was similar to the one observed on the weeks 9 and 10 gram stains for specimen 1. This organism was hard to identify and did not appear to utilize sugars. It was presumptively identified as an aerobic, gram positive rod, possibly a diphtheroid.

DISCUSSION

As RBCs age, they lose their ability to resist oxidative damage. Oxidative damage has been linked to changes in intracellular constituents and membrane proteins (45,84). When these changes occur in RBCs used for transfusion they have been termed storage lesions. These changes lead to the affected cells losing membrane integrity, antigenicity and ultimately post-transfusion viability (58,84). The loss of the membrane integrity leads to an increasingly fragile cell which is prone to lysis under stress and may be sequestered by the spleen (84). Further, changes in membrane proteins due to oxidative damage may change the antigenic characteristics of the cell and thus allow the cell to be identified as nonself by the body and targeted for removal. This removal is generally antibody mediated complement lysis (58).

The loss of cellular oxidative resistance is primarily due to a decline in reducing equivalents which quench or consume oxidatively active species. In RBCs, reducing equivalents are primarily generated from the glycolytic and HMP pathways as glucose is consumed and lactate is produced. Alternatively, they can be generated by the utilization of adenine, it's derivatives or PPRP recycling via the purine salvage pathway (43). Since, the RBC lacks xanthine oxidase, hypoxanthine is the byproduct of this pathway (67).

Numerous reports have described the loss of oxidative resistance in stored RBCs used for transfusion (9,30,45,66). The decrease in the concentration of reducing equivalents in the form of GSH is important to this process (31,47,60,61). GSH serves as a direct or indirect antioxidant in many biochemical reactions because it contains free thiols (33,82). Therefore, if an exogenous agent could be found to maintain or replace the reducing equivalents lost during storage this may augment cellular oxidative resistance. If successful, the final product could present with a decrease in oxidatively produced storage lesions and thus, better viability and longevity. This lead to testing the hypothesis, that the inclusion of an anolog to GSH in the makeup of the RBC perservative solution, Adsol, will increase RBC oxidative resistance.

The above hypothesis was tested by evaluating effects on the production of Heinz bodies, a storage lesion caused by oxidative stress (80), after addition of a thiol reducing agent. The chemical chosen for this study was dithiothreitol (DTT) (82,83). This compound was chosen for several reasons. Dithiothreitol is a reducing compound with a high degree of specificity for oxidized thiol groups (47,82,83). Thiols are a class of functional groups which are prone to oxidation (47,70,82). Most proteins, including hemoglobin, contain thiols which are susceptible to oxidation (40,53).

Oxidation of thiol groups has been implicated as a precursor to Heinz Body formation (38,49). Furthermore DTT was chosen because it is water soluble and cell permeable (82).

Experimental data demonstrates the percent Heinz Bodies was almost undetectable until after week 3. From the third week on the Heinz Body content of both the control and DTT treated groups rose consistently. However, the Heinz Body content of the control group was higher at all points except week 9 and 10 than the DTT treated group. At week 10, the values of both the control and the treatment groups declined from the week nine value where they were equal. Since no methodology for the dispersal of formed particulates was employed and the Heinz Body count was made on intact RBCs, the only possible explanation might be the increased lysing of the RBCs at this point. At week 6 (42 days) the control content was 32.5% and that of the DTT treat group was 21.0% (Figure 5). A difference of 11.4% corresponding to a 1.7 fold reduction in Heinz Body production.

This correlates well with the analysis of the methemoglobin data (Figure 6). At week 2, the content of both samples began to rise, peaking at week 3. This would yield a high concentration of precursor substance from which Heinz bodies could be produced. This in fact is evidenced by the gradual decrease in methemoglobin content at weeks 3 though 6 and the comittant rise in percent Heinz bodies. Implied in this observation is that methemoglobin concentrations decrease due to further oxidation and the subsequent formation of Heinz bodies. That is to say that the consumption of methemoglobin to form Heinz Bodies exceeds the production of methemoglobin. Here the DTT treated group yielded less Heinz Body production than the control group. Both the production of Heinz Bodies and the consumption of methemoglobin are indicators of decreased cellular oxidative resistance.

