IN VITRO STUDY OF ERYTHROPOIESIS IN RAINBOW TROUT (SALMO GAIRDNERI) USING $^{59}FeCl_3$

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ABSTRACT

IN VITRO STUDY OF ERYTHROPOIESIS IN RAINBOW TROUT (*SALMO GAIRDNERI*) USING $^{59}FeCl_3$

By

Richard Laurence Walker

Trout red cells were incubated in vitro with $^{59}FeCl_3$ for varying lengths of time. The cells were washed three times with phosphate buffered saline (PBS) and the amount of activity remaining was expressed as μg ^{59}Fe/g hemoglobin (Hb). Red cells from normal trout were found to incorporate 0.005 μg/g Hb/48 hr via two rate constants representing attachment of radioiron to the red cell membrane and incorporation into intracellular storage areas or heme. Erythrocytes incorporated 1.5 times more radioiron when suspended in plasma than when suspended in PBS. Trout red cells incorporated 6 to 7 times as much iron as dog red cells for the same time period. Erythrocytes from trout bled 20 to 30% of their whole blood volume incorporated (over a 48-hour period) 30 times as much activity as control trout red cells. The increase in iron incorporation was attributed to the increase in reticulocytes in the peripheral

circulation. Reticulocytes stained with New Methylene Blue N were apparent as early as 5 days following hemorrhage of 20 to 30% whole blood volume, and counts remained high at day 20 as shown by the significant increase in radioiron incorporation from 0.080 $\mu g^{59}Fe/g$ Hb on day 1 to 0.350 $\mu g^{59}Fe/g$ Hb on day 20.

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DEDICATION

I would like to dedicate this thesis to Dr. Ronald O. Kapp and Dr. Arlan Edgar, Department of Biology at Alma College, whose interest and encouragement made my graduate education possible.

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INTRODUCTION

The production of red cells by some organ or tissue and their appearance in the peripheral circulation is termed erythropoiesis. Hemorrhage and hypoxia are two stresses which can initiate erythropoiesis in homeotherms. Immature red blood cells increase in number in the peripheral circulation in response to a lowering of blood P_{O_2} and/or a decrease in blood volume.

The physiology of the red blood cell of aquatic species such as rainbow trout may be influenced by the presence of pollutants in the water such as insecticides, herbicides, polychlorinated biphenyls, and the heavy metals mercury, chromium, lead, cobalt, etc. Lead and cobalt are known to affect the oxygen-carrying capacity of the red cell. Jandl et al. (1959) state that lead interrupts the biosynthesis of heme within the immature red cell, while cobalt (Co^{+2} or Co^{+3}) in the ionic state is more tightly bound to immature red cells than iron. Also trivalent Co is not displaced from transferrin by Fe^{+3}, thus disrupting the transport of iron to the red cell. Mercury and other heavy metal ions may have a similar effect on the oxygen transport mechanism, thus stimulating

the production and release of immature red cells in the peripheral circulation. Therefore toxicants as they are present in aquatic environments may affect the erythropoietic activity of aquatic animals.

If this is actually the case it should be possible to measure changes in the erythropoietic activity by measuring the number of radiolabeled immature red cell forms, such as reticulocytes or polychromatocytes, present in the peripheral circulation. Radio-iron (^{59}Fe) incorporation by immature red cells may be a useful parameter or monitor in the detection of the effects of pollutants on red cell production in aquatic animals. This precipitated the study of red cell production rate and the feasibility of measuring erythropoiesis in trout with the use of $^{59}FeCl_3$.

LITERATURE REVIEW

Much of the literature on red blood cells pertains to mammalian species. Some authors have attempted to study teleost red cell production and maturation, but most find it difficult due to the inability to distinguish many of the stages in teleost red cell maturation. The information reviewed here pertains to either mammalian or lower vertebrate forms.

Origin of Erythrocytes

Theories of Human Red Cell Production

The final or myeloid period of hematopoiesis begins during the fifth month of human fetus development (Wintrobe, 1961), and it is initiated with the establishment of placental circulation. At first the liver is the chief organ of erythropoiesis, while leukocyte development occurs in the bone marrow. Soon the bone marrow becomes hematopoietically active and the function of the liver as the site of red cell production disappears. Myeloid cells in bone marrow are involved in hematopoiesis, the production of blood cells of all

types. Growth of myeloid cells leads to reduction of the mesenchymal cells of the liver and other tissues to a very scant reticular stroma which remains throughout life with its erythropoietic potentialities intact (Wintrobe, 1961). Multipotential cells of the mesenchyme are found in bone marrow, the intralobular capillaries of the liver, the venous sinuses of the spleen and lymph nodes, in the walls of the intestine, in serous membranes, and possibly in the adrenals. Together they form the reticulo-endothelial system. Wintrobe (1961) has reviewed the two major theories of blood cell production, the monophyletic and polyphyletic theories, and cites several references which deal with the subject of blood cell origin from myeloid cells and the reticulo-endothelial system. The classical monophyletic theory proposes that lymphocytes of lymphatic tissue are identical with primitive blood cells and are totipotential. With the proper stimulus erythrocytes, leukocytes, granulocytes and monocytes are said to all arise from lymphocytes.

The polyphyletic theory essentially states that formation of erythrocytes and various forms of leukocytes arise from specific blast cells instead of from a common progenator. There are a number of corollaries to this theory which deal with specific origins of granulocytes, agranulocytes and erythrocytes.

Maximow (1924) proposed a common stem cell which differs in size and structure from lymphocytes and is capable of producing both red and white blood cells. Doan et al. (1932) proposed two independent lines for blood cell development: erythrocytes from endothelial cells of the bone marrow and white cells from reticular cells. Jordan and Speidel (1924) studied stem cells in elasmobranchs and teleosts and found them to be indistinguishable morphologically from lymphocytes and capable of developing into red or white blood cells.

Theories of the Red Cell Line in Teleosts

Most of the theories of teleost red cell origin can be classified as monophyletic in nature. A stem cell or hemoblast is proposed as the common ancestor of both red and white cells. Yoffey's (1929) data supports the theory of Jordan and Speidel (1924); and he states that the lymphoid tissue of fishes, composed of small, round cells with large nuclei and little or no cytoplasm, gives rise to all cellular components of blood. Duthie (1939) found the main center of blood cell formation to be in the kidney where erythrocytes and leukocytes develop from intertubular tissue; however, he states that the lymphoid centers in the submucous layer of the intestine and mesentery are mainly for leukocyte formation.

Jakowska (1956) stated that a large hemoblast was the first identifiable form in hematopoietic tissue or in the circulating blood of teleosts. She proposed that this large hemoblast is a stem cell which can be either leukogenic or erythrogenic and give rise to all types of blood cells in the teleost.

Klontz et al. (1963), in an unpublished manuscript, also support the totipotential free stem cell theory. They state that there is no justification in making a distinction between a reticulum cell and an endothelial cell. In their scheme the hemocytoblast, which is derived from a reticulo-endothelial cell, gives rise to all blood cells in rainbow trout (Figures 1 and 2). Large and small lymphoid hemoblasts arise from the hemocytoblast and are pluri-potential. The large lymphoid hemoblast produces granulocytes, especially neutrophils and macrophages, while the small lymphoid hemoblast forms erythrocytes, thrombocytes and lymphocytes (Figure 1). The proerythroblast is known to undergo mitosis and is the direct descendant of the small lymphoid hemoblast (Figure 2). Following the proerythroblast are the erythroblast, in which mitosis may occur, the early polychromatocyte, with no noticeable mitosis, and the mid-polychromatocyte, where chromatin is compacted into thick, radially arranged strands. The late polychromatocyte is the youngest cell in the series to appear in the circulatory system

