1980

Assisted and non-assisted hemoglobin recovery from phlebotomy.

Ronald Gerard. Weese
University of Windsor

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ASSISTED AND NON-ASSISTED HEMOGLOBIN
RECOVERY FROM PHLEBOTOMY

by

Ronald Gerald Weese

A thesis
submitted to the Faculty of Human Kinetics
of The University of Windsor in partial
fulfillment for the degree of
Masters of Human Kinetics

Windsor, Ontario, Canada
1980
ASSISTED AND NON-ASSISTED HEMOGLOBIN
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ABSTRACT

It was hypothesized that an experimental group of healthy male subjects who received slow-release iron and folic acid supplement after phlebotomy of 450 ml of whole blood would exhibit a significantly elevated erythropoiesis rate, when compared to a similar group who received no supplement and underwent the same phlebotomy.

The hypothesis was tested by randomly assigning eight healthy male subjects to two equal groups after control data of serum ferritin, serum iron, and hematologic values were collected. Each subject underwent phlebotomy and removal of 450 ml of whole blood which was then stored in an Acid-Citrate-Dextrose anticoagulant medium at +4°C. The experimental group was provided with a twenty-one day supply of an iron and folic acid supplement (Slow Fe-Folic-CIBA) and instructed as to dosage. The other group received no supplement. Training schedules of either were not interrupted.

Serum iron concentration and hematologic data on all subjects were collected on days four, six, eight, twelve, fifteen, eighteen and twenty-one.
Red Cell lysis data on the stored blood consisting of plasma-potassium, sodium and hemoglobin were collected on days four, eight, fifteen, eighteen and twenty-one to determine when red cell in vivo lysis became significantly different from day one of storage.

A final serum ferritin determination was made at the conclusion of the experiment to estimate changes in subject body iron stores.

Statistical analysis indicated no significant differences between groups on subject serum iron and subject hemoglobin concentrations (p < .05). A complete hemoglobin return to pre-phlebotomy values did occur within the twenty-one day duration of the experiment (p > .05). Serum ferritin values were slightly elevated from control values at the conclusion of the experiment for the iron supplemented group and slightly lower for the non-supplemented group. These differences were not statistically significant (p < .05).

Hemolysis indicators from the stored blood showed that red cell lysis remained low until after day fifteen post-phlebotomy. At that time, plasma potassium levels increased and plasma hemoglobin levels were significantly elevated from control (p < .05).

The data did not support the hypothesis that oral iron and folic acid supplements would increase the erythropoietic rate in healthy male donor subjects after blood loss of 450 ml.
It is concluded from the data that the iron and folic acid supplement of this dosage prevented any depletion of iron from body stores as a result of the 450 ml blood loss and increased erythropoietic stimulus. The supplement, however, did not affect the rate at which erythropoiesis occurred in the supplemented subjects and did not produce a rapid hemoglobin rebound post-phlebotomy.
To W2
ACKNOWLEDGEMENTS

I would like to acknowledge the cooperation of the laboratory personnel at Windsor Western Hospital Centre and Grace Hospital of Windsor who assisted in data collection and assay. Also, a special thanks is extended to Mr. Roger Laplante of Clinical Chemistry at The University of Windsor for his unsolicited interest and guidance in the project.

Finally, I would like to thank Dr. R. T. Hermiston and the members of my committee; Dr. Draisey, Dr. Taylor and Dr. Thibert for their expert assistance in the research.
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CHAPTER I

INTRODUCTION

The technique commonly known as blood doping or blood boosting (Spreit, 1978) has been investigated as an ergogenic aid (Ekblom, 1972; Ekblom, 1976; Spreit, 1978). Ergogenic aids are defined as: any substance which tends to increase work output, and as such are of interest to both coaches and athletes. Indeed, the technique has been given international attention due to allegations that blood doping has been used in Olympic competitions. As a result, the International Olympic Committee has ruled that blood doping is an illegal technique and is therefore disallowed in international athletic events.

Blood doping is, however, a poorly defined term. The literature identifies two distinct techniques designed to produce a polycythemic state (Ekblom, 1972; Spreit, 1978). These two techniques, while reporting somewhat similar results in work output, vary considerably in the methods used to induce polycythemia. The ramifications of the two methods, in terms of the technical skill required, potential for aerobic augmentation and expense, lend themselves to controversy.

Each technique invokes an erythropoietic response as a result of acute tissue hypoxia resulting from the phlebotomy
of a volume of the subject's blood components. The re-infusion of the venedected blood after an adequate period of erythropoiesis rebound produces the polycythemic circulatory state.

The important consideration relevant to this study is the period of erythropoiesis rebound. This period is a critical step in the blood doping technique as the rapidity of the rebound is paramount in importance for the development of a significant polycythemia.
CHAPTER II

REVIEW OF LITERATURE

Polycythemia has been produced in the laboratory by autologous transfusions (Ekblom, 1972; Ekblom, 1976; Spreit, 1978; Weese, 1977). In those experiments the effects of the induced polycythemia on work output was investigated. It has been shown that oxygen transport to the tissues has been increased (Ekblom, 1976). Also, data exist that indicate aerobic work parameters have been affected in that increased time to exhaustion on treadmill runs have been observed, and maximal oxygen uptake ($V\dot{O}_2$) measurements have increased (Ekblom, 1972; Ekblom, 1976; Spreit, 1978). While no definitive explanation of the exact mechanism for the increases in aerobic metabolic tolerance has been proven, the data suggest that athletes can perform at higher levels of work as a result of the induced polycythemia.

A difference in experimental technique should be noted among these studies. The differences were based on the mode of production of the polycythemia in the test subject. The method of blood storage was the technical variant responsible.

Similarities among the techniques have aided in elucidation of the physiologic factors necessary for the production of polycythemia in subjects via blood loss and reinfusion.

In all cases of induced polycythemia, the infusion of
a volume of blood was preceded by the venesection and storage of blood or blood components by the donor subject (Ekblom, 1972; Robertson, 1978; Spreit, 1976; Weese, 1977). This resulted in the loss of erythrocytes and a reduction in arterial oxygen concentration (Ekblom, 1976). Plasma losses were also observed in venesections where erythrocyte separation and plasma reinfusion were not performed (Ekblom, 1972; Spreit, 1976; Weese, 1977). This combination of circulatory trauma produced hemodynamic adjustments in order to return the hypovolemic anemic subject to a normal circulatory environment.

Starling (1896) recognized that the loss of fluid from the vascular system or a drop in blood volume altered the homeostatic forces governing fluid dynamics. It was also observed that the reduction of intracapillary filtration pressure resulting from fluid loss and the increased oncotic resorptive pressure from accelerated lymphatic involvement combined to draw fluid from the interstitial spaces to replace lost plasma volume (Adamson, 1968; Cope, 1962). Evidence also exists which indicates that the drop of blood pressure and loss of vascular fluid volume which accompanies phlebotomy, initiates the Renin-Angiotensin-Aldosterone fluid retention cycle in the kidney (Mountcastle, 1974). The time course for in vivo plasma replacement has been shown to be dependent on the severity of the volume loss, and is approximately 25 ml per hour (Williams, 1973).
The loss of 500 ml of blood results in the loss of approximately 250 ml of red blood cells. As the plasma volume is replaced, hemoglobin concentration, red cell concentration and hematocrit have all been shown to be reduced (Ekblom, 1972; Ekblom, 1976; Spreit, 1978; Weese, 1977). This indicates unequal replacement time courses for plasma and red cells. The normovolemic anemia which results from the unequal replacement times for the two blood components invokes the erythropoietic responses.

The erythropoiesis response produces physiologic changes in the maturation rate of red blood cells. This rate occurs with varying rapidity in response to acute blood loss (Ekblom, 1972). The erythropoiesis rate is a critical factor in the blood doping technique, and the duration reported to return a subject to control hematologic values post-venesection is varied (Ekblom, 1976; Spreit, 1978; Weese, 1977).

Ekblom (1972) failed to report complete hemoglobin recovery in twenty-eight days after venesection of 800 ml of blood from one group and 1200 ml from another. The 1200 ml group were significantly retarded in erythropoiesis recovery in comparison to the 800 ml group. This prompted Ekblom (1976), in a follow-up study, to utilize 800 ml venesections as the erythropoiesis stimulus and he again failed to observe a complete hemoglobin recovery twenty-eight days post-venesection.

Spreit (1978) observed a return to control hemoglobin
values after venesection of 1000 ml of blood and plasma replacement, which required forty-two days.

In contrast, Weese and Hermiston (1977) reported a complete hemoglobin recovery in fourteen days post-venesection of 500 ml of blood.

Serious experimental differences exist among these studies, particularly in the experimental control of training and diet. Ekblom (1972) used trained subjects but gave no report of dietary restrictions or exercise regimen. Spreit (1978) also made no report of training or diet. Weese and Hermiston (1977), however, reported dietary iron maintenance and monitored training regimen. These experimental differences may account for the discrepancy in reported erythropoiesis rates.

It is a well established fact that erythropoiesis is dependent on the production of a glycoprotein hormone, erythropoietin, in response to lowered arterial oxygen concentration at the kidney (Guyton, 1971; Hamstra, 1969; Jokl, 1968; Nathan, 1964). While lowered arterial oxygen concentration and the resultant increased erythropoietin production initiates the erythropoiesis response, the actual production of red cells occurs at the bone marrow provided the erythropoiesis maturation factors of iron and folic acid are available for red cell anabolism (Guyton, 1971; Hausman, 1976; Nixon, 1970).

The importance of iron in anemic conditions was
recognized decades ago but its absorption and utilization in the body is not well understood (Conrad, 1963; Crosby, 1968).

The total iron content of the body has been found to be approximately 4-5 gr (Guyton, 1971; Mountcastle, 1974; Tietz, 1970). Seventy-five per cent of this total body iron has been measured to be in functional combination with heme compounds (hemoglobin and myoglobin), and twenty per cent as storage iron chiefly as ferritin in the kidney, liver, spleen, intestinal mucosa and the bone marrow (Tietz, 1976). A small amount of approximately 0.1 per cent is constantly in transport in a dynamic equilibrium between the actively functioning and the reserve fractions (Tietz, 1976).

The absorption of iron in the gut has been found to be dependent on physiologic need (Conrad, 1963; Crosby, 1968). No active elimination mechanism is evident in the body so absorption appears a rate determining step. Conrad and Crosby (1963) have determined that the control of iron absorption lies within the mucosal cell of the duodenum; however, how the absorptive cells are informed of corporeal iron requirements remains unclear. It has been hypothesized that the circulating plasma iron ion content is the regulating mechanism for iron transfer from mucosal cells to plasma, as its concentration has been observed to fluctuate in accordance with bodily needs (Conrad, 1963; Guyton, 1971; Tietz, 1976). Figure 1 illustrates a concept of iron control by the intestinal mucosa.
FIGURE 1. A Concept of Iron Control by Intestinal Mucosa
It is predicted that iron absorption is regulated primarily through the columnar epithelium of the small intestine. In normal iron replete subjects the mucosal cells may contain a variable amount of iron supplied from the body store. This deposit regulates (within limits) the quantity of iron which can enter the cell from the gut lumen. After the iron has entered the cell it may proceed to the body to fulfill a requirement. Alternatively, a portion of the iron may become fixed in the epithelial cytoplasm to be lost when the cell is sloughed at the end of its life span. Iron deficient subjects apparently possess little or no mechanism to inhibit entrance of iron to the villous epithelial cells or to return it. Thus, dietary iron readily proceeds into the body. In iron loaded subjects, the body iron incorporated in the epithelial cells is eventually lost, but during the life span of the cells its presence inhibits the entrance of iron to the cells (Conrad, 1963).