Having shown an association between methemoglobin content and Heinz body formation, energy metabolism was evaluated (Figures 7 and 8). Knowing that oxidative resistance is associated with energy metabolism, glucose consumption and lactate content were evaluated. Over the entire experimental period, the glucose content of the control group declined gradually in a linear fashion (Figure 7). The DTT treated group however, at weeks 2 - 4 showed decrease in glucose utilization, then increased causing concentrations to fall below that of the control. This decrease is most probably due to the consumption of the added thiol reducing equivalents. This finding is consistent with the given hypothesis. As glucose utilization is increased by the glycolytic pathway and the HMP shunt the result is an increase in oxidative resistance. This would result in methemoglobin remaining reduced and therefore a decline in the concentration of Heinz bodies formed (16).

On the other hand, with the consumption of DTT, both concentrations of methemoglobin and Heinz bodies would increase. This is further supported by the lactate content of the control and treated RBCs over the experimental period (Figure 8). The lactate content of the DTT treated group presented lower than that of the control. As reducing equivalents are consumed it is possible that more glucose is drawn into the glycolytic pathway and channeled into the HMP shunt. This is suggestive of glucose being diverted away from lactate production to generate reducing equivalents.

Membrane integrity, in terms of elasticity, was evaluated in control and DTT treated RBCs. This evaluation was accomplished by stressing the cells in a gradient of hypotonic NaCL solutions and plotting the resulting values as a function of percent stress hemolysis over time (Figures 10 and 11). From this data, percent nonstressed hemolysis was also indirectly evaluated as a rise in isotonic (0.8%) baseline (Figure 12). It was found that as RBCs age, they do become increasing fragile (21). The addition of DTT lead to a 2-fold increase in hemolysis in both cases. From this, the speculation can be made that although DTT protects the cell from intracellular oxidative damage, it acts as a competitor for high energy phosphates, drawing these key compounds away from the glycolytic pathway. This reduction in high energy phosphates may lead to a decrease in ATP production. Decreases of ATP concentrations has been linked to deleterious effects which lead to the eventual loss of cellular integrity and cell death (10,45).

In conclusion, 5 mM DTT gave limited success in reducing Heinz body formation. However, this minor windfall appears to have come at the expense of creating a more fragile cell. Therefore DTT has failed to produce a better product although it apparently kept methemoglobin from further oxidation and thus temporarily reduced Heinz body formation.

PROBLEMS AND SOLUTIONS

1. Experimental Design

In this experiment, 3 ml of sample was stored in 150 ml bag for each specimen. This may have caused the extreme nonstress (baseline) hemolysis seen due to increased surface area. This increase in surface area may have allowed a larger percentage of the cell population to be exposed to atmospheric oxygen and temperature extremes. It is suggested for subsequent testing to use a smaller custom made bag or increase the sample size to at least 25-50 ml for each 150 ml bag.

2. Osmotic Fragility

It is suggested that a hematocrit reading be taken, the test samples then washed once in physiologic saline and resuspended to standard hematocrit for testing. This might eliminate result values over 100% and should remove excessive nonstress hemolysis to give cleaner baselines. This would provide a more accurate assessment of the shift to the right of the remaining RBCs after accounting for the level of nonstressed hemolysis.

3. Glucose

With this particular kit (SIGMA 16-UV) high hemolysis may cause false readings. Although the manufacturer supplied a protocol for use with hemolyzed samples, some values were questionable. In particular, the week 10 results which show a rise in glucose, which cannot occur. It is suggested that a different procedure be use in subsequent assays. This procedure should utilize a protein free filtrate to allow for extended evaluation.

4. Adenine and Hypoxanthine

Results predict that the addition of thiol reducing equivalents effect glucose content and lactate accumulation. Adenine may also play a part in the maintenance of reducing equivalents and/or ATP concentration. In subsequent assays adenine and hypoxanthine should be evaluated. An HPLC method for nucleotides and nucleosides is discussed by Hammer et al: ANAL BIOCHEM. 1988. 169. p. 300.

APPENDICES

APPENDIX A

Adsol RBC Preservative Solution

The standard Adsol solution is 100 ml containing 2.2 g dextrose monohydrate U.S.P., 27 mg Adenine, 900 mg Sodium Chloride usp, 15.4 mEq Sodium, and 750 mg Mannitol usp.