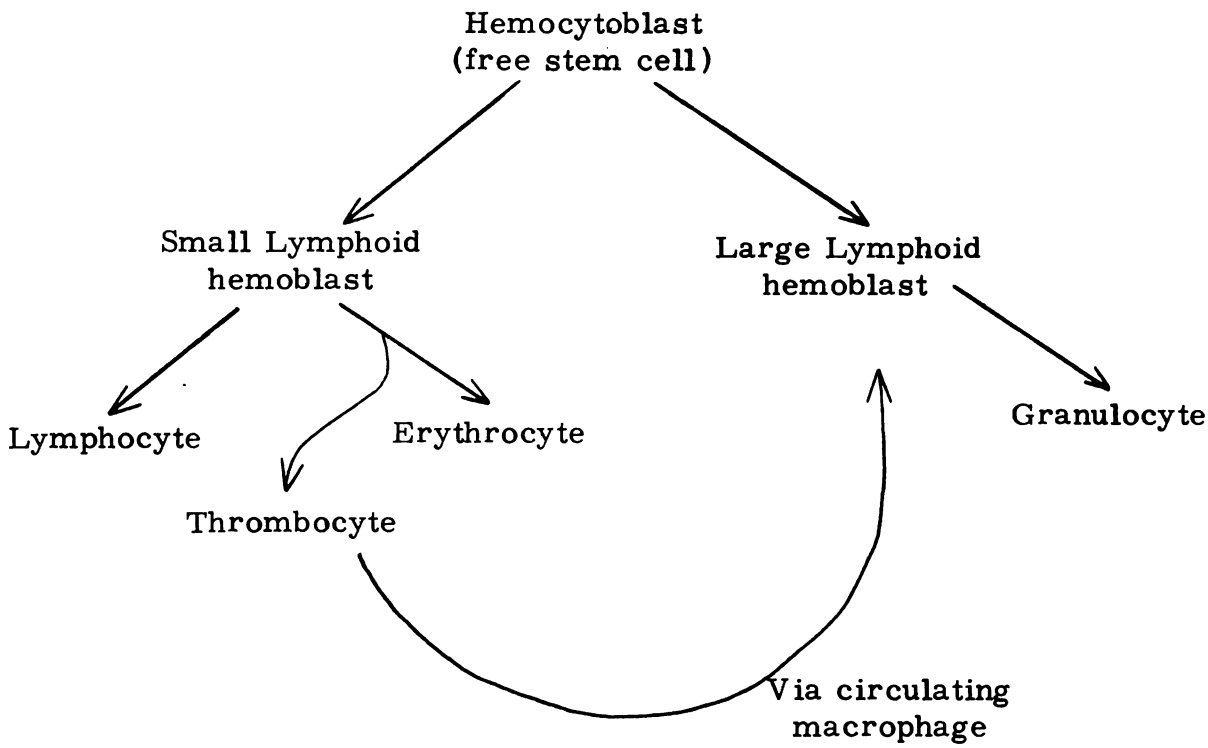

Figure 1. --Diagram showing the fate of the hemocytoblast (free stem cell) after Klontz et al. (1963).

Erythrocytic series

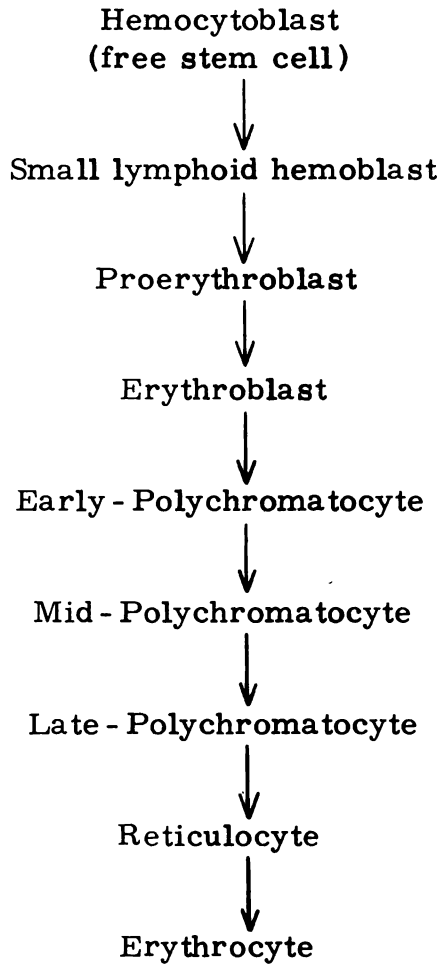

Figure 2. --Maturation scheme for development of rainbow trout erythrocytes (Klontz et al., 1963).

in small numbers. No nuclear membrane is visible at this stage and mitosis does not appear to occur. The reticulocyte is next in order of maturation and has an oval or elongate, compacted nucleus. The ribosomal RNA inside the cytoplasm stains dark blue with New Methylene Blue N. This characteristic is important in distinguishing reticulocytes from mature erythrocytes when reticulocyte counts are made. A mature erythrocyte is characterized as having an elongate, compact nucleus, and mitotic division is not apparent. However, fish on certain diets have a high incidence of amitosis, the significance of which is undetermined. In mitosis (or direct cell division) there is first a simple cleavage of the nucleus without change in the structure (such as the formation of chromosomes) followed by the division of the cytoplasm.

Recently a form of amitotic division of erythrocytes has been discovered in eight species of avians and two poikilothermic species (Engelbert and Young, 1970 a and b). Deutsch and Engelbert (1970) describe this type of red cell formation as sac-like protuberations or nuclear blebs which enlarge and then differentiate into red blood cells. Chromatin is shed into the extensions of the nuclear membrane which eventually come free of the cytoplasm of the mother cell and form sacs. These sacs are attached to the mother cell by fine threads which eventually break, setting the sacs free

as clone cells. The clone cell first appears as a naked nuclear mass which later develops cytoplasm, RNA and hemoglobin.

Red Cell Maturation

Yuki (1957) proposed that fluctuations of the blood cell types are usable as a biological indicator in studies of fish physiology. He noted, however, that studies based on this idea never advanced because, as Jakowska (1956) and Klontz et al. (1963) found, differential identification of blood cells in fish is very difficult due, in part, to the fact that even in normal fish undifferentiated blood cells are found in the peripheral circulation. Yuki reports that such a state resembles the beginning of the third stage of hemopoiesis in the fetus of higher vertebrates. Deutsch and Engelbert (1970) mention Jordon (1938) and Andrew's (1965) observations that red blood cell proliferation and maturation take place largely in circulating blood of teleosts. Despite the common appearance of immature red cell forms in the peripheral circulation, Altland and Brace (1962) estimated the mean erythrocyte life span in the turtle to be between 600 and 800 days.

Erythropoietic Stimuli

Hypoxia

According to Rosse and Waldmann (1966) the erythropoietic response to environmental hypoxia appears only among homeotherms.

Erythropoiesis in birds and mammals may be due to the fact that other physiological adjustments, such as increased respiratory exchange, increased circulatory rate, decreased body temperature, or shift to anaerobic metabolism are not enough to compensate for the increased oxygen deficit caused by hypoxia. Production of the erythropoietic stimulating factor, and therefore erythropoiesis, is controlled by the balance between tissue oxygen supply and the requirements of the body for oxygen.

Jandl et al. (1959) state that maturation of the red cell and incorporation of iron into heme in vitro are somewhat inhibited, rather than stimulated by anoxia. Iron uptake by reticulocytes is least under anaerobic conditions and increases slightly with increasing oxygen tensions. There is no evidence of a direct anoxic stimulus to red cell metabolism in terms of the ability of immature red cells to take up iron, to incorporate iron into heme and to mature morphologically. In other words, low ambient P_{O_2} may cause an increase in reticulocytosis but the uptake of iron by these immature forms does not result in a concomitant increase in the hemoglobin concentration.

Fried, Johnson, and Heller (1970) report an increase in the rate of erythropoiesis and an increase in the hematocrit of rodents after exposure to hypoxia. Erythropoietin levels reach a peak after

8 hours of exposure to hypoxia but then fall to barely detectable levels after 72 hours. They hypothesize a feedback inhibition where low blood P_{O_2} triggers the release of erythropoietin which in turn results in elevated red cell production and increased hematocrit (Hct). The increase in reticulocytes and Hct then may cause the noticeable drop in blood erythropoietic stimulating factor (ESF) level. Therefore elevation of blood ESF titers is the initial response to hypoxia.

Rosse and Waldman (1966) state that poikilotherms, such as frogs and turtles, do not increase their rate of erythropoiesis when exposed to reduced ambient P_{O_2}. Altland and Parker (1955) noted this to be true in box turtles exposed to low P_{O_2} for 53 days at 25,000 ft. altitude where they reported no significant difference in Hct or Hb values, erythrocyte or leukocyte concentration of control or exposed turtles. There was no evidence of erythroblastosis in spleen smears. This confirmed the findings of Sokolov (in Altland and Parker, 1955) that fish, frogs, toads and turtles show no increase in erythrocytes when exposed to a barometic pressure of 200 mm Hg (P_{O_2} = 42 mm Hg) for 7 days. Bonnet (in Altland and Parker, 1955) also found that exposure of fish and frogs to a pressure of 10 to 20 mm Hg for 1 to 2 hours daily for 6 days failed to stimulate erythropoiesis. Evidence in favor of hypoxia stimulating erythropoiesis in poikilotherms is reported by Gordon (1935) who found

exposure of the salamander (Necturus maculosus) to 330 mm Hg pressure for 7 weeks resulted in an increase in numbers of immature red cells and hematocytoblasts in the peripheral circulation.