The incorporation of plasma iron into the hemoglobin molecule of the red cell has been found to occur in the bone marrow by the red cell precursors, i.e., normoblasts and reticulocytes (Dacie, 1975; Guyton, 1971; Tietz, 1976). The rate of this red cell production is dependent not only on the amount of circulating erythropoietin and iron stores, but also on the availability of adequate supply of other erythrocyte maturation factors such as folic acid (Guyton, 1971; Nixon, 1970; Tietz, 1976). Deficiency of folic acid
results in abnormal maturation of erythrocytes due to impaired Deoxyribonucleic acid (DNA) synthesis within the red cell precursors (Nixon, 1970). As a result, red cell division is retarded and the normal maturation rate reduced in response to the erythropoietic stimulus (Nixon, 1970).

It appears from the literature that optimal conditions for erythropoiesis exist under conditions of hypoxia where sources of iron and erythrocyte maturation factors are available.

The rate of erythropoiesis is critical in the blood doping technique as the blood stored for autologous infusion undergoes physical changes which inhibit its potential for effective respiratory gas transport and exchange, post transfusion.

Under traditional liquid Acid-Citrate-Dextrose (ACD) or Citrate Phosphate Dextrose (CPD) storage, red blood cells have been found to increase in oxygen affinity and undergo accelerated lysis with time in storage (Bailey, 1975; Benesch, 1967; Bowen, 1974; Bunn, 1969; Dawson, 1972; Valtis, 1954).

Measurements of plasma hemoglobin and plasma potassium levels have shown that under conditions of ACD storage at +4°C red cell lysis occurs slowly for a period of fourteen days and more rapidly beyond that time span (Gibson, 1961). As well, measures of oxygen affinity and 2,3-diphosphoglycerate (2,3-DPG) levels have shown that red blood cells
increase in their affinity to oxygen slowly for seven days, and more rapidly after that period of time (Bailey, 1975; Dawson, 1972; Gibson, 1961; Valtis, 1954). Red cell (2,3-DPG) levels, however, have been rejuvenated in vivo, indicating a reversal of the oxygen affinity towards normal values within twenty-four hours post-transfusion (Chanutin, 1967; Valeri, 1972; Valtis, 1954).

The inability of some investigators (Ekblom, 1974; Ekblom, 1976) to produce significant polycythemia due to inadequate erythropoiesis rates and high red cell lysis of stored blood in vitro, has led others to experiment with the freeze-thaw method of blood storage (Robertson, 1978; Spreit, 1978).

The freeze-thaw method has been shown to have distinct advantages over traditional liquid storage for purposes of producing polycythemia for exercise purposes. The freeze-thaw method allows the subject to return to normal hemoglobin values post-venesection as the blood can be stored almost indefinitely with little lysis or increased oxygen affinity (Teleschi, 1976; Valeri, 1975). In fact, this method has resulted in polycythemia which are significantly higher than when traditional methods were used (Robertson, 1978; Spreit, 1978). As such, for experimental purposes where a large blood banking facility is available and equipped for the freeze-thaw methodology, the technique is effective and results in significant polycythemia.
Significant polycythemia\textsuperscript{s} have, however, been produced in this laboratory using traditional blood storage methods utilizing highly trained subjects under controlled iron intake and a monitored intense exercise regimen (Weese, 1977). Thus, it appears that significant polycythemia can be produced without the intensive handling and expense of the freeze-thaw methodology. For those scientists who wish to produce polycythemias in experimental subjects for exercise purposes, and who do not have the availability of a large blood banking facility or the budget for the expense, a technique which is safe and produces significant polycythemias at reasonable expense is desirable.

The following experiment is designed to identify the role that oral iron and folic acid supplement contribute to the production of a rapid erythropoietic response which was reported in a previous study (Weese, 1977).

**Purpose of the Study**

The study described herein is designed to determine what effects the oral dosage of erythrocyte maturation factors (iron and folic acid) has on the rate of erythropoiesis of subjects who have undergone a venesection of 500 ml of whole blood. Previous work in this laboratory has shown that subjects under iron supplementation post-phlebotomy of 500 ml whole blood and constant exercise regimen have experienced a rapid erythropoiesis rate as
measured by whole blood hemoglobin and hematocrit response. It is the intention of this paper to identify the contribution of the oral iron and folic acid supplement to the erythropoiesis rate.

Also of importance to the study is the lysis rate of the stored blood. This lysis rate allows for the identification of the effects of time on the stored blood and makes an extrapolation of the optimal reinfusion date for that particular storage condition possible.

It is hypothesized that the oral iron supplement will have a significant positive effect on the hemoglobin rebound rate due to elevated serum iron concentrations and that the assisted recovery from phlebotomy will occur prior to significant lysis of the stored blood.
CHAPTER III

METHODS

Eight healthy male subjects volunteered for the study. The subjects were members of the Windsor YMCA Roadrunners Club. Their aerobic training schedules were uninterrupted over the duration of the experiment.

Preliminary Data Collection

Tables 1 and 2 contain pertinent data collected for each subject prior to the beginning of the experimental schedule. All measures were taken in the post-absorptive state at 08:00 hours and constitute control values for the experiment.

A screening procedure was utilized prior to resumption of the experiment to ensure that each subject had no medical history which would confound the study. The questionnaire appears in Appendix B.

An informal consent form was completed by each subject (see Appendix C).

Prior to resumption of the experiment, a request for approval to complete the investigation was filed with the Human Ethics Committee of the University of Windsor (Appendix D).
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<th>Potassium mEq/L</th>
<th>Sodium mEq/L</th>
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Control Hematologic Data for Experimental Subjects

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<th>Red Blood Cells K/mm³</th>
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<th>Mean Cell Volume µm³</th>
<th>Mean Cell Hemoglobin µg</th>
<th>Mean Corpuscular Hemoglobin Conc. % conc/cell</th>
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<td>1.34</td>
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<td>41.2</td>
<td>87</td>
<td>29.6</td>
<td>33.9</td>
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<td>15.5</td>
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<td>5.37</td>
<td>16.5</td>
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<td>4.81</td>
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<td>16.7</td>
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<td>90</td>
<td>30.9</td>
<td>34.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Technical Assistance

Due to the necessity of repeated venepuncture and bloodletting, all invasive techniques were performed by trained technicians at the outpatient area of the Laboratory Department of Windsor Western Hospital Centre. This procedure was necessary to ensure aseptic patient conditions and an optimal environment for the sampling, storage and assay of blood and its components.

Control Data Collection

Each subject who would act as his own control was asked to report to the outpatient area of the hematology department after a ten-hour fast at 08:00 hours the day of control sampling. At this time, each subject had 3, 5 ml samples venesected from the antecubital vein of the arm using the vacutainer collection technique.

A whole blood sample was used to obtain hemoglobin concentration, red cell count, hematocrit, mean cell volume, mean cell hemoglobin concentration and white cell count by the Coulter method (Simmons, 1976). A reticulocyte smear was obtained from the same sample using the Cresyl blue method of Dacie (1975). The second sample was allowed to clot for fifteen minutes in a 37°C water bath and centrifuged to obtain serum which was respun and assayed for: serum iron ion concentration using the method of Peters (Dacie, 1975); serum potassium and sodium ion using the method
prescribed for the Ilford Flame Photometer; and ferritin concentration using the Radioimmune Assay of Clinical Assays (Appendix D). The third sample containing whole blood was double centrifuged to obtain plasma for the purpose of assaying for plasma hemoglobin concentration using the method of Crosby and Furth (Dacie, 1975).

Phlebotomy and Blood Storage

Each subject returned four days later in order that a 500 ml phlebotomy of whole blood could be performed.

Five hundred millilitres of blood were drawn from the antecubital vein of each subject into a Fenwal Bag containing 46 ml of Acid-Citrate-Dextrose anticoagulant storage medium. The blood was then clearly identified and placed in a quarantine refrigerator at the Windsor Western Hospital Centre blood bank at +4°C.

Assignment to Experimental and Control Groups

Each subject was randomly assigned to either the control or experimental group in a manner designed to produce equal group sizes.

The experimental group was given a twenty-one day supply of Slow Fe Folic (Appendix F) and instructed as to dosage. The control group was given no supplement.

Each subject was advised to continue a normal dietary pattern throughout the experimental schedule and to be
cognizant of the dietary restriction necessary for the ten-hour fast prior to sampling on subsequent days. Aerobic training schedules were not interrupted.

Subject Data Collection

Data for the experiment were collected on day four, six, eight, twelve, fifteen, eighteen, and twenty-one, post-venesection. At each collection 2, 5 ml samples were collected by the Vacutainer method.

Sample one was used to obtain measures of hemoglobin concentration, red cell count, white cell count, hematocrit, mean cell volume, mean cell hemoglobin, and mean corpuscular hemoglobin concentration by the Coulter Method (Simmons, 1976). A blood smear for reticulocyte count was made from the remainder of the sample using the brilliant Cresyl blue method (Dacie; 1975).

Sample two was collected in a Cor Vac Serum Separator® tube. The sample was allowed to clot and was double centrifuged. The supernatant from this sample was used in the determination of serum iron concentration (Dacie, 1975).

Stored Blood Data Collection

A five- to seven-ml sample from each unit of stored blood was obtained and used for assay of plasma hemoglobin (Dacie, 1975) and plasma potassium and sodium concentration on day one, four, eight, fifteen, eighteen and twenty-one.
The sampling technique was as follows:

Each unit of stored blood was removed from the blood bank refrigerator and allowed to approach room temperature for approximately fifteen minutes. This procedure was followed to allow the blood to more approximate an *in vivo* physiologic viscosity in order that a representative aliquot could be obtained. After the fifteen-minute period each unit was gently tumbled to mix the blood components.

The sample was obtained by inserting a sterile 10cc syringe fitted with a large bore needle into the sampling line of the Fenwal bag. The syringe plunger was withdrawn slowly to ensure no red cell lysis would occur due to sampling trauma. After the required sample was obtained, the sampling line was clamped directly above the site of needle insertion. This technique was necessary to prohibit septicemia from developing over the repeated sampling dates.