The Standard Preparation of Adsol Units

From the donor 450-500 ml of whole blood is drawn into the primary bag containing 63 ml of Citrate Phosphate Dextrose solution. This unit is centrifuged. After centrifugation the plasma is expressed off to satellite bag number 1 and the adjoining tube is heat sealed. The hematocrit at this point is approximately 80 -90%. Next 100 ml of the above formulation is then expressed onto the remaining packed red cells from satellite bag number 2. The hematocrit at this point is approximately 60 - 65%. Further satellite bags may be added to this system to remove other components such as white blood cells and platelets.

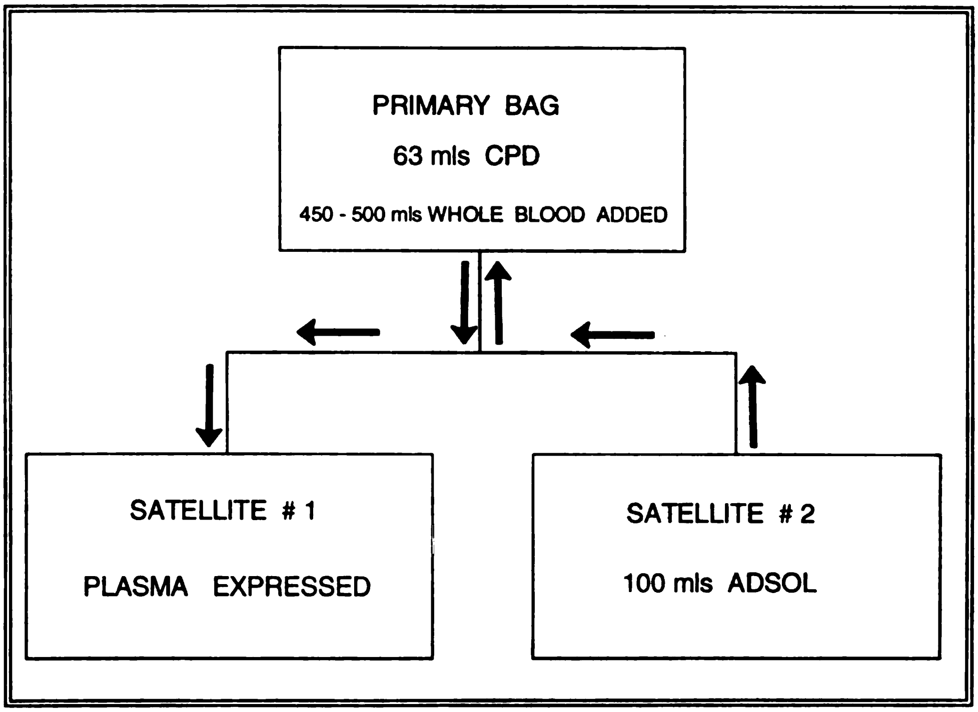

Figure 14. The Standard Preparation of Adsol Stored RBCs.

APPENDIX B

Donor Selection

In keeping with AABB standards and procedures used at the American Red Cross for donor selection (23,24), donors were selected and screened (Table 1).

TABLE 1. Criteria for Donor Selection

	Ranges	Means ± SD	AABB Standards
Weight			
Female	111-130 lbs	120 ± 0.5	>110
Male	172-230 lbs	196 ± 30.4	>110
Hematocrit			
Female	40-42%	41 ± 1.2	38-47%
Male	45-47%	46 ± 1.2	40-54%
Blood Pressure			
Female			
systolic	90-128	112 ± 9.7	90-180
diastolic	65-80	73 ± 7.6	50-100
Male			
systolic	120-140	130 ± 10.0	90-180
diastolic	70-80	75 ± 5.0	50-100

Following the donor procedure used by the Red Cross (23,24), the equivalent amount of the anticoagulant CPD and the storage solution Adsol were calculated to maintain the ratios as outline by AABB standards. Under sterile conditions, the CPD and Adsol were individually collected and pooled from 12 Red Cross donor bags. From these pools, 14 ml CPD was added to 100 ml of whole blood and 15.4 ml of Adsol was added to approximately 30 ml of packed RBCs to acquire approximately 45 ml total volume.