Rosse, Waldmann, and Hull (1963) found that in frogs, sublethal levels of hypoxia do not stimulate erythropoiesis as measured by the incorporation of thymidine-2-^{14}C into peripheral erythrocytes. No reticulocytosis is seen in response to acute or chronic anoxia in the turtle, frog or lizard and it appears that environmental hypoxia does not grossly alter the oxidative metabolic rate of the frog. However, chronic hypoxia may induce polycythemia in newts and fish.

Prosser et al. (1957) noted that acclimation of the goldfish to low P_{O_2} lowered the oxygen consumption from an average of 0.239 to 0.163 ml O_2/g/hr and shifted the critical P_{O_2} from approximately 3.1 to 1.5 ml O_2/liter. This shift may have resulted from an increase in Hb concentration in the blood. The Hb values of control goldfish averaged 9.8 g/100 ml, compared to 11.9 for fish exposed to 2.0 ml O_2/liter. The statistical significance of these two values was not high ($0.1 \leq p \leq 0.2$) but they concluded that acclimation of the goldfish to low P_{O_2} results in an increase in circulating Hb. They also noted a 30% increase in the normal RBC count after acclimation of fish to 0.92 ml O_2/liter.

Hemorrhage

Rosse et al. (1963) noticed that even though sublethal hypoxia did not stimulate erythropoiesis, hemorrhage of 1/3 of the blood volume of frogs resulted in a 70-fold increase in erythropoiesis as measured by incorporation of thymidine-2-^{14}C into erythrocytes in the peripheral circulation. They concluded that either the erythropoiesis-stimulating response of bleeding resulted from some effect other than tissue hypoxia, or that hypoxia induced by anemia is somehow different from that induced by changes in the environmental P_{O_2}. Somehow, the poikilothermic animal makes adjustments to environmental hypoxia that are not made to anemia. This is contrary to what is believed to be the physiological response of mammals to hypoxia and hemorrhage.

Altland and Thompson (1958) observed that a marked reticulocytosis was induced in turtles by repeated bleedings. High reticulocyte counts persisted for 3 weeks following hemorrhage, and 4 months were required for restoration of normal erythrocyte values. Following hemorrhage of turtles, Hirschfeld and Gordon (1965) noted decreases in RBC and plasma volumes, Hct, Hb and serum iron concentration. An indication of the cellular response to bleeding was the appearance of radioiron-labeled erythroblasts in the circulation 2 days after ^{59}Fe administration, signifying

erythropoiesis, and the release of immature RBC's into the peripheral circulation.

Zanjani et al. (1969) reported that after bleeding, RBC regeneration rapidly occurred in gourami, a tropical fish. By as early as the fifth day following hemorrhage in both fed and starved fish, considerably greater values were obtained for radioiron incorporation into circulating erythrocytes, as compared to non-bled fish.

Starvation

Lack of food causes a significant decrease in the ability of poikilothermic vertebrates to respond to hypoxia or hemorrhage. Zanjani et al. (1969) in their study of humoral factors influencing erythropoiesis in the blue gourami, found that although total blood volume was not altered significantly, RBC counts, Hb, Ht and percentage incorporation of radioiron into circulating RBC's were all lowered due to the lack of food. Full replacement of the diet completely reversed the inhibitory effects of food deprivation on erythropoiesis in the gourami.

Hirschfeld and Gordon (1965) found that starved-bled turtles were incapable of increased erythropoiesis as measured by ^{59}Fe incorporation by reticulocytes. Erythroblast levels were not elevated in those turtles subjected to starvation and bleeding.

Starvation of turtles for 6 weeks prior to hemorrhage prevented increases in benzidine-staining (used to detect Hb) and also radioiron incorporation by erythroblasts, both of which increase in fed-bled turtles. Starvation led to a decrease in serum iron concentration, and these researchers warn that the inability of some workers to detect changes in erythropoiesis in lower vertebrates following application of stimuli effective in mammals may be due to an inadequate nutritional state.

Erythropoietic Response

Erythropoietic Stimulating Factor (ESF)

Summarized below is the excellent review of erythropoiesis and the origin and function of ESF by Gordon (1959). Erythropoietin, "Carnot serum," hematopoietine, plasma erythropoietic stimulating factor, plasma erythropoietic factor (EPF) and erythropoietic stimulant are all terms referring to ESF, which has been classified as a hormone that is specifically responsible for increased RBC production.

ESF is a glycoprotein (M.W. 60,000) formed by the combination of the Renal Erythropoietic Factor (REF) with a globular protein produced by the liver. REF is produced exclusively by the kidney in dogs; but in rats, rabbits and humans ESF is found in the

blood even after removal of the kidneys. REF is secreted by cells in the glomeruli in response to hypoxia and at the same time the liver increases its production of the specific globulin which reacts with REF (Figure 3). Combination of the two occurs in the plasma to form ESF which in turn increases the production of proerythroblasts by the erythropoietin-sensitive stem cells.

Zanjani et al. (1967) in a study of the role of ionic and vasoactive agents on erythropoietin formation detected the existence of an erythropoietic inhibitor. Exogenous ESF was inactivated when mixed with kidney homogenates, and it was speculated that REF containing extracts may have an enzyme which in the presence of a serum-borne metal ion, destroys newly formed ESF under in vitro conditions. ESF levels may be restrained due to the presence of an ESF-inhibitor which destroys circulating erythropoietin and maintains a normal plasma ESF concentration. Angiotensinase, the enzyme which destroys angiotensin II, requires Ca^{+2} for its effectiveness and was thought to be the ESF-inactivating factor in the REF-containing extracts. However, the presence of Ca^{+2} and angiotensinase failed to activate destruction of ESF and actually stimulated ESF production in vitro. Zanjani et al. (1967) concluded that REF required Ca^{+2} at a lower concentration for ESF production than does the erythropoietin inactivator.

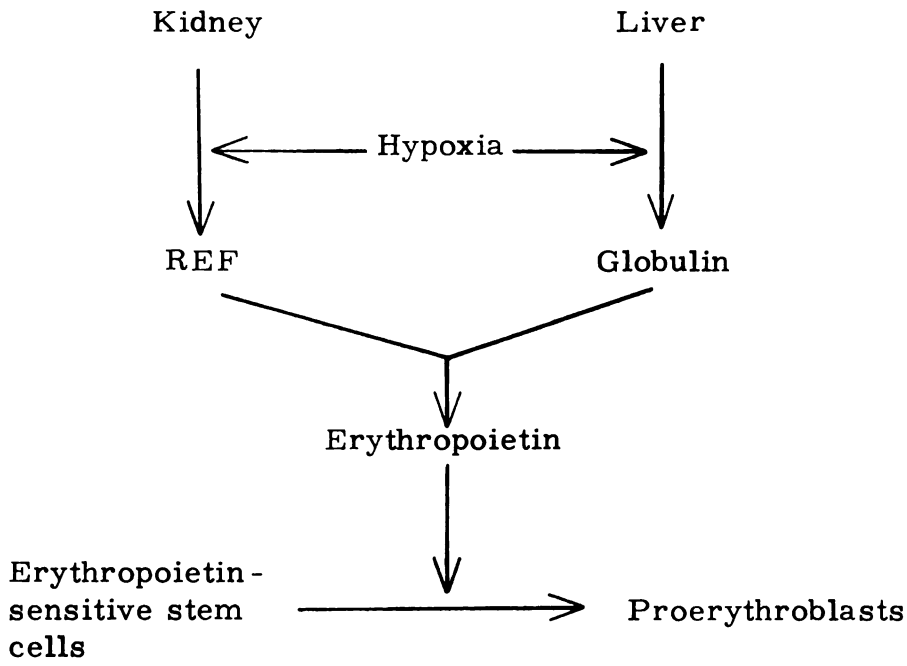

Figure 3. --Proposed scheme for formation of erythropoietin (Ganong, 1969).