The sample from each bag was transferred to a clean test tube and double centrifuged. Three 20-lambda samples were obtained from the supernatant using Sahli pipets and transferred to clean test tubes for assay of plasma hemoglobin concentration. The remaining supernatant was used in the determination of plasma potassium and sodium ion concentration.
These techniques for subject and stored blood data collection were quantitatively and qualitatively repeated at each of the collection days. All pertinent data were recorded and statistically analysed.

Statistical Analysis

A two-way repeated measures Analysis of Variance was calculated to determine the effects of time and the iron supplement on subject serum iron concentration, subject hemoglobin concentration and ferritin concentration.

A one-way repeated measures Analysis of Variance was calculated on all stored blood data to determine the effects of time on each.

All experimental group means for the time variable were compared individually with group control values using the method of Dunnett (Winer, 1971).
CHAPTER IV

RESULTS

Subject Data

The results of blood assays taken from each subject on control day plus each subsequent test day for Serum Iron and Hemoglobin are presented in Tables 3, and 4 in Appendix A. Mean values and the standard error of the mean for subject serum iron concentration under iron supplement and no supplement appear in Table 5. Mean serum iron values for the iron supplement group are consistently elevated from control values while the no supplement group show slight serum iron reduction except for days twelve and twenty-one.

No significant effect on the serum iron concentration for the iron supplement appears from statistical analysis. (p < .05). There is also no significant effect of time on the serum iron concentration over the entire duration of the experiment (p < .05).

Mean subject hemoglobin concentrations and the standard error of the mean for both experimental conditions are shown in Table 6. From these data it can be seen that a complete return to control hemoglobin values post-phlebotomy did occur in both condition of supplement or no supplement. Figures 2, and 3 show the mean hemoglobin values for both
Table 5
Mean Serum Iron Concentrations (mcg/dl) Plus Standard Error by Experimental Group

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
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<tr>
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<td>Standard Error</td>
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<td>14.3</td>
<td>14.2</td>
<td>15.8</td>
<td>11.9</td>
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<td>12.4</td>
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<table>
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<th>Days</th>
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<th>8</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
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<tbody>
<tr>
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<td>94.2</td>
<td>107.2</td>
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<td>116.3</td>
</tr>
<tr>
<td>Standard Error</td>
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<td>16.7</td>
<td>14.5</td>
<td>8.9</td>
<td>7.5</td>
<td>11.5</td>
<td>11.2</td>
</tr>
</tbody>
</table>
Table 6
Mean Hemoglobin Concentrations (gm/100ml) Plus Standard Error by Experimental Group

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>15</th>
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<td>13.7</td>
<td>13.6</td>
<td>13.7</td>
<td>14.0</td>
<td>14.0</td>
<td>14.1</td>
<td>14.4</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.64</td>
<td>0.82</td>
<td>0.94</td>
<td>0.82</td>
<td>0.84</td>
<td>0.94</td>
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<table>
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<th>6</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
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<tr>
<td>Mean</td>
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<td>14.3</td>
<td>14.3</td>
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<td>15.0</td>
<td>14.4</td>
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<tr>
<td>Standard Error</td>
<td>0.48</td>
<td>0.42</td>
<td>0.44</td>
<td>0.38</td>
<td>0.37</td>
<td>0.38</td>
<td>0.33</td>
<td>0.33</td>
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</tbody>
</table>
FIGURE 2. Subject Hemoglobin Values (gm/100ml) for No Supplement Group.
FIGURE 3. Subject Hemoglobin Values (gm/100ml) for Iron Supplement Group
iron supplement and no supplement over the duration of the experiment and indicates the relative rates of hemoglobin appearance as red blood cells are synthesized and released to the blood stream after phlebotomy.

Statistical analysis of subject hemoglobin values for each group show no significant effect of iron supplement on hemoglobin concentration between groups (p < .05). A significant effect of time post-phlebotomy was observed in both iron supplement and no supplement groups (p < .05). Subsequent Dunnett's t statistical analysis on the time variable showed that a significant difference existed between control values and days 4, 6, 8, 12, 15, and 18 for the iron supplement group (t < .975). Day 21 was not significantly different from control (t > .975). For the no supplement group, Dunnett's t showed a significant difference between control and days 4, 8, 10, 15, and 21 but no difference between control and day 12 and 18 (t > .975).

Serum ferritin concentrations for each subject, group mean values, plus standard error of the mean for each group are recorded in Table 7. An increase of 36 per cent between control and final ferritin determinations (79.5 - 107.5 ng/l) for the iron supplement group and a decrease of 25 per cent (260.0 - 194.5 ng/l) for the no supplement group was not significantly different upon statistical analysis for either the supplement effect or the time effect (p > .05).
### Table 7

Subject Serum Ferritin Concentrations (ng/l) Plus Group Means and Standard Error for Each Experimental Condition

<table>
<thead>
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<td></td>
<td></td>
</tr>
<tr>
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<td>65.8</td>
<td>81</td>
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<td>2</td>
<td>61.7</td>
<td>162.0</td>
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<td>21.0</td>
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<tr>
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</tr>
<tr>
<td>$\bar{X}$</td>
<td>78.5</td>
<td>107.5</td>
</tr>
<tr>
<td>$S_E$</td>
<td>33.2</td>
<td>34.8</td>
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</tbody>
</table>

<table>
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<th>Subject</th>
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<th>Day 21</th>
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</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
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<td>180.0</td>
<td>137</td>
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<tr>
<td>2</td>
<td>231.0</td>
<td>97</td>
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<td>3</td>
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<td>31.0</td>
<td>87</td>
</tr>
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<td>$\bar{X}$</td>
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<td>194.5</td>
</tr>
<tr>
<td>$S_E$</td>
<td>120.15</td>
<td>88.2</td>
</tr>
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</table>

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---
Stored Blood Data

Plasma sodium, plasma potassium ion and plasma hemoglobin data from the assay of aliquots sampled from each subject's stored blood over the duration of the experiment are recorded in Tables 8, 9, and 10 in Appendix A. The mean values plus the standard error of the mean for each of these parameters are shown in Table 11.

These data are graphically presented in Figure 4, and 5; Plasma Potassium and Plasma Sodium and in Figure 6; Plasma Hemoglobin. The subsequent days revealed a significant difference for the time effect over the twenty-one day duration of the experiment (t < .975).
Table 11

Mean Stored Blood Data Plus Standard Error
of Mean for All Subjects

<table>
<thead>
<tr>
<th>Days</th>
<th>Pre Phleb.</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>15</th>
<th>18</th>
<th>21</th>
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<tbody>
<tr>
<td>Plasma Potassium Ion (mEq/l)</td>
<td>$\bar{X}$</td>
<td>3.63</td>
<td>3.88</td>
<td>8.19</td>
<td>12.16</td>
<td>14.97</td>
<td>16.99</td>
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<tr>
<td></td>
<td>$S_{E\bar{X}}$</td>
<td>+ 0.21</td>
<td>0.13</td>
<td>0.37</td>
<td>0.85</td>
<td>0.50</td>
<td>0.83</td>
</tr>
</tbody>
</table>

| Plasma Sodium Ion (mEq/l) | $\bar{X}$ | 170.0 | 169.8 | 163.4 | 164.3 | 164.1 | 161.6 | 161.6 |
|                          | $S_{E\bar{X}}$ | + 0.81 | 3.43 | 0.64 | 0.5 | 0.6 | 0.78 | 0.46 |

| Plasma Hemoglobin (mg/l) | $\bar{X}$ | 23.25 | 46.5 | 63.2 | 59.7 | 48.0 | 89.3 | 92.9 |
|                         | $S_{E\bar{X}}$ | + 0.40 | 6.3 | 19.8 | 18.0 | 7.3 | 34.6 | 11.0 |
FIGURE 4. Mean Stored Blood Plasma Sodium Ion Values (mEq/L) for all Subjects.
FIGURE 5. Mean Stored Blood Plasma Potassium Ion Values (mEq/l) for all Subjects.
FIGURE 6. Mean Stored Blood Plasma Hemoglobin Values (mg/l) for all Subjects.
CHAPTER V

DISCUSSION

In the present study, all subjects selected for blood donation had hemoglobin values above 13.0 gm/100 ml. None had donated blood in the previous four months and all were free from hematologic disorders.

Prephlebotomy serum ferritin values were above minimal values indicating normal total body iron stores were present in all subjects. A large range, however, existed between subjects (14.7 ng/ml to 597.2 ng/ml). This large interindividuval variance can be ascribed to previous dietary or age factors.

The amount of blood venesected in each case was 450 ml, which corresponds to a red cell loss of 225 ml. Iron loss from this venesection was approximately 250 mg assuming normal iron concentrations within the hemoglobin molecule of the red cells (Guyton, 1971; Hausmann, 1976; Hoffman, 1973).

The total body shifts of iron and the effects of these shifts are important to the discussion of the results. It is evident from the results of Norrby et al. (1974), Ekenved et al. (1976) and Hausmann et al. (1976) that trauma such as acute blood loss, and iron deficiency, alters individual responses to iron mobilization, absorption, and
utilization. It is also evident from the work of Mendel et al. (1961) that these iron dynamics are affected by the severity of the trauma.

Figure 7 shows the comparison between the mean serum iron ion values for the iron supplement and no supplement groups over the duration of the experiment. Both the iron supplement and no supplement serum iron response were similar post-venesection of 450 ml of blood to various authors (Ekrenved, 1976; Magnussen, 1975; Norrby, 1971). Oral iron supplementation increased serum iron levels over control values (mean increase 12.5 mg/l), and the absence of iron supplement resulted in a decrease of circulating serum iron ion (mean decrease of 7.8 mg/l). In the present study, the difference between the experimental groups and between control and subsequent days were not, however, statistically significant. As a result, it can be concluded that the oral supplement had no effect on serum iron ion concentration.

Serum ferritin results of the experiment are consistent with the findings of other experiments (Finch, 1975; Magnussen, 1975). The no iron supplement ferritin results (Table 7) are similar to the results reported by Finch et al. (1975), in that a reduction of ferritin levels occurs as a result of blood donation. Finch (1975) reported a 50 per cent reduction in serum ferritin levels in subjects undergoing repeated blood donations over a three-year period of time. Similarly, results from the present study show a reduction
FIGURE 7. Mean Subject Hemoglobin Values (g/100 ml) for Iron Supplement and No Supplement Groups.
in serum ferritin levels of approximately 25 per cent after a single blood donation and no iron supplement.

No recent research could be found for iron supplemented ferritin response to blood loss, however, the work of Magnussen et al. (1975) using bone marrow hemosiderosis as evidence of iron storage, indicates iron supplemented blood donation results in maintained or elevated iron storage after blood donation. These results agree with the data presented in the present study in that ferritin values were found to increase approximately 36 per cent from 78.5 ng/ml to 107 ng/ml under conditions of iron supplement.