For each donor, two 60 ml syringes were loaded with 7 ml of CPD. The puncture site was cleansed according to Red Cross procedures with a solution of betadine and allowed to dry for 3 to 5 minutes. A single puncture was performed using a 16 gauge 1' infusion set and 50 ml was drawn. To draw the second syringe, the infusion set was pinched off with a pair of hemostats and the syringes were sterilely exchanged. In a sterile hood, both syringes were gently mixed and the contents were delivered to 2 sterile 50 ml conical centrifuge tubes. These tubes were then spun at 2700 to 3000 rpm for 30 minutes. The plasma was removed using sterile pipets (one for each sample). The plasma was removed and frozen. The final product was packed RBCs (pRBCs).

To the pRBCs, 15.4 ml of Adsol was added to each control tube and 15.4 ml of Adsol containing 2.2 g of DTT was added to each treatment tube. The final concentration of the treatment tubes was approximately 5 mM DTT. The final volume of all tubes was 45 ml +/- 2 ml. Each tube was mixed by gentle inversion and 3 ml were dispensed by syringe into sterile transfer bags which were then placed into cold storage (4^{0}C).

APPENDIX C

Blood Bag Culturing Protocol

(Donna Duberg M.A., M.S., MT(ASCP)SM)

1. At 5 weeks as much blood as possible was removed from the storage bag aseptically, 1 mL was used for testing. The remaining blood in the syringe was immediately injected into a blood culture set (BBL, 1 aerobic bottle, 1 anaerobic bottle) using aseptic technique and equally dividing the amount in the syringe between the two bottles (approximately 0.2 to 0.5 mL of blood/bottle). The two blood culture bottles comprise the standard culturing medias used to isolate most aerobic and anaerobic bacteria and yeast (95).

   At 10 weeks only blood bag #1 had approximately 1 mL of blood left for culturing after the first 1 mL was transferred to the test situation. The blood from bag #1 was cultured as indicated above for the 5 week bags. The remaining bags (#2 thru #12) were rinsed with 1 mL of sterile 0.15 M phosphate buffered saline (PBS), pH 6.6. The 1 mL of PBS and blood was then distributed equally between the two blood culture bottles.

2. The bottles were incubated at 37^0C in a non-CO_2 incubator for 5 days. A non-CO_2 incubator is appropriate at this stage of the culturing since the bottles are sealed with no exchange of gases with the external environment. Blood culture bottles were

observed at 24 hours and before subculturing on Day 5 of incubation, due to the age of the cells and possibly the effect of the treatment of the cells, slight visible hemolysis and "fluffy" turbidity in several of the aerobic bottles was noted. These observations did not resemble the hemolysis and turbidity usually associated with positive blood cultures. Since these were not patient specimens and there was no urgency in determining the presence or absence of bacteria/yeasts, it was decided to remain on the proposed culture schedule. After the 24 hour observation, each bottle was gently inverted twice to resuspend the blood that had settled on the bottom. This would allow any organisms trapped by the blood cells access to the nutrients in the broth increasing the probability of recovering them.

3. After 5 days an aliquot was subcultured from the blood culture bottles to solid media (see A,B & C below). All transfers were made using a sterile syringe to enter the bottle after the stopper had been cleansed with a sterile 70% ethyl alcohol wipe. The bottles were gently inverted twice until the blood was completely resuspended in the broth. After insertion, the syringe was rinsed twice in the blood-broth mixture to remove any alcohol that may have adhered to the stopper after cleansing. Approximately 0.1 mL was placed on each of the solid media and the inoculum was

allowed to soak into the media before the plate was inverted.

A. From the aerobic bottle, an aliquot was placed on a chocolate agar plate (BBL) and left at room temperature for 72 hours to enhance the chances of recovering *Yersinia* spp. Subcultures were examined for growth each 24 hours of the 72 hour period. (Jim Ivey, Laboratory Manager and Microbiology Supervisor, St. Lawrence Hospital).

B. Also from the aerobic bottle, an aliquot was placed on another chocolate agar plate and incubated at 35-37C in 5% CO_2 for 72 hours "to detect aerobic, capnophilic (CO_2 loving) and facultatively anaerobic bacteria. Subcultures were examined for growth each 24 hours of the 72 hour period.