Fried et al. (1970) noted a rise in rat plasma erythropoietin levels to a peak after 8 hours of hypoxia. ESF levels were barely detectable at 72 hours and upon injection of erythropoietin no decrease in the rate of ESF clearance was noticed. Therefore it is likely that production of ESF decreased even though the erythropoietic response to hypoxia was not concluded. ESF initiates the erythropoietic response, but apparently the continued high plasma ESF titers are not necessary and may be inactivated after delivering the initial stimulus to the stem cells.

Species Specificity of ESF

Several authors have attempted to stimulate erythropoiesis by injecting one species with anemic plasma or ESF-containing plasma of another species. Rosse et al. (1963) report that the serum of bled frogs increases the incorporation of thymidine-2-^{14}C into erythrocytes of frogs, but anemic frog serum does not stimulate erythropoiesis in the polycythemic mouse. Serum taken from anemic humans containing large amounts of erythropoietin does stimulate RBC production in the polycythemic mouse, but does not do so in the frog. Rosse concludes that stimulating factors in frogs and mammals are chemically different.

Erythropoietin (ESF) from mammalian serum requires a protein structure and sialic acid for biological activity. Rosse and

Waldman (1966) discovered that bird ESF has a similar protein structure but lacks sialic acid since it is not inactivated with sialidase. This is why hypoxic quail serum and anemic chicken serum stimulate erythropoiesis in quail but fail to do so in mice, while anemic human serum which increases erythropoiesis in mice is without effect in polycythemic quail. Erythropoiesis in birds and mammals is induced by the same stimuli, but is mediated by different protein humoral factors.

Goldberg et al. (1956), while studying the biosynthesis of heme by avian erythrocytes in vitro, discovered that the erythropoietic factor which might exist in anemic chicken serum does not act directly on heme synthesis. Two chickens were bled daily for one week, while two others were injected with phenylhydrazine, resulting in severe anemia and increased reticulocytosis. The plasma from the anemic chickens was added to a hemolysate solution prepared from lyzed red cells which had been centrifuged and the stroma removed. Plasma was also taken from two normal chickens and added to the hemolysate as a control. Goldberg found that the uptake of radioiron into the heme portion of the red cell was no greater in the presence of extracts of anemic bird plasma than in the presence of extracts from normal birds.

Zanjani et al. (1969) report erythropoiesis in gourami after administration of anemic fish plasma. The addition of 0.15 ml of plasma obtained from anemic gourami to fish with suppressed erythropoiesis, due to starvation, resulted in a highly significant increase in the rate of ^{59}Fe incorporation into the immature red cells. Plasma obtained from normal fish had minimal erythropoietic activity. Administration of mammalian ESF to starved fish also caused a significant increase in radioiron incorporation into circulating red blood cells, but the erythropoietic effects did not occur until large doses of ESF were used. The first clear response was obtained only when 0.80 International Reference Preparation (IRP) units of ESF were administered (16 units/100 g body wt.). Krzymowska et al. (1960) showed that plasma concentrates from anemic sheep stimulated erythropoiesis in carp. In this regard, the reported failure of mammalian ESF to stimulate erythropoiesis in birds and frogs may have been due to the use of insufficient amounts of this factor.

When hypoxia-induced polycythemic mice were injected with anemic gourami plasma no erythropoietic response occurred (Zanjani et al., 1969). The fish plasma was "toxic" to mice even when very dilute amounts were injected.

An in vitro study was conducted by Zanjani et al. (1969) to determine if anemic fish plasma and mammalian ESF stimulated

erythropoiesis in the gourami by direct effect on the circulating erythrocytes. While gourami reticulocytes incorporated radioiron in vitro, no stimulatory effect on this process occurred upon the addition of anemic gourami plasma or mammalian ESF to the RBC samples. In fact, red cells from starved gourami accumulated radioiron in vitro to a smaller degree than cells obtained from normally fed fish.

Role of the Spleen in Erythropoiesis

Yoffey (1929) describes the spleen of fish as a capsule consisting of a single layer of epithelium with a thin stratum of fibrous tissue underneath, and pulp consisting of spongy cellular reticulum through which blood percolates. The arteries of the spleen of most teleosts terminate by dividing into three or four short, thick-walled capillaries which open directly in the spaces of the pulp reticulum. In his review, Gordon (1959) points out that splenectomy prevents the appearance of increased reticulocytosis in animals subjected to hemorrhage, and speculates that the spleen may be one of the sites of ESF production. Meints (1969) reported an increase in spleen size and a shift of red cell maturation from the spleen to the peripheral circulation in frogs injected with phenylhydrazine, an erythropoietic agent. The spleen of fish may

be a storage and production site for immature red cells and leukocytes (Duthie, 1939). In cases of severe hemolytic anemia the spleen may be forced to release more erythrocytes and play a larger role in manufacture of red cells. The bone marrow in mice normally has the greatest responsibility for red cell production, the spleen having only a minor role. Hemorrhage and hypoxia produce a marked increase in splenic radioiron incorporation while the increase in bone marrow radioiron uptake is not as great (Bozzini et al., 1970). Splenectomy in a normal adult mouse produces a mild anemia and total circulating red cell volume is decreased by about 15%. Bozzini concludes that red cell production in bone marrow is normally not sufficient in the mouse to meet the demand for erythrocytes and requires the assistance of extramedullary erythropoiesis, the spleen being the secondary site of erythropoiesis along with liver and lymph tissue.

Transferrin

When immature red cells which lack iron are present in increased numbers in the plasma, the condition is referred to as reticulocytosis. Iron may be obtained by simple equilibrium between ferrous and ferric ions in the extracellular fluid and the intracellular fluid of the red cell or from an iron-binding protein

molecule (transferrin) present in the plasma which acts as a carrier of iron atoms. Jandl and Katz (1963) state that immature red cells acquire most of their iron for Hb formation by the second method. Radioactive iron has been shown to be taken up directly from the plasma or from solutions of purified transferrin by reticulocytes both in vitro and in vivo. After injection of transferrin-bound radioiron, radioactivity is present in bone marrow within a few minutes.

Transferrin is a B_1 globular protein (M.W. 86,000) with two separate iron binding sites, each capable of binding one atom of ferric iron (Jandl et al., 1959; and Fletcher and Huehns, 1968). The half-life of transferrin in humans is approximately 12 days. There are more transferrin iron-binding sites than atoms of iron in normal plasma, thus no free iron is present. This is not unexpected since even small quantities of free iron are toxic. Transferrin iron is very tightly bound to the protein molecule and only very concentrated EDTA solutions will remove this iron. Binding proteins have been found in lamprey and fish blood, but it is not known whether they have one or two iron-binding sites per molecule (Fletcher and Huehns, 1968).

Iron is transferred directly from the iron-binding protein to specific iron-binding receptors on the cell surface of reticulocytes

(Jandl et al., 1959). Iron transfer is an energy-dependent process (Wintrobe, 1961); however, it is improbable that transfer is directly dependent upon oxidative mechanisms since uptake of ^{59}Fe is only moderately diminished by anoxia in vitro. Uptake is stimulated by the presence of glucose and inhibited by agents which block intermediary glucose metabolism (Jandl et al., 1959). Anaerobic glycolysis appears to be the main source of the energy required from the transfer process. The specific membrane receptor sites are present only in developing red cells and after hemoglobin synthesis is complete, they are lost (Fletcher and Huehns, 1968). Not only is there an iron carrier protein in the plasma, but also one inside the immature red cell. The ease with which red cell precursors release iron from transferrin, compared with the difficulty of achieving this in vitro with the use of EDTA at physiological pH, suggests that the attachment of transferrin to the receptor site produces a change in conformation of a specific protein inside the cell (Fletcher and Huehns, 1968). To summarize the transfer process, ferric iron is bound to transferrin in the plasma and is transported to specific receptor sites on the reticulocyte membrane where it is actively transported to the inside of the cell by a process involving a change in the structure of a specific iron-carrying protein inside the cell.

Upon completion of the transfer process the transferrin molecule, minus one atom of iron, detaches from the reticulocyte membrane receptor site. A new transferrin molecule, with both iron binding sites saturated, then attaches to the vacant receptor site and the transfer process is repeated. Jandl and Katz (1963) report a specific dynamic equilibrium between cell attached transferrin and free transferrin for attachment to the immature red cell. The rate of transferrin detachment is much the same as the rate of attachment, indicating that free transferrin exchanges quite rapidly with cell-attached transferrin, the half-time of exchange being approximately one minute.