The results of the serum ferritin data could possibly explain the non-significant difference in serum iron concentration between the groups and could support the iron absorption model of Crosby (Figure 1). According to the data, and the model, the iron stores (as ferritin) in the presence of inadequate dietary iron could have been liberated to the plasma in response to the trauma of blood loss and increased iron requirement at the bone marrow. Thus, serum iron values are maintained but storage iron is lost as is the case in the non-iron supplement group. For the iron supplement group, dietary iron is likely absorbed in the mucosal epithelium and is released to the plasma in response to corporeal requirements. As a result, no iron is drawn from storage to meet erythropoietic requirements, and a slightly
elevated serum iron concentration is observed. Other iron storage tissues (spleen, liver, bone marrow) may, in fact, increase iron storage due to the elevated serum iron concentration as evidenced by serum ferritin values post-phlebotomy for the iron supplement group being above control values.

Figure 8 shows the comparison of subject circulating hemoglobin concentration by experimental group. From the data, it can be seen that the phlebotomy of 450 ml of whole blood reduced the mean subject hemoglobin concentration in the iron supplement and no iron supplement groups by 1.2 gm/100 ml and 1.1 gm/100 ml, respectively. These values represent consistent drops in hemoglobin, with the venesections reported by Ekblom (1972, 1976) and Spreit (1977). As a result, identical erythropoietic stimuli via similar tissue hypoxia could be expected.

As identified previously, erythrocyte maturation and the rate at which it ensues is dependent on humoral stimulus via erythropoietin and the presence of erythrocyte maturation factors such as iron and folic acid.

The data presented show that both experimental groups are exposed to similar erythropoietic stimulus via identical phlebotomies and hemoglobin reduction.

Figure 8 presents Serum Iron concentrations for both experimental groups. Group I (iron supplement) exhibits serum iron increases above control values due to increased
absorption from the supplement. Group II data (non-supplement) show slightly depressed serum iron concentrations as a result of corporeal demands for erythrocyte maturation. The two groups, however, are not significantly different with respect to serum iron concentration throughout the experiment.

The reduction of circulating hemoglobin concentration is equivalent for the two groups and the serum iron concentrations are not significantly different. As such, the erythropoiesis rate which is dependent on these two factors is not significantly different between groups (Figure 8) (p<.05).

Serum ferritin values, however, indicate that body iron stores are reduced in Group II (non-supplement) and increased in Group I (supplement) (Table 7), but are not statistically different.

Stored blood parameters, plasma hemoglobin, plasma potassium, and plasma sodium were monitored in order that an estimation of in vitro cell lysis could be obtained. This was necessary to establish an optimal reinfusion date based on minimal red cell destruction in storage.

Table II plasma hemoglobin, plasma potassium and plasma sodium, previously presented, show data from all eight subjects in the experiment. These results are generally consistent with the findings of reported studies of ACD stored blood.

The decline in plasma sodium as sodium entered the erythrocyte was less profound than the results of Bailey et al. (1975) but more closely paralleled the results of
Lysis indicators; plasma hemoglobin and plasma potassium were slightly lower than those reported by Bailey and Bove (1975). Plasma hemoglobin showed a rise from 46.5 mg/ml to 96.9 mg/ml at twenty-one days compared to a rise from 3.1 mg/100 ml to 19.1 mg/100 ml reported by Bailey (1975). Similarly, plasma potassium levels were slightly less elevated than those reported in the Bailey study (1975). The present experiment reports a rise of 14.22 mEq/l (range, 3.88 - 18.66) compared to a 17.1 mEq/l rise (range, 5.4 - 22.5) in the data reported by Bailey et al. (1975). The results reported by Gibson et al. (1961) and Orlinda and Josephson (1969) show more profoundly elevated lysis rates using plasma potassium and plasma hemoglobin as determinants in ACD stored blood.

Statistical analysis of the stored blood data indicates red cell lysis differed significantly from control values at day 18. This suggests that plasma hemoglobin liberated by in vitro red cell lysis remains relatively unchanged for the first fifteen days of storage and experiences a rapid rise beyond that time. Similar results have been noted in studies of red cell fragility due to adenosine triphosphate (ATP) depletion in the stored red cell (Dawson, 1972). Thus, it appears that blood stored in ACD anticoagulant medium should be reinfused to donor subjects within 15 days of the phlebotomy date if maximal hemoglobin values are to be realized post reinfusion.
CHAPTER VI

SUMMARY AND CONCLUSION

It was hypothesized that a group of subjects who received oral iron and folic acid supplements post-phlebotomy of approximately 450 ml of whole blood would exhibit a significantly increased erythropoiesis rate, as evidenced by elevated hemoglobin concentration, when compared to a similar group who received no supplement and exactly the same venesection.

To test this hypothesis, eight healthy active males were randomly assigned to two equal groups after control data of serum iron, serum ferritin, and hematologic values were collected. Each subject had 450 ml of whole blood venesected and stored in an ACD anticoagulant medium. One group was provided with a twenty-one day supply of an iron and folic acid supplement and instructed as to dosage. The other group received no supplement.

Serum iron, and subject hematologic data were collected throughout the duration of the twenty-one day experiment as well as lysis data consisting of plasma hemoglobin, plasma potassium and plasma sodium on the stored blood. A final serum ferritin determination was made at the conclusion of the experiment.
Statistical analysis indicated no significant difference between experimental groups on serum iron and subject hemoglobin values. Complete hemoglobin recoveries to pre-phlebotomy levels were observed in both groups. The rate of this return however, was independent of the iron supplement variable and contrary to the hypothesis that the supplemented group would return to control values prior to the iron supplement group (Figure 7).

Stored blood data showed red cell lysis remained relatively low until after day fifteen post-venesection. At that time, cell lysis rose dramatically as indicated by elevated plasma hemoglobin values and plasma potassium ion concentration.

The data do not support the hypothesis that oral iron supplements of this particular dosage significantly increase the erythropoietic response to a 450 ml phlebotomy from healthy male donor subjects. The results show that while absorption of iron appears greater in subjects receiving iron supplements, the serum iron levels are not significantly affected. This could be due to incomplete absorption of the supplemented iron in the gut, however, this parameter was not directly measured. A contributing factor which was measured however, was serum ferritin levels. These results indicated that storage iron throughout the body was likely mobilized after phlebotomy, utilized for
corporeal requirements in erythropoieses and not replaced in non-iron supplemented subjects. Iron supplemented subjects, in contrast, showed no reduction of total body iron stores.

Stored blood lysis indicators, i.e., plasma potassium, plasma hemoglobin and plasma sodium showed that blood stored in Acid-Citrate-Dextrose at +4°C undergoes significant lysis after 15 days of storage. Thus, for purposes of inducing maximum levels of polycythemia, conditions for autologous transfusions using this storage method would be optimal prior to 15 days post-venesection.

The present research indicates that for healthy iron replete males undergoing aerobic training, the supplementation of oral iron and folic acid does not augment the erythropoietic return to normal hemoglobin values post-phlebotomy of 450 ml of whole blood.

While the iron supplement resulted in no increase in erythropoiesis rate as hypothesized, the erythropoiesis rate of both groups was more rapid than reported by Spreit, et al. (1978).

Further, the results show that significant red cell lysis occurs during in vitro storage of whole blood after 15 days at +4°C in Acid-Citrate-Dextrose anticoagulant medium.

Thus, for purposes of producing significant polycythemias using liquid ACD storage techniques, iron supplementation...
appears to be of no value providing that adequate subject storage iron is present.

As well, the study shows that the autologous infusion of previously veseected blood stored in ACD should occur at or about two weeks post-phlebotomy.

Suggestions for Further Research

The results of a previous study in this laboratory which produced a rapid erythropoietic response using similar phlebotomy and storage techniques and subject iron supplementation were not duplicated.

This discrepancy could possibly be due to two factors which were not controlled in the present study. Firstly, the subjects in the previous study were adolescent athletes as opposed to the post-adolescent subjects in the present study. Iron stores were not estimated in the previous study, nor were subject serum iron values. Iron shift dynamics are known to change with age and this could account for the different hemoglobin response.

Secondly, the type of training employed during the erythropoiesis rebound period was different in the two experiments. A very intensive program of anaerobic and aerobic training during swimming comprised the work regimen in the first study. In the present study, aerobic training via cross-country running was used as the training mode. It is possible, therefore, that the high intensity program of the
previous study could have provided an additional stimulus for erythropoiesis. In the present study the training program may have been significantly less intensive and, as a result, erythropoiesis may not have been affected.

Further research focusing on the erythropoietic humoral response to the hypoxia of training could provide information which would be beneficial in producing a rapid hemoglobin rebound post-phlebotomy. As well, this research could provide evidence toward elucidating the factors causing athletic hematological changes such as elevated hematocrit, hemoglobin concentration, and blood volumes, which are evident even in the absence of post-exercise dehydration.

Additional implications of the study must include the scrutiny of the beneficial use of iron supplements in normal iron replete subjects. It appears that the trauma of blood donation results in a slight total body iron loss which is replenished by a twenty-one day supplement of a slow release iron preparation. However, even under the traumatic conditions of blood loss, no control subject became depleted of iron stores. This indicates that dietary iron is perhaps absorbed more rapidly after blood loss in order to reserve bodily iron stores. Therefore, the use of oral iron supplements when no pathology of iron deficiency is apparent is superfluous and could be potentially hazardous.

The limitations of the study must include the close scrutiny of the sample size used in the statistical analysis.
It is difficult to test hypotheses with confidence with such small sample sizes and large interindividual differences. Thus, a much larger sample size with a more homogenous population in relation to the variables studied would increase the confidence in testing significant effects of the manipulated variables. With respect to this study, it appears that a hemoglobin rebound rate was effected which could well have produced a significant polycythemia had the stored blood been reinfused in either group at day 14. It appears that provided subjects are healthy, they have adequate iron stores, and are not subjected to large amounts of blood loss (above 450 ml) the erythropoiesis rate may be augmented significantly through high intensity exercise.

In conclusion, the results of the study show that while iron availability for erythropoiesis is necessary, it does not appear to be a critical factor in erythropoiesis rebound rate of healthy males with adequate iron stores. It appears that iron is mobilized to meet corporeal demands for hemoglobin synthesis from body storage of these subjects and thus it is not a rate limiting factor. Rather, an important factor in producing a rapid hemoglobin synthesis response could be the mode of training used during the post-phlebotomy period prior to blood reinfusion. While hypoxia during training has not been well documented, the reduced oxygen concentration coupled with high oxygen utilization could
provide additional stimulus for erythropoiesis. Hence, an augmented erythropoiesis response coupled with optimal blood loss and reduced stored blood lysis may produce polycythemias which parallel those observed using the freeze-thaw methodology at a cost which is practical.
Table 3

Individual Subject Serum Iron Concentrations (mg/l)

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### Table 4

Individual Subject Hemoglobin Concentrations (gm/100 ml)

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### No Iron Supplement Group

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Table 8

Individual Subject Stored Blood Plasma Sodium Ion

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Table 9: Individual Subject Stimulation of Blood Potassium Ion Concentrations (mEq/l) by Experimental Day
Table 10

Individual Subject Stored Blood Plasma Hemoglobin

Concentrations (mg/l) by Experimental Day

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Test Subject Questionnaire

Please read and answer the following questions to the best of your knowledge. The questions are designed in order that certain health factors can be identified which could be significant to the study.