C. From the anaerobic bottle, an aliquot was placed on a blood agar plate (BBL) and incubated at 35-37^{0}C for 1 week in an anaerobic jar with conventional Gas Pak envelope and indicator system to generate and confirm anaerobic conditions. At the end of 1 week, the jar was opened and the plates observed for growth.

This regimen was modified from the standard procedure to maximize isolation of fastidious bacteria (95). Since these were not patient specimens and treatment was not pending results obtained, additional time in the bottle and extending the 37^{0}C and room temperature incubations were acceptable. Terminal cultures at 5-7 days were recommended for maximizing the isolation of *Neisseria gonorrhoeae* and yeasts. Also since we did not need to view the anaerobic plates at 48 hours to observe for growth and report out patient results, the anaerobic jar was kept closed for a full week to maintain optimal anaerobic conditions for the more fastidious anaerobic organisms such as *Peptococcus* and several of the anaerobic gram positive rods.

All media used in this protocol was "in-date" and had been appropriately quality controlled according to the College of American Pathologists guidelines (96). Except for the initial transfer of blood from the blood bags to the blood culture bottles, all work was performed at the Michigan State University Clinical Center Laboratory using hoods and incubators that are operated according to the College of American Pathologists guidelines. Daily monitoring and routine preventative maintenance is performed and documented.

Final Report

At week 5, all culture bottles and subcultures were negative for growth of any kind. However, at week 10, growth was observed on subcultures from the aerobic and anaerobic bottle sets of samples #1, #5 and #11. The bottles had appeared slightly turbid with a tint of hemolysis which could have been attributed to the age of the cells before culturing. Since the appearance of set #1 was similar to sets #5 and #11, there does not seem to be an effect attributable to the phosphate buffered saline added to the blood bags to increase recovery of cells for culturing.

The chocolate agar subcultures stored at 37^0C and RT were observed daily but growth was not evident until 72 hours. There were approximately 30 to 60 colonies per plate. The 72 hour time frame for growth was also observed when the colonies were transferred to blood agar. Transfer to MacConkey agar (BBL) resulted in no growth. The colonies on chocolate agar at 37^0C were slightly larger than those isolated on the chocolate agar at room temperature but the overall gram stained colony morphology and appearance of the organism from both plates were identical. The anaerobic transfer plates were first observed after one week incubation and the same colonial and gram stain morphology was observed. The number of colonies isolated corresponded to the number seen on the aerobic media.

Final Analysis

Aerobic, nonsporforming, gram positive long thin rods, possibly Diphtheroids; no further identification available.

All culture work was performed by Donna M. Duberg, M.A., M.S., MT(ASCP)SM and Kathleen MacDonald MT(ASCP).

APPENDIX D

Sample Size

The estimation of the sample size (N) was determined by an independent source (97). The determination of the required sample size utilized preliminary data generated in this laboratory for methemoglobin controls. The use of the appropriate paired difference formula (Below) presented with the apparent problem that several factors required for it's solution were unknown.

$$N = \frac{(Z_{\alpha/2})^2(\sigma d)^2}{(\omega/2)^2}$$

The $Z_{\alpha/2}$ term, the standard gaussian variable, was defined as 2, consistent with a 95.5% confidence interval. ω is the margin of error. This value was not directly available with control data only in a pair method experiment. σd represents the standard deviation of the differences in a pair method design and was also unavailable with control data only. To estimate N with this preliminary data several assumptions were made. The first assumption was that the differences in the paired design are normally distributed. The second was that the variance in the treatment group was the same as that of the control group. With these assumptions, a sequential analysis was performed using a series of paired T-statistics with a variable N. Explained briefly, a T-statistic was acquired from a published table, (two sided, $\alpha = 0.05$) at an assigned N value for the mean difference (X_a-X_b) was back calculated.

The results are depicted below (Table 1). It was found that as sample size (N), increased, the difference of the means (X_a-X_b) needed to establish significance, decreased. Using matrix analysis it was determined that beyond N=5 no significant difference in the rate of decrease of (X_a-X_b) was observed. From this an N=6 was chosen.

TABLE 2. Comparisons of the difference of control and test means at variable sample sizes (N).

N	Xa - Xb
3	1.261
4	0.744
5	0.685
6	0.643
7	0.609

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