To illustrate the importance of transferrin in formation of hemoglobin, comparison was made between incorporation of ^{59}Fe by immature and mature RBC's from a saline solution and from plasma containing transferrin. Jensen et al. (1953) found that uptake of iron by duck erythrocytes suspended in 0.15 M NaCl was approximately 35% less than that of cells suspended in plasma. The decrease in iron incorporation was due to the reduction in heme-bound iron, the nonheme ^{59}Fe fraction being the same for both media. Jandl et al. (1959) and Walsh et al. (1949) reported that both mature and immature washed red cells took up iron from a saline solution at a greater rate than from plasma containing transferrin.

Approximately 90% of the ^{59}Fe adsorbed from saline remains in the stroma or membrane fraction upon hemolysis and only 10% is in the heme portion. However, when placed in plasma containing the iron-binding protein only the immature forms were able to incorporate the iron. Upon hemolysis of these cells approximately 40 to 90% of the ^{59}Fe was recovered in the heme fraction and 10 to 60% was associated with the reticulocyte membranes. Addition of a metallic ion chelator, EDTA, to a cell suspension of iron-binding protein and washed red cells did not affect the transfer of iron from the iron-binding protein to reticulocytes until the EDTA/Fe ratio exceeded 10. Free iron in saline was completely blocked from entering reticulocytes and mature red cells alike with the addition of EDTA.

Jensen et al. (1953) compared the uptake of radioiron by human and duck erythrocytes in vitro. They discovered that cells from normal human subjects take up negligible quantities of iron, whereas in blood from a patient with hemolytic anemia, containing 33% reticulocytes, appreciable quantities of iron are taken up. Finch (1957) states that all immature red cells through reticulocytes are able to take up iron from plasma while mature erythrocytes do not. As determined by autoradiographs, human pronormoblasts and basophilic normoblasts have the greatest ability to assimilate

iron. Jandl et al. (1959), in their study on iron transfer from serum iron-binding protein to human reticulocytes, found that the incorporation of ^{59}Fe increases with increasing reticulocyte numbers and confirmed Finch's statement that mature red cells do not acquire iron from transferrin. According to Jandl and Katz (1963), reticulocytes have 100 times the affinity for transferrin that mature RBC's have. Walsh et al. (1949) added support to this hypothesis in their study on heme synthesis in vitro by immature erythrocytes. They state while blood containing less than 1% reticulocytes took up no measurable quantity of radioiron, uptake was demonstrated repeately in blood with a high reticulocyte content. That radioactivity was localized in immature cells was shown by correlation of reticulocyte count and radioactivity in various fractions of this blood separated by the albumin floatation technique.

Jandl et al. (1959) found that not only does ^{59}Fe incorporation increase with increasing reticulocyte numbers but incorporation is also related to the amount of iron-binding protein present in the plasma. Increasing amounts of radio-labeled iron-binding protein (IBP) (33% saturated with iron) were added to a series of incubation vials containing a constant percentage of reticulocytes. Radioiron incorporation increased in proportion to the iron-binding protein concentration until a concentration of 160 μM was exceeded; thereafter,

further increase in ^{59}Fe IBP resulted in no further incorporation by the reticulocytes.

Percentage saturation of the iron-binding sites on transferrin is another important factor in iron incorporation by reticulocytes. Iron transfer from iron-binding protein to immature erythrocytes in vitro is reported to be decreased when the percentage saturation of transferrin drops below 30% (Finch, 1957). Jandl et al. (1959) report when transferrin is less than 20% saturated, iron uptake of reticulocytes diminishes despite an amount of free iron in the system well in excess of that utilizable by reticulocytes. Only when the transferrin iron-binding capacity was exceeded did free iron non-specifically attach to red cells. Jandl and Katz (1963) report that reticulocytes have a greater affinity for iron-saturated transferrin than to the iron-unsaturated form. Fletcher and Huehns (1968) state that iron is taken up by reticulocytes from molecules containing two atoms of iron at roughly 10 times the rate that it is taken up from molecules carrying only one atom. They speculate that this is a result of a difference in shape or charge of the transferrin molecules.

Heme Synthesis

After transfer of iron to the carrier protein inside the reticulocyte, the iron accumulates in the particulate matter of the

cell, the stroma, mitochondria and microsomes (Wintrobe, 1961). Upon release from the particulate matter the iron combines with a non-hemoglobin protein. The iron can be incorporated into a hemoglobin molecule as the link between the porphyrin, heme, and the protein molecule, globin, or it can remain permanently as a non-heme fraction within the erythrocyte. Jensen et al. (1953) found that approximately 85% of the iron entering avian erythrocytes becomes irreversibly incorporated into heme and only 15% is held within the cell as the non-heme RBC iron pool. Equivalent values have been reported for human RBC's by Jandl et al. (1959). Jensen reports that duck erythrocytes are normally supplied with a reserve of Hb precursor, substances such as glycine and preformed porphyrins, therefore little additional material other than iron is required for hemoglobin synthesis. Based on the hypothesis of Neuberger (1951), that globin synthesis parallels the rate of porphyrin synthesis, the assumption is made that all newly formed heme is converted to hemoglobin.

Iron Storage in Red Cell

Kaplan, Zuelzer and Mouriguard (1954), in developing a stain for nonhemoglobin iron, noticed the presence of iron granules in the cytoplasm of normal red cells. If the presence of this iron is

not due to the degradation of Hb, two possibilities exist: either the iron granules represent an intracellular depot for heme synthesis, or an excess of iron entered the cell and was not utilized in Hb formation. Kaplan explains that the appearance of iron granules in normoblasts prior to maturation, and the evidence of adequate Hb synthesis, might indicate that the first of these hypotheses is true. The fact that mature red cells, produced under the condition of iron deficiency anemia, do not contain stainable iron suggests utilization of iron granules during the maturation process. Kaplan concludes that stainable nonhemoglobin iron in the red cell precursors is a normal phenomenon, and might be used in the recognition of immature red cells.

Iron Pool

Iron from catabolized red cells is not lost by excretion from the body, but, for the most part, is retained in an iron pool. Some authors have reported that iron released from catabolized hemoglobin is re-utilized in preference to other sources of iron for hemoglobin synthesis. However, Sanchez-Medal, Duarte, and Labordini (1970) found that re-utilization of ^{59}Fe-labeled hemoglobin released from radio-labeled red cells decreased by dilution in an iron pool. They gave daily intraperitoneal injections of

^{59}Fe-labeled hemoglobin to two dogs recovering from anemia (due to hemorrhage). Over a period of 14 days only 20.8% of the ^{59}Fe-hemoglobin was re-utilized, accounting for only 12.9% of the total iron present in the hemoglobin produced during this period. Thus most (87%) of the iron for hemoglobin synthesis was derived from iron stores. Ross (1946) suggested that once iron is introduced into the body it enters the iron pool and is utilized in a uniform fashion. Sanchez-Medal et al. (1970) concluded that plasma Hb is rapidly catabolized, mainly in the liver, and iron is released and handled in the same manner as iron absorbed through the gut.

Effect of Multivalent Ions on Erythropoiesis

Lead

Goldberg et al. (1956) mention that ionic lead inhibits the biosynthesis of heme by interrupting some intermediate state in heme formation such as the conversion of protoporphyrin to heme. Jandl et al. (1959) state that lead suppresses incorporation of ^{59}Fe by reticulocytes through inhibition of heme synthesis. Incorporation of ^{59}Fe into Hb is almost entirely prevented, but RBC radioactivity is only slightly decreased. This indicates that a very concentrated amount of radioactivity must be found somewhere near the red cell membrane. Jandl found that the increase in stromal ^{59}Fe incorporation

increases tremendously when lead is present within the red cell, and there is no evidence of iron accumulation in the microsomes or mitochondria, the temporary storage areas for iron before it is incorporated into Hb and nonhemoglobin protein.