Name: ________________________  Birthdate: __________

1. Are you presently being treated for a medical disorder?

If so, What? A) ________________________

B) ________________________

Medications? ________________________

2. Do you regularly take vitamin or mineral supplements?

If so, what? ________________________

3. Are you presently on any form of restrictive diet?

Describe ________________________
4. Are you now being treated for allergies?

What are they?

5. Have you recently had a medical examination?

When?

6. Are you aware of any medical reason not previously described which would or could preclude your participation in the study?

Comments

It is advised that each subject inform his family doctor of the nature of the study in order that there are no contraindications.
CONSENT FORM FOR TEST SUBJECTS

Windsor Metropolitan Hospital
Windsor, Ontario
(University of Windsor)

Name: ____________________________
Address: __________________________
City: ______________________________
Telephone No.: _____________________
Next-of-Kin:
  Mother: __________________________
  Father: __________________________

The following is to be signed by the research subject, except in the case of a minor or of a subject who is not competent mentally, in which case it is to be signed by the spouse, parent, guardian, or next-of-kin.

The following is to be read over and explained to the signatory who stated that he/she understood same and offered his/her signature agreeing thereto.

1. RELEASE OF INFORMATION

I, the undersigned, hereby authorize Windsor Metropolitan Hospital to release any and all information from my medical record to any medical, social and/or educational authority where in the opinion of the hospital, this information will be used for the benefit of the subject.

I also authorize Windsor Metropolitan Hospital to release information as to the nature of my participation in the research study, for scientific or teaching purposes.

2. OBTAINING OF INFORMATION FROM OTHER AGENCIES

I, the undersigned, hereby authorize Windsor Metropolitan Hospital to obtain information from any medical, social and/or educational authority including medical records at any hospital which previously treated me.

3. MEDICAL PROCEDURES

I, the undersigned, understand that this study necessitates venipuncture to obtain the appropriate blood samples, and hereby consent to this procedure.
4. GENERAL INFORMATION

I, the undersigned, understand that this study may necessitate the occasional interview where details of diet and medication are divulged, and hereby consent to release such information.

5. GENERAL CONSENT

I, the undersigned, have been made aware of the relevant details of the study in which I am going to participate. The procedures to be followed in this study have been fully explained to me, and I agree to participate in the study.

Date: ______________ I have read 1, 2, 3, 4, and 5 above and give my consent/authorization to all five (5) sections.

Father ___________________________ Witness ___________________________
Mother ___________________________ Witness ___________________________
Guardian __________________________ Witness __________________________
Other ___________________________ Witness ___________________________
Request for Support of the Ethics Committee

University of Windsor

To:  Dr. R. T. Hermiston
From: Ronald G. Weese
Advisor: Dr. R. T. Hermiston
Committee: Dr. P. Taylor
Dr. R. J. Thibert
Dr. H. S. Asselstine

June 28, 1978
Purpose of the Study

The effects of an assisted recovery from a 500 ml phlebotomy using ferrous sulphate, folic acid and vitamin B12 on the erythropoiesis response will be investigated. Red cell in vitro survival rates will be measured in order that an optimal reinfusion date can be calculated. No re-infusion of the previously venuesected volumes will take place.

A rapid erythropoiesis response is anticipated in the assisted recovery group to allow for a maximal post-infusion hemoglobin response on the order of other investigators using freeze-thaw blood storage techniques.
Methods

Ten healthy males have volunteered for the study. (See Appendix for study proposal.) They have been informed of the nature of the experiment and have agreed to all procedures described herein. A copy of the informed consent form appears with this submission.

Prior to phlebotomy of 500 ml. of blood, each subject will report to the Pathology Department of Windsor Western Hospital Centre at which time control hematologic data will be collected. All blood samples will be via venepuncture of the anticubital vein by trained Registered Technologists at Windsor Western Hospital Centre. Appropriate blood samples will be obtained for the following determinations:

a) Complete Blood Count (CBC) Coulter (42)
b) Serum Potassium and Sodium Ion (56)
c) Plasma Hemoglobin (57)
d) Serum Iron Concentration (57)
e) Total Iron Binding Capacity (57)
f) Reticulocyte Count (56)

All assays will be performed by the experimenter, Mr. R. Weese, under the supervision of the members of the Thesis Committee.

After control hematologic values are determined and recorded, each subject will be randomly assigned to one of
two experimental groups by an assistant of the experimenter as follows:

Group 1 Assisted Recovery
Group 2 Non-Assisted Recovery

According to the experimental schedule, 500 ml. of blood will be removed from each subject using standard aseptic techniques for blood withdrawal by Registered Technologists at Windsor Western Hospital. The entire procedure will be under the supervision and responsibility of an attending physician.

The blood will be labelled and stored in a quarantined blood bank refrigerator at +4°C. Aliquots of whole blood from storage will be sampled according to the experimental schedule and the following determinations performed:

a) CBC Coulter (42)
b) Serum Sodium and Potassium Ion (57)
c) Plasma Hemoglobin (56)

During the entire experiment, Group 1 subjects will be required to inject daily a vitamin and mineral supplement containing ferrous sulphate, folic acid, and vitamin B12.

The supplement type will be approved by the physician in charge.

A lactose placebo will be taken orally daily by the non-assisted recovery group to complete the double blind experimental design.
Experimental Schedule

Day 1
Control data collection

Day 4
Repeat subject control data
500 ml. phlebotomy
control data on stored blood

Days 7, 11, 15, 19, 21
subject data collection

Days 8, 12, 16, 20, 22
stored blood data collection

Data collection will proceed until subject control
values are reached for hemoglobin and red cell count.
APPENDIX E
(1) **Red Cell Determination**

**Coulter Counter, Model S**

**Principle.** The counter is designed on the same principle as other earlier model Coulter counters. Using a mercury siphon, a specific volume of an electrolyte suspension of particles can be drawn through an aperture of specific dimensions. An aperture current exists between an electrode inside of the tube and another electrode outside the aperture, thus, the particles change the resistance between the two electrodes and produce a voltage drop. The magnitude of the voltage drop is proportional to the volume of the particle. The voltage pulses are fed into a threshold circuit, which discriminates between them by generating voltage count pulses for only the particles that exceed the threshold level, thus counting the number of particles in passage. The voltage pulses are amplified and displayed on an oscilloscope screen as distinct vertical spikes. Relative size is indicated by the relative height of the spikes. Pulses are also fed to a threshold circuit, allowing selection of a level which if reached by a pulse causes the pulse to be counted.

The Counter consists of five separate interconnected units: diluter, analyzer, power supply, pneumatic power supply and printer. The blood is aspirated into the diluter, mixed, lysed, diluted and sensed. The information obtained is processed by the Analyzer where the counting, measurement, and computing functions take place. From the Analyzer unit, signals in the form of voltages representing values are applied to the electronic circuits located in the power supply. These signals are then converted from voltage information to digital data for use by the printer where a numerical printout takes place.

The power supply provides the electrical power for all circuits as required, while the pneumatic power supply provides vacuum pressure for the diluter.

**Description of Model S Coulter Counter**

EDTA anticoagulated blood is placed under the aspirator and the touch control bar is depressed. Approximately 1.0 ml of blood is aspirated into the system.

In the blood sampling valve, a measured amount of blood and 10.0 ml of diluent are forced into the WBC Mixing Chambers. The blood and diluent are slowly mixed in the
chamber, providing a first dilution of 1:224. A portion of this first dilution is siphoned out of the WBC Mixing Chambers into the Blood Sampling Valve, where a measured amount of the first dilution is forced out by 10.0 ml of diluent into the RBC Mixing Chambers. Here, the first dilution slowly mixes with diluent to provide a second dilution of 1:50,000.

From the WBC Mixing Chambers, the first dilution is forced into the Lysing Chamber, where it is mixed with 1.0 ml of lysing agent, producing a 1:250 dilution. This dilution remains in the chamber for a sufficient amount of time for the red cells to hemolyze and release hemoglobin. The suspension is then passed into the WBC Aperture Counting Bath containing three aperture tubes. By means of a constant fluid vacuum, the sample is drawn into all three aperture tubes simultaneously for 4 seconds. Each aperture tube is provided with an internal electrode, and there is a common electrode in the bath acting as a ground. Three signals are obtained simultaneously by the passage of the WBC's into each aperture tube. In this manner, each count is done in triplicate and the average of the three counts recorded. Each aperture has its own set of electronics. Electronic circuitry contained in the analyzer unit provides an output from these circuits which directly displays an average white cell count.

Because free hemoglobin is present in the WBC count suspension, the hemoglobin measurement may be accomplished in the same bath. A beam of light is passed through the suspension and into a photosensitive device which measures the amount of light passing through the fluid. Electronic circuitry to the output of the photocell provides the hemoglobin reading.

Simultaneously, the red cell suspension passes into a similar bath having three aperture tubes. As in the WBC arrangement, the scanning occurs for a 4-second period representing a flow of a given volume of liquid into all three aperture tubes. The counting is carried out in the same manner as the white count; in triplicate and the results averaged. The mean cell volume measurements are electronically derived from the red count and are recorded.

The remaining red cell indices are electronically derived from the hemoglobin, red cell count and mean red cell volume. The hematocrit is calculated from the formula:

\[ Hct = \text{Red Cell Count} \times \text{M.C.V.}/100. \]
The model S is also capable of testing capillary blood. A small volume (44.7 ml) of capillary blood is diluted with 10.0 ml of filtered buffered saline or Isoton diluent. The whole blood 1:224 dilution switch is turned into position, and the blood aspirated through the capillary blood aspirator. The results are obtained as previously outlined.

The Pneumatic Power Supply provides for the filling and emptying of the various vessels as well as for discharging waste. Once started, the operations are continuous and automatic, and the respective samples do not contaminate each other.

Reagents and Apparatus.
1. Model S Coulter Counter
2. EDTA anticoagulated blood
3. Cell diluting fluid (Isoton)
4. Cleaning reagent (Istoterge)
5. Lysing agent (Lyse S)

Method
1. The instrument is set up as in the manufacturer's instructions.
2. A tube of EDTA anticoagulated blood is placed under the aspirator tip, and the touch control gently pressed.
3. Approximately 1.0 ml of whole blood is aspirated and in 20 seconds the results are recorded by the printer.

(2) Reticulocyte Counts

Reticulocytes are young red cells, containing, within the cell membrane, a proportion of ribonucleoprotein which was present in larger amounts in the cytoplasm of red cell precursors. This basophilic material has the property of reacting to certain dyes and forming a blue granular precipitate. The most mature cells are those with the least granulation.

Methods of Counting

Reagents and Apparatus (Indirect Methods)
1. Diluting fluid: 0.4 g of brilliant cresyl blue or new methylene blue are added to 80 ml of normal saline and 20 ml of 3% sodium citrate. Filter before use.
2. Pasteur pipet
3. 1% Aqueous eosin
4. Eyepiece stocks
5. Tube - 10 x 75 mm

Method

1. Approximately 0.01 g of brilliant cresyl blue or new methylene blue is added directly to anticoagulated whole blood.

2. Mix the blood and stain, and leave at room temperature for 10 minutes.

3. Blood smears are prepared in the same way as in the previous method, and step 5 is carried out as previously described.