Cobalt

Cobalt is reported to stimulate the erythropoietic response to hypoxia in mammals. Miller and Hale (1970) found that daily intraperitoneal injections of cobalt chloride (4 to 8 mg/kg) depresses oxygen consumption in rats and may affect their metabolic response to cold. The stimulating effect of cobalt on the erythropoietic centers is probably caused by the brief periods of tissue hypoxia following each injection. Jandl et al. (1959) mention that, like most multivalent metals, cobalt combines with the membranes of normal washed RBC's; however, unlike multivalent iron or chromium, cobalt is taken up more by reticulocytes than by adult cells. Trivalent cobalt (Co^{+3}) had 1.47 times the affinity for reticulocytes and 1.38 times the affinity for mature RBC's than divalent cobalt (Co^{+2}), thus the more highly charged cations are more extensively bound by red cell membranes. When iron-binding protein, less than half saturated with ^{60}Co, is placed in a vial with a reticulocyte-rich red cell suspension, ^{60}Co incorporation by reticulocytes is from 5 to 12 times greater than incorporation by mature red cells. Uptake

of ^{60}Co from ^{60}Co-transferrin in vitro by reticulocytes is approximately 10% of the uptake of ^{59}Fe from ^{59}Fe-transferrin at the same specific activity, which demonstrates the preference for transferrin-bound iron by the immature red cells. However, once Co^{+3} is bound to transferrin it is not displaced by Fe^{+3}. Cobalt will remain bound to transferrin as long as there is a suitable oxidizing agent present to maintain the trivalent state. The erythropoietic effect of cobalt may be due to the inability of red cells to obtain iron for Hb synthesis. A form of tissue hypoxia, as proposed by Miller and Hale (1970), may result from the substitution of cobalt for iron on the transferrin molecule. The erythropoietic response to tissue hypoxia is not seen in poikilotherms, and cobalt administration to turtles did not cause erythropoiesis (Altland and Thompson, 1958).

RESEARCH RATIONALE

Since reticulocytes and polychromatocytes are present in the peripheral circulation as forms lacking the essential iron, it should be possible to provide the necessary iron required to complete the synthesis of Hb and convert the reticulocytes into mature erythrocytes. This can be accomplished by adding radio-iron ($^{59}FeCl_3$) to the RBC pool. The radioactive tracer will be incorporated into the Hb structure of the reticulocyte as ferrous iron.

The rate of radioiron incorporation into the RBC's will be used as an indication of the circulating level of reticulocytes. The greater the number of immature forms in the blood, the greater the amount of ^{59}Fe incorporated.

The objectives of this study were:

1. To determine if trout red cells could incorporate ^{59}Fe in vitro.
2. To determine if red cells would retain this label when dialyzed against a substance of equal binding capacity.

3. To establish the importance of transferrin or iron-binding protein in the plasma.
4. To measure the kinetics of ^{59}Fe incorporation by red cells.
5. To determine the effect of hemorrhage on red cell production.
6. To determine the practicality of use of red cell ^{59}Fe incorporation as a monitor of the erythropoietic effects of environmental pollution on animal physiology.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) weighing 150-200g were obtained from the Michigan Department of Natural Resources fisheries research station at Grayling, Michigan. They were transported to Michigan State University in an 80 gallon galvanized metal tank lined with non-toxic paint. The tank was contained within an insulated box fitted with a telethermometer and an agitator for aeration. The water was maintained at 13 ± 2° C during transit. At the university fish were held in 120 gallon fiberglass tanks (Frigid Units, Toledo, Ohio) equipped with aeration stones and flow-through water supply (2 liters/min.) and held at 13 ± 1° C under 14 hours of light and 10 hours darkness per day. The water was filtered through activated charcoal to remove iron and chlorine.

Fish were divided into two groups: one starved and the other fed (100g Feed/10 kg of Fish) commercial trout pellets (3/16 inch, Zeigler Bros. Feed Mills Inc., Gardners, Penn.) every other day for the duration of the study. Fungal problems, such as

fin rot, were controlled by cleaning tanks once or twice weekly and treating infected fish with a solution of malachite green.

Bleeding Technique

Fish were bled from the caudal vein at a point just posterior to the anal fin using a syringe fitted with a $1\frac{1}{2}$ inch long 21 gauge needle. The syringe and needle were rinsed with sodium heparin (1,000 USP units/ml) to prevent clotting. The needle was inserted through the ventral surface into the haemal arch, and the plunger was pulled back slowly until the desired amount of blood was obtained. This method is a quick and easy way to obtain 2 - 3 ml of blood, with the majority of fish surviving.

Hematocrit, Hemoglobin and Red Cell Counts

Blood was drawn into heparinized capillary tubes (Biological Research, St. Louis, Mo.) and centrifuged (IEC Model MB Micro-hematocrit Centrifuge, Needham Heights, Mass.) at 11,500 rpm for 2 minutes. The percentage of packed red cells was determined with an IEC Circular Microcapillary Tube Reader.

Hemoglobin determinations were made spectrophotometrically by the cyanmethemoglobin method (Oser, 1965) using standards and reagents (Hycel, Houston, Tex.) and a Bausch and Lomb Spectronic 20 Colorimeter (Rochester, New York). A standard

curve of optical density (OD) and per cent transmittance (%T) versus concentration (g%) was plotted from dilutions of the prepared standard. Hemoglobin solutions were prepared by adding 20 µl of whole blood to 5 ml of cyanmethemoglobin reagent. Optical densities for each corresponding %T read from the spectrophotometer were calculated using equation 1:

$$O\ D = \log \frac{1}{T} \tag{1}$$

Hemoglobin concentrations in gram% were calculated from equation 2:

$$\frac{\text{Concentration (unknown)}}{\text{Concentration (standard)}} = \frac{O\ D\ \text{(unknown)}}{O\ D\ \text{(standard)}} \tag{2}$$

where gram% and corresponding O D from the standard curve were used for the concentration and O D of the standard.

Red cell counts were made using a blood diluting pipette and an A.O. Spencer hemocytomer (Buffalo, New York). Hendrick's diluting solution (Appendix A) was described by Hesser (1960) as satisfactory for trout blood, whereas Gower's and Hayem's diluting solutions result in distortion of the red cells of salmonid species. To obtain a red cell count, blood is drawn up to the 0.5 mark on the blood-diluting pipette followed by the addition of Hendrick's solution to the 101 mark and then placed on a vibrating shaker for 2-3 minutes.

Before filling the counting chambers of the hemocytomer, 5 or 6 drops of fluid were removed from the pipette to ensure delivery of an accurately diluted mixture. Samples were counted in two grids and the average RBC count of five secondary squares of each grid were calculated and multiplied by 50,000 to give the number of RBC's per cmm of blood.

Area of 1 secondary square = 0.04 mm^2; depth = 0.1 mm; volume = 0.004 mm^3 (cmm)

Pipette dilution = 1:200. $\frac{1 \text{ cmm}}{0.004 \text{ cmm}} \times 200 = 50,000$

Radioiron and Isotope Technique

The radioiron (as $^{59}Fe\ Cl_3$ in HCl) was obtained from Abbott Laboratories (North Chicago, Ill.). Iron^{59} has a half-life ($T\frac{1}{2}$) of 45 days and is a strong gamma emitter with photopeaks at 0.191, 1.097 and 1.289 MEV. The primary stock solution was acidic with a pH of 1.5 to 2.5; therefore a dilute solution was prepared by adding from 0.1 to 0.4 ml of $^{59}FeCl_3$ to a glass vial (depending on desired concentration) and diluting up to 2.0 ml with PBS (Appendix A). The pH was maintained by the PBS after adjusting to 7.2-7.6 with 10% Na OH, which was the optimum range for uptake of ^{59}Fe reported by Jandl et al. (1959). A known amount of ^{59}Fe PBS was drawn into a microburet syringe and added directly

to each blood sample. A Scientific Industries (Springfield, Mass.) Variable Speed Rotator set at 6 rpm was used to keep the blood sample mixed during the incubation periods. Red cell uptake of ^{59}Fe is temperature dependent (Jandl et al., 1959; and Jensen et al., 1953); therefore, all experiments with trout blood were run at 13° C while dog blood samples were maintained at 37° C to insure the proper "in vivo" range of metabolic activity. After incubation samples were removed, radioactivity measured, and the cells washed three times with 1 ml of PBS. Radioactivity of the packed RBC mass after washing was measured and per cent ^{59}Fe incorporation was calculated from equation 3:

$$^{59}\text{Fe incorporation} = \frac{\text{counts/minute after wash}}{\text{counts/minute before wash}} \times 100 \quad (3)$$

All counting was done with a Nuclear Chicago (Des Plaines, Ill.) NaI Crystal Well Scintillation Detector. Counting efficiency was estimated at 15 to 20%. The scaler/analyzer (Model 8725) was set to count above 900 KEV, thus counting all photoelectric energy at or above this level which includes the two highest ^{59}Fe photopeaks. This setting was preferred due to the 50% reduction in background counts which resulted when the lower (900 KEV) photo energies were deleted. Although background was reduced, photoelectric energy due to ^{59}Fe gamma radiation was not significantly reduced; therefore,

the Rs/Rb ratio (uncorrected sample rate/background rate) was increased and the necessary counting time shortened, resulting in a more accurate quantitative estimate of the ^{59}Fe present.