Normal Values

Adults ........ 0.2 - 2%
Infants ........ 2.0 - 6%

Notes:

1. An area of the smear should be counted where the cells are undistorted and where the staining is good.

2. The cells should be counted using a high power oil immersion lens 97x.

3. If the brilliant cresyl blue is increased in strength, the reticulum fragments appear larger and more easily defined.

4. If the stain is warmed, the reticulum appears with fine granulation and can be easily missed.

5. Ensure that the stain has an alkaline pH; an acid pH results in a fine granular reticulum.

6. The presence of glucose or sodium salts inhibits the staining reaction.

(3) Estimation of Plasma Haemoglobin

Principle. The catalytic action of haem-containing proteins brings about the oxidation of benzidine by hydrogen peroxide to give a green colour which changes to blue and finally to reddish violet. The intensity of the colour may be compared in a photoelectric colorimeter with that produced by solutions of known Hb content. Methaemalbumin and Hb are measured together.
Every effort must be made to prevent haemolysis during the collection and manipulation of the blood. A "clean" venepuncture is essential.

Reagents

**Benzidine Reagent.** 1 g of pure benzidine base is dissolved in 90 ml of glacial acetic acid and made up to 100 ml with water. The solution will keep for several weeks in a dark bottle at 4°C.

**Hydrogen Peroxide.** One volume of 3% ("10 vols") H₂O₂ is diluted with two volumes of water before use.

**Acetic Acid.** 100 g/l glacial acetic acid.

**Standard.** A blood sample of known Hb content is diluted with water to a final concentration of 200 mg/l. It is convenient to use a HiCN standard solution (p 31) as the source of Hb.

Method

20 ul of plasma are added to 1 ml of the benzidine reagent in a large glass tube. A control tube, in which 20 ul of water are substituted for the plasma, and a standard tube, containing 20 ul of the Hb standard, are also set up. 1 ml of the H₂O₂ solution is added to each tube and the contents are well mixed.

The mixtures are allowed to stand at c 20°C for 20 min and then 10 volumes of the acetic acid solution are added to each tube and, after mixing, the tubes are allowed to stand for a further 10 min. The colours developed are compared at 515nm or in a photoelectric colorimeter provided with a green (e.g., Ilford 624) filter, using the colour developed by the control tube as a blank. If the Hb content of the plasma to be tested is abnormally high, the plasma should be diluted until it is just visibly tinged red. As benzidine is potentially carcinogenic, the reagent must be handled with care; its manufacture is limited, and in some countries it is no longer readily available. A modified method has been described in which orthotolidine is substituted for benzidine. However, orthotolidine may be equally dangerous and there seems to be no advantage in its use. In the laboratory, the use of benzidine presents no greater hazard than do many other chemicals, provided that it is carefully used.
(4) Estimation of Serum Iron

The method to be described for the estimation of serum iron is that recommended by the International Committee for Standardization in Haematology. It is based on the development of a coloured complex when ferrous iron is treated with bathophenanthroline (4:7-diphenyl-1:10-phenanthroline).

Reagents and Materials

Protein Precipitant. Trichloracetic acid 100 g/l and thioglycollic acid 30 ml/l in 2 m-HCl. This solution should be stored in a dark brown bottle; it is stable for 2-3 months.

Chromagen Solution. 2 m-sodium acetate, containing 250 mg of sulphonated 4:7-diphenyl-1:10-phenanthroline (bathophenanthroline sulphonate, BDG or Sigma).

Iron Standard. Stock. 100 mg of freshly cleaned pure iron wire are dissolved in 4 ml of 500 ml/l HCl and the volume made up to 1l with water.

Iron Standard, Working. 2 ml of stock are diluted to 100 ml with water (= 2 mg/l).

Preparation of Glassware. It is essential to avoid contamination by iron. All glassware, including reagent bottles, should therefore be washed in a detergent solution, soaked in 500 ml/l HCl for 24 h and finally rinsed in iron-free water.

Iron-Free Water. De-ionized, double-distilled water is used for the preparation of all solutions and for rinsing glassware.

Method

2 ml of serum (free of haemolysis), 2 ml of working iron standard and 2 ml of iron-free water (as a blank) are placed, respectively, in three separate iron-free test tubes. 2 ml of protein precipitant are added to each. The contents are mixed vigorously, e.g., with a vortex mixer, and allowed to stand for 5 min. The tube containing the serum is centrifuged to obtain an optically clear supernatant. To 2 ml of this supernatant and to 2 ml of each of the other mixtures are added 2 ml of chromagen solution with thorough mixing. After standing for 5 min., the absorbance is
measured in a spectrophotometer against water at 535 nm.

Calculation

\[
\text{Serum iron (mg/l)} = \frac{A_{535}^{\text{test}} - A_{535}^{\text{blank}}}{A_{535}^{\text{standard}} - A_{535}^{\text{blank}}} \times 2
\]

To convert to umol/l, the result in mg/l must be multiplied by 18 (i.e., 1000/55.8).

(5) **(125I) Ferritin Radioimmunoassay**

I. **Proprietary Name**

GammaDab (125I) Ferritin Radioimmunoassay Kit

II. **Intended Use**

The GammaDab (125I) Ferritin Radioimmunoassay Kit is to be used for the quantitative determination of the concentration of ferritin in serum or heparinized plasma.

III. **Explanation of the Test**

Ferritin, an iron-containing protein with a molecular weight of approximately 450,000 is predominantly found in the cytoplasm of hepatic and reticuloendothelial cells. It is the primary storage compound from which iron is mobilized to the transferrin bound plasma pool. While most of the body's ferritin is intracellular, small but clinically significant amounts are found in serum. Serum ferritin is secreted in constant proportion to the total iron stores of the body except in certain conditions listed in Section IX.B. Therefore, the measurement of serum ferritin is believed to reflect total iron stores of the body. The detection and quantitation of serum ferritin has been made practical through immunoradiometric technology. A number of investigators have confirmed this relationship and clinically validated serum ferritin determinations in normal patients, patients with iron deficiency, and iron overload. The serum ferritin determination has been suggested as a reliable means of monitoring iron levels and the amount of iron therapy required by patients on hemodialysis.
The advantages of the serum ferritin measurement, in comparison with other means of assessing iron status (e.g., hemoglobin, serum iron, transferrin saturation, red cell protoporphyrin, etc.) are that it can resolve differences within the physiological range and provide data on certain iron deficiency states that previously were only detectable after body iron stores were totally depleted. The serum ferritin determination provides a direct measurement or iron stores, and thus aids in the differentiation between absolute iron deficiencies due to chronic blood loss (in which iron stores are absent) and relative iron deficiencies due to impaired release of iron from stores.

IV. Principles of the Method

The GammaDab (125I) Ferritin Radioimmunoassay is a competitive binding assay, which utilizes a precipitating antiserum reagent to separate antibody-bound tracer from unbound tracer.

The procedure is based on the competitive binding principles of radioimmunoassay as developed by Yalow and Berson. Non-radioactive ferritin from patient samples, ferritin standards, and controls compete with a constant amount of (125I) ferritin tracer for binding sites on the ferritin antibody, which is held at a limiting concentration. The amount of (125I) ferritin tracer which will bind to the antibody is inversely proportional to the amount of non-radioactive ferritin present in the assay tube.

The precipitating reagent solution containing a second antibody in a polymer solution is used to separate the antibody-bound (125I) ferritin from unbound (125I) ferritin by immunoprecipitation. The assay tubes are then centrifuged and the supernatants are decanted. The antibody-bound (125I) ferritin, which is located in the centrifugal pellet, is counted in a well type, solid crystal gamma counter. A standard curve is constructed and the ferritin concentrations of the patient samples are read from the standard curve.

It is important to note that recent research indicates that the concentration of serum ferritin measured by radioimmunoassay is markedly affected by the isoferritin
and subunit composition of the ferritin sample, as well as by the nature of the ferritin antiserum employed. Drysdale and co-workers proposed that crystalline human liver ferritin be used as the immunogen, labelled antigen, and reference standards for serum ferritin radioimmunoassay determinations used to evaluate iron status.

Crystalline human liver ferritin has a subunit composition almost identical to that of serum ferritin. It also exhibits a consistently homogeneous isoferitnin population. The GammaDab (125I) Ferritin Radioimmunoassay Kit utilizes crystalline human liver ferritin for the preparation of standards, serum controls, tracer, and antibody.

V. Reagents

A. (125I) Ferritin Tracer

Each vial contains approximately 2 uCi of Tracer in Phosphate Buffered Saline with 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

B. Rabbit Anti-Ferritin Serum

Each vial contains Rabbit Anti-Ferritin serum in Phosphate Buffered Saline with 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

C. Precipitating Solution

Each vial contains a water soluble polymer in Phosphate Buffer with 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

D. Assay Buffer

Each vial contains Phosphate Buffered Saline with 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

E. Precipitating Antiserum (Lyophilized)

Upon reconstitution, each vial contains Goat Anti-Rabbit Serum in Phosphate Buffer with 0.003 M Sodium Azide as a preservative. Store at 2-8°C.
F. Ferritin Control Level 1 - 10 ng/ml
Each vial contains Ferritin in Phosphate Buffered Saline, Bovine Serum Albumin, and 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

G. Ferritin Control Level II - 100 ng/ml
Each vial contains Ferritin in Phosphate Buffered Saline, Bovine Serum Albumin, and 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

H. Ferritin Blank
Each vial contains Phosphate Buffered Saline, Bovine Serum Albumin, and 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

I. Ferritin Standards
Each vial contains Ferritin in Phosphate Buffered Saline, Bovine Serum Albumin, and 0.003 M Sodium Azide as a preservative. Store at 2-8°C.

The source materials from which the controls, standards, and tracer were derived were found non-reactive for HB Ag by a FDA licensed third generation test. No known test method can assure that a product derived from human source does not contain the hepatitis virus.

CAUTION: HANDLE AS IF CAPABLE OF TRANSMITTING HEPATITIS. For in vitro diagnostic use only.

VI. Specimen Collection and Handling
A venous blood sample is collected aseptically using an evacuated glass tube without additives. Heparinized plasma collected in a green-top evacuated glass tube is also suitable for assay.

The serum fraction is separated for use in assay. Samples containing a visible precipitate may be used in the assay only if the precipitate is separated and removed by centrifugation. Do not use grossly hemolyzed or dipemic specimens.

Samples may be stored up to seven days at 2-8°C. If
the length of time between sample collection and analysis is greater than seven days, the sample should be stored frozen at -20°C. Samples may be stored frozen for as long as six months without altering the ferritin content. Freeze-thaw cycles must be avoided since they may denature the ferritin molecule.

Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37°C bath. Do not vortex or sharply agitate samples since this may result in ferritin denaturation.

VII. Test Procedure

A. Quality Control

The reagent components supplied in this kit are intended for use as an integral unit. Do not mix various lots of any component reagent within an individual assay. Do not use reagents beyond the expiration date shown.

Ferritin Controls at 2 levels are provided in the kit; the nominal values are:

Control Level I: 10 ± 2 ng/ml

Control Level II: 100 ± 20 ng/ml

Each laboratory should utilize controls at levels to monitor assay performance. The controls should be treated as unknowns. Quality control charts should be maintained to follow the performance of the controls. Appropriate statistical methods should be used to evaluate trends. Acceptable performance limits should be ascertained for the individual laboratory using methods such as those of Tonks and Rodbard et al.

B. Equipment and Supplies Required

The following items which are not provided with the kit are required to perform the assay.

1. Test tube racks (e.g., Fisher, Cat. No. 14809-A)
2. Plastic or glass test tubes (12 x 75 mm)
3. Precision pipets (25, 50, 100 and 500 ul)  
4. Constant temperature water bath, 37°C  
5. Semi-automatic adjustable pipet (0.1 to 1.0 ml delivery). Used for pipetting Precipitating Antiserum Reagent.  
6. Refrigerated centrifuge (or centrifuge in cold room)  
7. 500 ml beaker (for decanting of supernatant after centrifugation of precipitate)  
8. Gamma counter  
9. Distilled water  
10. Plastic-backed absorbing paper (optional, for blotting tubes following decantation)

C. Assay Procedure

All reagents except the Tracer-Buffer Reagent and the Precipitating Antiserum Reagent are supplied in a ready-to-use form.

1. Tracer-Buffer Reagent: Add the entire contents of one vial of the (125I) Ferritin Tracer (Cat. No. CA-674) to the 56 ml container of Assay Buffer (Cat. No. CA-744) and mix gently. A final rinse of the tracer vial (Cat. No. CA-674) with the Tracer-Buffer Reagent will maximize transfer of the tracer.

The auxiliary Tracer-Buffer Reagent label (supplied) is filled out and affixed to the container for proper identification. May be stored 60 days at 2-8°C.

2. Precipitating Antiserum Reagent: Reconstitute the Precipitating Antiserum (Cat. No. CA-139) by carefully pipetting 6.5 ml of room temperature distilled water into the vial. Allow vial to stand for 15 minutes without mixing. Then swirl gently or invert capped vial to mix.

Add the entire contents of the reconstituted Precipitating Antiserum to the bottle containing 58.5 ml of the Precipitating Solution (Cat. No. CA-746). A final rinse of the Precipitating Antiserum Reagent will maximize transfer of the Precipitating Antiserum.
To insure a homogeneous mixture of the Precipitating Antiserum Reagent, it should be stirred continuously with a magnetic spinbar and stirring apparatus or be hand-swirled every 10 to 15 pipettings.

The auxiliary Precipitating Antiserum Reagent label (supplied) is filled out and affixed to the container for proper identification.

3. Standards and controls should be mixed by inversion or gentle swirling rather than vortexing.

Storage and Stability: All reagents, including the Tracer-Buffer Reagent, are stable for at least two months, when stored at 2-8°C.

The assay procedure includes the preparation of a standard curve where known amounts of ferritin are used to compete with a fixed amount of (125I) ferritin for sites on the ferritin antibody. This standard curve is used to determine the unknown ferritin content in the sample.

4. Label the tubes in duplicate according to the following scheme. Allow all reagents to reach ambient temperature and gently mix before using.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Contents of Tube</th>
<th>Ferritin Added (ng in 0.025 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1, T2</td>
<td>Total Counts (Tracer)</td>
<td>--</td>
</tr>
<tr>
<td>1, 2</td>
<td>Ferritin Blank, 0 ng/ml B</td>
<td>0 ng</td>
</tr>
<tr>
<td>3, 4</td>
<td>Ferritin Standard, 5.0 ng/ml</td>
<td>.125 ng</td>
</tr>
<tr>
<td>5, 6</td>
<td>Ferritin Standard, 20 ng/ml</td>
<td>.05 ng</td>
</tr>
<tr>
<td>7, 8</td>
<td>Ferritin Standard, 50 ng/ml</td>
<td>1.25 ng</td>
</tr>
<tr>
<td>9, 10</td>
<td>Ferritin Standard, 200 ng/ml</td>
<td>5.0 ng</td>
</tr>
<tr>
<td>11, 12</td>
<td>Ferritin Standard, 500 ng/ml</td>
<td>12.5 ng</td>
</tr>
<tr>
<td>Tube No.</td>
<td>Contents of Tube</td>
<td>Ferritin Added (ng in 0.025 ml)</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>13,14</td>
<td>Ferritin Control Level I 10 ng/ml</td>
<td>0.25 ng</td>
</tr>
<tr>
<td>15,16</td>
<td>Ferritin Control Level II 100 ng/ml</td>
<td>2.5 ng</td>
</tr>
<tr>
<td>17,18</td>
<td>Patient &quot;X&quot; Serum Sample</td>
<td>&quot;X&quot; ng</td>
</tr>
</tbody>
</table>

5. Add 25 microliters of Ferritin Blank, 0 ng/ml (Cat. No. CA-235) to the blank tubes (1,2).

6. Add 25 microliters of each Ferritin Standard to the appropriate tubes in duplicate:

<table>
<thead>
<tr>
<th>Standard Concentration</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 ng/ml</td>
<td>CA-236</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>CA-237</td>
</tr>
<tr>
<td>50 ng/ml</td>
<td>CA-238</td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>CA-239</td>
</tr>
<tr>
<td>500 ng/ml</td>
<td>CA-240</td>
</tr>
</tbody>
</table>

7. Add 25 microliters of each Ferritin Control, Level I (Cat. No. CA-417) and Level II (Cat. No. CA-418), to the appropriate tubes in duplicate.

8. Add 25 microliters of sample to the appropriate tubes in duplicate.

9. Add 100 microliters of Rabbit Anti-Ferritin Serum (Cat. No. CA-138) to all tubes except Total Counts tubes (T1, T2). Mix reagents by carefully shaking test tube rack or gently mixing each tube.

10. Add 500 microliters of Tracer-Buffer Reagent to all tubes. Mix reagents by carefully shaking test tube rack or gently mixing each tube.

11. Incubate tubes in a constant temperature water bath for three hours at 37°±2°C.
12. Add 500 microliters of Precipitating Antiserum Reagent to all tubes (except Total Counts). Mix reagents by carefully shaking test tube rack or gently mixing each tube.

13. Incubate all tubes in a constant temperature water bath for thirty minutes at 37±2°C.

14. Immediately centrifuge all tubes (except Total Counts) for fifteen minutes at a minimum relative centrifugal force (RCF) of 850 x g in a refrigerated centrifuge at 4°-12°C.

15. Carefully decant each tube (except Total Counts). Tap the rim of the tubes to an absorbent surface to remove any adhering supernatant. Failure to remove adhering supernatant may result in poor replication and spurious values.

16. Count all tubes, including Total Counts, in a gamma counter adjusted for (125I) for one minute.

17. Calculate results; refer to Section-VIII.

18. If the initial ferritin determination is "off-scale" (500 ng/ml), the sample may be reassayed by making a one to ten (1:10) dilution of the serum sample. This dilution is performed by adding 50 microliters of patient serum to 450 microliters of the Ferritin Blank 0 ng/ml (Cat. No. CA-235).

VIII. Results

A. Standard Curve

Construct a standard curve by plotting the CPM BOUND for each standard point against the corresponding Ferritin concentration (ng/ml) using the 3-cycle semi-logarithmic paper supplied.

1. Record the CPM Bound for each tube.
B. Controls and Patient Samples

Locate the CPM BOUND for each tube which corresponds to Control Levels I and II and each sample on the vertical axis and draw a horizontal line intersecting the standing curve. At the point of intersection, read Ferritin Concentration (ng/ml) from the horizontal axis.

IX. Limitations

A. Procedural

A thorough understanding of this package insert is necessary for successful use of the product. Reliable and optimal results will be obtained by using careful laboratory techniques and strict adherence to the package insert.

The user should note that erroneous results can be caused by improper handling of patient samples. Serum samples can be stored at 2-8°C for up to seven days prior to assay. If there is a longer time interval between collection of sample and the assay, the sample should be stored frozen. When thawing a frozen sample, allow sample to thaw at room temperature. Do not thaw samples in 37°C water bath. Avoid freeze-thaw cycles. After removing a serum sample from the refrigerator, allow it to equilibrate at room temperature and then mix before pipetting. Improper storage of samples may result in decreased assay accuracy and precision.

Moderate hemolysis will not interfere with the assay. Grossly hemolyzed or lipemic serum samples will interfere with the assay. Samples containing a visible precipitate may be used only if the precipitate is separated and removed by centrifugation. Samples which reflect ferritin values of >500 ng/ml may be reassayed after dilution. Refer to Section VII D (Radioimmunoassay Procedure, Item 19).

After removing assay reagents from the refrigerator, allow them to equilibrate to room temperature before pipetting. Assay reagents should be stored at 2-8°C and freeze-thaw cycles should be
avoided. Standards and controls should be mixed by inversion or gently swirling rather than vortexing. Mix reagents by carefully shaking the test tube rack or carefully mixing each tube.

Failure to attain the appropriate serum ferritin values for the controls indicates deterioration of reagents, imprecise manipulations, or improper sample handling. Failure to adequately blot tubes following decantation may result in poor replication and spurious values. A standard curve must be established for every run. The ferritin controls must be included in every run.

Incubation conditions should be checked routinely. Temperature in the water bath must be constant at 37°C ± 2°C and the water level kept above that of the level of the solution in the tubes.

To insure a homogeneous mixture, the Precipitating Antiserum Reagent should be stirred continuously with a magnetic spinbar and stirring apparatus or be hand-swirled every 10 to 15 pipettings.

B. Interpretative

It has been shown that the concentration of ferritin in male sera is 2 to 3 times higher than that found in female sera. This difference is consistent with the known sex difference in iron stores. However, this difference is not apparent during childhood, adolescence, and old age. Elevation of serum ferritin values above normal is observed commonly in anemic states from causes other than iron deficiencies. Any decrease in serum ferritin level may be interpreted as iron depletion, but an increased level does not necessarily mean iron overload.