Dialysis

Cellulose dialysis tubing (A. H. Thomas, Phil., Pa.) with a $\frac{1}{4}$ inch diameter and having an average pore diameter of 4.8 mμ was prepared by boiling $2\frac{1}{2}$ inch strips in distilled water, washing 3 times with distilled water and then storing in PBS. One end was tied off with heavy thread, then one solution was added by means of a syringe and needle through the open end. The open end was tied off and the dialysis sacs were placed in disposable glass culture tubes containing the second solution. The 4 ml culture tubes were stoppered and placed on a variable speed rotator at 6 rpm to ensure thorough mixing of each solution.

To determine the uptake of ^{59}Fe, whole trout blood was dialyzed against PBS containing radioiron. Two ml of PBS containing 0.265 μc ^{59}Fe were added to each culture tube. Approximately 0.5 to 1.0 ml of pooled whole blood from starved trout was added to a series of dialysis sacs and 0.25 ml of blood pooled from several fish bled 30% of their blood volume a month previously was added to another series of sacs. Sacs were placed in the culture tubes and incubated for 48 hours. At the end of this period the sacs

were removed and equal volumes of the saline and whole blood were counted and the percent incorporation of ^{59}Fe by RBC's was determined.

In a second experiment labeled and non-labeled whole blood samples were dialyzed against each other to determine how tightly the radioiron was bound by blood. One ml aliquots of whole blood which had been incubated with ^{59}Fe for 48 hours were injected into dialysis sacs and dialyzed against 1.5 ml of pooled whole blood. After 24 hours incubation the dialysis sacs were removed and 0.2 ml samples of the two blood pools were taken and counted.

In the third experiment dialysis sacs containing 0.265 μc ^{59}Fe and 1 ml of plasma were placed into culture tubes containing 1 ml of PBS. After 24 hours incubation the activity in each solution was determined. Acetone was then added to the plasma to precipitate protein. Following centrifugation both supernatant and precipitate were counted and the per cent of activity remaining in each was calculated.

Uptake Studies

Studies were conducted (a) to determine if plasma has a significant effect on the rate and amount of activity incorporated by the red cells and (b) to test the hypothesis that a peripheral blood

sample from a trout should incorporate more iron than a mammalian blood sample due to a greater number of immature forms in the circulation of the trout.

In both studies, 8 to 10 ml samples of pooled blood were incubated with ^{59}Fe for various times in a 10 ml microburet syringe attached to a variable speed rotator at 6 rpm. Triplicate 0.2 ml samples of whole blood were removed at various time intervals from 15 minutes to 48 hours, Hct and Hb determined, and the amount of radioactivity measured before washing. The RBC's from each sample were then washed with PBS and centrifuged three times. The counts/minute after washing was converted to μg ^{59}Fe/g Hb based on the average g% Hb for the pooled blood sample (Appendix B). Jensen et al. (1953) state that in duck red cells no significant increase in hemoglobin concentration occurred with the incorporation of iron, thus ^{59}Fe uptake can be accurately expressed in terms of μg ^{59}Fe/g Hb.

Radioiron Uptake from Plasma and PBS

Ten ml of pooled whole trout blood was divided equally into two tubes and one was centrifuged for 5 minutes at setting 4 on the IEC Clinical Centrifuge. After the plasma had been removed, an equal volume of PBS was added. After the addition of 0.265 μc

of radioiron to each sample, they were incubated in microburet syringes for varying lengths of time. The rate and amount of radioiron incorporation as μg ^{59}Fe/g Hb was determined for both samples.

Radioiron Uptake by Dog and Trout RBC's

A study was designed to test whether nucleated red blood cells from the peripheral circulation of rainbow trout would incorporate radioiron to a greater extent than a sample of mammalian red cells. Ten ml of blood was removed from the femoral vein of a normal dog and incubated at 37° C in vitro with 0.265 μc of ^{59}Fe. A 10 ml sample of blood from 5 trout, which had been starved for at least one month, was pooled and incubated at 13° C with 0.265 μc ^{59}Fe. Samples were removed at various time intervals, washed, and the amount and rate of radioiron incorporation determined.

Uptake Equilibrium Curve

The concentration of ^{59}Fe within the RBC measured over a 24- to 48-hour period was plotted as an equilibrium curve. A two-component analysis of this curve is given in Appendix B.

Reticulocyte Study

A study was conducted to investigate the effect of hemorrhage on the number of immature red cell forms in the peripheral

circulation of trout and the incorporation of ^{59}Fe by red cells. Fish were kept in the large storage tanks for several weeks and were fed commercial trout pellets every other day. Feeding was continued on a regular basis throughout the duration of the study. Fed trout were in better physical condition than starved trout and were better able to withstand the stress of bleeding. On the first day each trout was tagged through the lower jaw with a piece of small plastic tape inscribed with a code number and weighed. It was necessary to anesthetize the fish before tagging or weighing by placing them into a solution of MS-222 prepared by adding 0.2 to 0.3 g of the anesthetic to 7 to 8 liters of water.

Ten per cent of the total blood volume (estimated to be 3.5% of the total wet weight) was removed on day one, another 10% on day 3 and then 0.5 ml blood samples were taken every other day for one week and again at 2 and 3 weeks. Hematocrit, hemoglobin, red cell counts and ^{59}Fe incorporation were measured for each sample obtained. The Student-Newman-Kuels test for multiple comparison for significant difference among means of unequal sample sizes was used to analyze the data obtained (Sokal and Rohlf, 1969a and b).

Blood from control and bled fish were compared using New Methylene Blue N Stain for reticulocytes (Appendix A). The

red cells were stained by placing a few drops of whole blood into a small glass tube containing about 1 ml of stain and allowed to sit for 5 minutes. After shaking the tube, two drops were removed, placed on a clean glass slide and coverslipped. Dry blood smears could not be used since the dye is a supravital stain. A series of slides were prepared and photomicrographs made using Köhler illumination.

Twenty-eight days after initial hemorrhage an uptake study was conducted using 4 ml of pooled trout blood to which 1.535 μc ^{59}Fe were added. RBC ^{59}Fe incorporation was measured over a 24 hour period and plotted as an equilibrium curve (Appendix B). From the 2-component analysis the amount and rate of RBC radio-iron incorporation were compared with the control values obtained from pooled blood samples of trout subjected to at least one month of starvation.

RESULTS

Dialysis

From the dialysis experiment it was evident that whole blood was able to take up and retain ^{59}Fe. (See Table 1.) Following 48 hours of dialysis, whole blood was found to contain from 54 to 62% of the total radioactivity. After separation and three washings with PBS, the packed red cells of the control group retained 31 to 36% while those of the bled group retained 57% of the activity contained in whole blood before washing.

In another experiment it was found that trout blood retained radioiron even after 24 hours dialysis against non-labeled blood. Table 2 shows that the non-labeled blood gained no activity from the radiolabeled blood; all activity remained within this sample and virtually no activity was detected in the unlabeled blood.

When radiolabeled trout plasma was dialyzed against PBS for 24 hours, 90% of the activity remained in the plasma. Acetone was then added to precipitate the protein in the plasma; and after centrifugation, 90% of the original plasma activity was found in the protein precipitate.

Table 1. --Activity in counts/minute (cpm) and % total activity incorporated (% TA) after 48 hours of dialysis of blood from control and bled trout against ^{59}Fe-labeled PBS at 13° C. Twenty per cent of whole blood volume was removed from fish in the bled group one month previously.

Sample	Total cpm after 48 hrs.	PBS		Whole blood (WB)		Washed blood	
		cpm after 48 hrs.	(% TA)	cpm after 48 hrs.	(% TA)	RBC cpm	% WB ^{59}Fe in RBC
Control	11,134	4,256	38.3	6,878	61.7	2,120	30.8
Control	4,979	2,304	46.3	2,672	53.7	962	36.0
Bled	4,094	1,887	46.0	2,207	54.0	1,251	56.7
Bled	3,959	1,588	40.0	2,371	60.0	1,353	57.1

Table 2. --Results of dialysis of ^{59}Fe-labeled whole blood against non-labeled blood after 24 hours of incubation at 13° C.