Certain disease states are known to elevate serum ferritin concentrations independent of the patient's iron stores. The following are examples of such disease states:
1. **Hepatic diseases including:**
   a) cirrhosis
   b) drug or viral induced hepatic necrosis
   c) hepatitis
   d) obstructive jaundice
   e) primary hepatoma
   f) metastatic carcinoma to the liver

2. **Hematopoietic diseases including:**
   a) anemia of chronic disease
   b) hemolytic anemia
   c) pernicious anemia
   d) leukemia

3. **Others:**
   a) Hodgkin's disease
   b) polycythemia
   c) rheumatoid arthritis
   d) fever, inflammation
   e) non-specific tissue damage
   f) general carcinomas

X. **Expected Values**

<table>
<thead>
<tr>
<th>Range</th>
<th>Normal Range</th>
<th>Iron Deficiency Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-300 ng/ml</td>
<td>less than 10 ng/ml</td>
</tr>
</tbody>
</table>

**Normals**

Results from several independent academic laboratories indicate that the normal range for serum ferritin is between 10 and 300 ng/ml. Normal values for both sexes are normally distributed on a logarithmic scale and the mean for the normal males varied from 52 to 103 ng/ml, while the mean for normal females was substantially lower, 29 to 36 ng/ml. The differences in serum ferritin concentration on the basis of sex, parallels the known differences in storage iron levels between males and females. Correlation data taken from quantitative phlebotomies, iron absorption measurements, urinary excretion of oxelated iron, bone marrow and liver biopsies, and hematological parameters suggest that 1 ng/ml of serum ferritin represents 8 tp 10 mg of storage iron. For a group of normal males and females with mean serum ferritin concentrations of 69 and 35
ng/ml, respectively, the corresponding mean iron stores are 552 to 690 mg in men and 280 to 350 mg in women. In normals, serum ferritin concentrations are inversely correlated with TIBC and iron absorption. Quantitation of serum ferritin has been found to be at least as reliable as iron absorption determinations in assessing iron stores.

Iron Deficiency

Serum ferritin concentrations less than 10 ng/ml are usually associated with transferrin saturation less than 15%. The reduction of transferrin saturation results as a consequence of impaired iron delivery to the plasma pool. The values stated above mark the lower limit of storage iron which is adequate to meet erythropoietic requirements.

Iron Overload

Patients with iron overload due to either transfusional siderosis or primary idiopathic hemochromatosis have greatly increased serum ferritin levels. Mean circulating concentrations of 2646 to 2930 ng/ml with a range of 940 to 4240 ng/ml have been reported.

Children (Normals and Iron Deficiency)

In normal infants and children, the concentration of serum ferritin parallels known changes in iron stores during development. Children with iron deficiency anemia have serum ferritin concentrations less than 9 ng/ml with little overlap in the normal child population.
Slow-Fe Folic

Ferrous Sulfate – Folic Acid  Hematinic-Hematopoietic

INDICATIONS: Prophylaxis of iron and folic acid deficiencies and treatment of megaloblastic anemia, during pregnancy, puerperium and lactation.

CONTRAINDICATIONS, PRECAUTIONS AND ADVERSE EFFECTS: The use of folic acid in the treatment of pernicious (Addisonian) anemia, in which Vitamin B₁₂ is deficient, may return the peripheral blood picture to normal while neurological manifestations remain progressive.

OVERDOSE: SYMPTOMS AND TREATMENT: Signs of toxicity from folic acid have not been observed even with doses several times higher than usual therapeutic levels.

DOSAGE: Prophylaxis: 1 tablet daily throughout pregnancy, puerperium and lactation. To be swallowed whole at any time of day regardless of meal times.
Treatment of megaloblastic anemia: during pregnancy, puerperium and lactation; in multiple pregnancy: 2 tablets, in a single dose, should be swallowed daily.

SUPPLIED: Each off-white, film-coated tablet contains: dried ferrous sulfate 160 mg and folic acid 400 mcg in a slow-release base.
Table 12

Analysis of Variance Summary for Subject Hemoglobin

Critical $F$ at $0.95 (1, 6) = 5.99$,

$F$ at $0.95 (7, 42) = 2.23$

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>5.46400</td>
<td>1</td>
<td>5.46400</td>
<td>0.43</td>
</tr>
<tr>
<td>Error</td>
<td>76.80133</td>
<td>6</td>
<td>12.80022</td>
<td></td>
</tr>
<tr>
<td>Across trials</td>
<td>8.94483</td>
<td>7</td>
<td>1.27783</td>
<td>10.86*</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.56484</td>
<td>7</td>
<td>0.08069</td>
<td>0.69</td>
</tr>
<tr>
<td>Error</td>
<td>4.94157</td>
<td>42</td>
<td>0.11766</td>
<td></td>
</tr>
</tbody>
</table>
Table 13

Analysis of Variance Summary for Subject Serum Iron

Critical $F$ at 0.05 (1, 6) = 5.99

$F$ at 0.05 (7, 42) = 2.23

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2747.08203</td>
<td>1</td>
<td>2747.08203</td>
<td>1.70</td>
</tr>
<tr>
<td>Error</td>
<td>9688.92188</td>
<td>6</td>
<td>1614.82031</td>
<td></td>
</tr>
<tr>
<td>Across trials</td>
<td>5163.58203</td>
<td>7</td>
<td>737.65454</td>
<td>1.42</td>
</tr>
<tr>
<td>Interaction</td>
<td>2880.26953</td>
<td>7</td>
<td>411.46704</td>
<td>0.79</td>
</tr>
<tr>
<td>Error</td>
<td>21826.26953</td>
<td>42</td>
<td>519.67285</td>
<td></td>
</tr>
</tbody>
</table>
Table 14

Analysis of Variance Summary for Subject Ferritin Concentrations

Critical $F$ at 0.95 (1, 6) = 5.99

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>72132.375</td>
<td>1</td>
<td>72132.375</td>
<td>1.56</td>
</tr>
<tr>
<td>Error</td>
<td>278016.000</td>
<td>6</td>
<td>46336.000</td>
<td></td>
</tr>
<tr>
<td>Across trials</td>
<td>1330.42578</td>
<td>1</td>
<td>1330.42578</td>
<td>0.49</td>
</tr>
<tr>
<td>Interaction</td>
<td>8944.41406</td>
<td>1</td>
<td>8944.41406</td>
<td>3.30</td>
</tr>
<tr>
<td>Error</td>
<td>16270.13281</td>
<td>6</td>
<td>2711.68872</td>
<td></td>
</tr>
</tbody>
</table>
### Table 15
Analysis of Variance Summary Tables for Stored Blood Parameters

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Across trials</td>
<td>28993.96</td>
<td>6</td>
<td>4832.32</td>
<td>3.28*</td>
</tr>
<tr>
<td>Error</td>
<td>61837.04</td>
<td>42</td>
<td>1472.31</td>
<td></td>
</tr>
</tbody>
</table>

Critical F at 0.95 (6, 42) = 2.32

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Across trials</td>
<td>1258.69</td>
<td>5</td>
<td>251.74</td>
<td>127.13*</td>
</tr>
<tr>
<td>Error</td>
<td>69.30</td>
<td>35</td>
<td>1.98</td>
<td></td>
</tr>
</tbody>
</table>

Critical F at 0.95 (5, 35) = 2.49

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Across trials</td>
<td>368.68</td>
<td>5</td>
<td>73.74</td>
<td>4.07*</td>
</tr>
<tr>
<td>Error</td>
<td>634.12</td>
<td>35</td>
<td>18.11</td>
<td></td>
</tr>
</tbody>
</table>

Critical F at 0.95 (5, 35) = 2.49
Dunnett's 't' Equation

\[ t = \frac{\bar{T}_C - \bar{T}_E}{\sqrt{MS \text{ error} / n}} \]

where:
- \( \bar{T}_C \) = mean of treatment cell compared to control
- \( \bar{T}_E \) = mean of control value

- MS error = mean square error of analysis of variance
- \( n \) = number of subjects in each treatment

Critical value for the collection of \( K-1 \) statistics of this form is obtained from the Dunnett's tables (Winer, 1971). The critical value for a two-tailed 0.05 level test is \( t_{.975} (K, df) \).
Table 16
Dunnett t Summary Tables for Subject Hemoglobin, Stored Blood Plasma Hemoglobin, Stored Blood Plasma Potassium Ion and Stored Blood Plasma Sodium Ion

A) Subject Hemoglobin

<table>
<thead>
<tr>
<th>Day Comparison to Control</th>
<th>t ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td>5.90*</td>
</tr>
<tr>
<td>Day 6</td>
<td>6.08*</td>
</tr>
<tr>
<td>Day 8</td>
<td>5.90*</td>
</tr>
<tr>
<td>Day 12</td>
<td>3.39*</td>
</tr>
<tr>
<td>Day 15</td>
<td>5.20*</td>
</tr>
<tr>
<td>Day 18</td>
<td>3.09*</td>
</tr>
<tr>
<td>Day 21</td>
<td>3.80*</td>
</tr>
</tbody>
</table>

Critical t \( .975 = 2.73 \)

B) Stored Blood Plasma Hemoglobin

<table>
<thead>
<tr>
<th>Day Comparison to Control</th>
<th>t ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.00</td>
</tr>
<tr>
<td>Day 4</td>
<td>2.08</td>
</tr>
<tr>
<td>Day 8</td>
<td>1.90</td>
</tr>
<tr>
<td>Day 15</td>
<td>1.29</td>
</tr>
<tr>
<td>Day 18</td>
<td>3.44</td>
</tr>
<tr>
<td>Day 21</td>
<td>3.63</td>
</tr>
</tbody>
</table>

Critical t \( .975 = 2.67 \)
Table 16 (cont'd)

Dunnet t Summary Tables

C) Stored Blood Potassium Ion

<table>
<thead>
<tr>
<th>Day Comparison to Control</th>
<th>t ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td>6.16*</td>
</tr>
<tr>
<td>Day 8</td>
<td>11.84*</td>
</tr>
<tr>
<td>Day 15</td>
<td>15.87*</td>
</tr>
<tr>
<td>Day 18</td>
<td>18.74*</td>
</tr>
<tr>
<td>Day 21</td>
<td>21.11*</td>
</tr>
</tbody>
</table>

Critical t .975 = 2.64

D) Stored Blood Sodium Ion

<table>
<thead>
<tr>
<th>Day Comparison to Control</th>
<th>t ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td>3.03*</td>
</tr>
<tr>
<td>Day 8</td>
<td>2.64*</td>
</tr>
<tr>
<td>Day 15</td>
<td>2.70*</td>
</tr>
<tr>
<td>Day 18</td>
<td>3.88*</td>
</tr>
<tr>
<td>Day 21</td>
<td>3.88*</td>
</tr>
</tbody>
</table>

Critical t .975 = 2.64
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VITA AUCTORIS

NAME: Ronald Gerald Weese

PLACE OF BIRTH: Dresden, Ontario, Canada

DATE OF BIRTH: December 11, 1950

ELEMENTARY EDUCATION: Dresden Public School, 1955-1964

SECONDARY EDUCATION: Lambton Kent Composite School, 1964-1969
   Diploma: Ontario Secondary School Honour Graduate

UNIVERSITY EDUCATION: University of Windsor
   Windsor, Ontario
   Faculty of Physical and Health Education 1973-1974
   Degree: Bachelor of Physical and Health Education

University of Western Ontario
London, Ontario
Faculty of Education 1973-1974
Diploma: Education

University of Windsor
Faculty of Human Kinetics
Windsor, Ontario

Expected Graduation: May 1980