Activity of labeled blood after dialysis (cpm)	Activity of original non-labeled blood after dialysis (cpm)	Percent activity remaining in blood after dialysis
4,752	0	100
5,104	0	100
5,194	2	99

Uptake Study

Radioiron Uptake from Plasma and PBS

Trout red cells suspended in plasma incorporated 1.4 to 1.6 times as much radioiron as RBC's suspended in PBS (Table 3 and Appendix B). Trials A and B are experiments using different pooled blood samples in which the y-intercept of component I represents the amount of activity incorporated between time zero and equilibrium. The y-intercept values of component I for plasma and PBS were 0.0086 and 0.004 μg ^{59}Fe/g Hb for trial A, and 0.0125 and 0.0082 μg ^{59}Fe/g Hb for trial B, a ratio of 1.4 to 1.6 in favor of iron incorporated by RBC's suspended in plasma.

The rate of iron incorporation was not dependent on the type of medium, since the half times were not different for plasma

or PBS. The half times measured for components I and II in trials A and B were similar.

Table 3. --Half time ($T\frac{1}{2}$) and y-intercept for components I and II (Appendix B) of ^{59}Fe incorporation by trout RBC's suspended in plasma and PBS over a 24 hour period at 13° C.

Trial	Medium	Component	$T\frac{1}{2}$ (hours)	y-intercept $\mu g^{59}Fe/g$ Hb
A	Plasma	I	2.10	0.0086
A	Plasma	II	35.17	0.0013
A	PBS	I	1.45	0.0049
B	Plasma	I	4.91	0.0125
B	Plasma	II	19.78	0.0013
B	PBS	I	5.37	0.0082
B	PBS	II	16.60	0.0014

Dog vs. Trout

Trout RBC's incorporated 6 or 7 times as much activity than dog RBC's could incorporate over a 24 hour period (Table 4). The trout component I y-intercept value was 0.0051 compared to 0.0007 $\mu g^{59}Fe/g$ Hb recorded for the dog between time zero and equilibrium, illustrating a much greater uptake of iron by the trout RBC's within the 24 hour period (Figure 4).

Table 4. --Half time and y-intercept for components I and II of trout and dog RBC ^{59}Fe incorporation over a 24 hour period at 13° and 37° C.

Sample	Component	$T_{\frac{1}{2}}$ (hours)	y-intercept ($\mu g\ ^{59}Fe$/g Hb)
Trout	I	2.97	0.0051
Trout	II	18.84	0.0044
Dog	I	18.06	0.0007

Iron incorporation occurred 6 times faster, initially, with trout RBC's compared to the slower uptake of ^{59}Fe by dog RBC's. Half times ($T_{\frac{1}{2}}$) of component I illustrate the difference in rate: a $T_{\frac{1}{2}}$ of 3 hours for trout compared to an 18 hour $T_{\frac{1}{2}}$ for dog RBC's. Near equilibrium there was no difference in uptake rate between trout and dog; both were within a $T_{\frac{1}{2}}$ range of 18 to 19 hours.

Reticulocyte Study

The hematological data in Table 5 show the effects of repeated bleedings on rainbow trout. The decrease in hematocrit from 38 to as low as 11 is evident in Figure 5. The numbers of RBC's in millions/cmm dropped from 1.113 to 0.363 and the Hb fell from 8.5 g% to as low as 2.2 g% 20 days after the initial hemorrhage. There was high correlation coefficient, +0.988, between the decrease in red cell count and g% Hb over the 20 day period.

Figure 4. -- Semi-log plot of components I and II for trout and dog RBC ^{59}Fe incorporation. The combined equation for components I and II of trout RBC ^{59}Fe incorporation is

$$C_T = 0.0051\, e^{-\frac{.693}{2.97} t_I} + 0.0044\, e^{-\frac{.693}{18.84} t_{II}}$$

Dog RBC ^{59}Fe incorporation was a one-component process expressed by the equation

$$C_T = 0.0007\, e^{-\frac{.693}{18.05} t}$$

The y-intercept value, representing the amount of ^{59}Fe incorporated in 24 hours, for trout RBC's is 6 to 7 times greater than the value measured for dog RBC's.

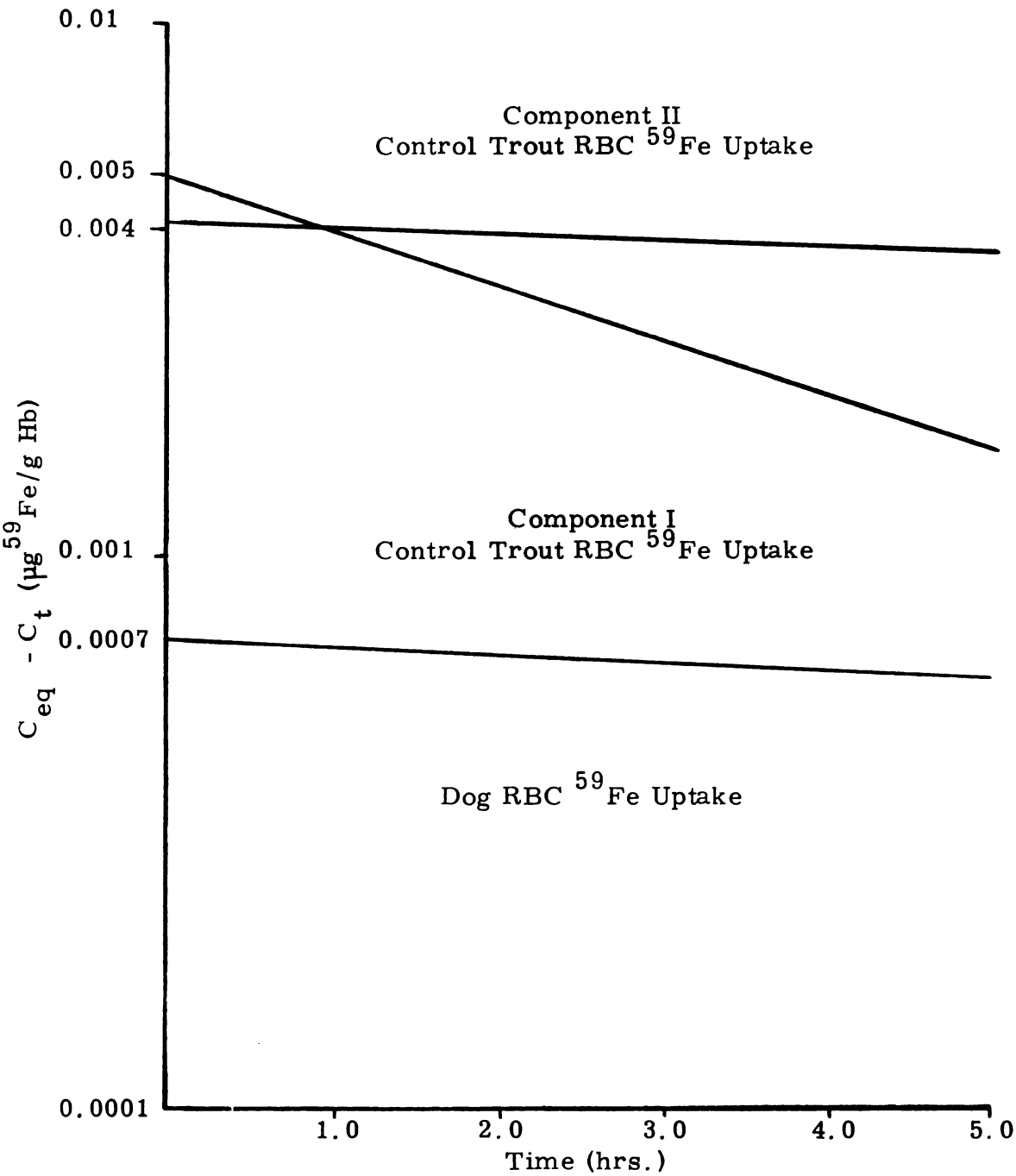
0.01
0.005
0.004
0.001
0.0007
0.0001
Component II
Control Trout RBC 59Fe Uptake
Component I
Control Trout RBC 59Fe Uptake
Dog RBC 59Fe Uptake
Ceq - Ct (μg 59Fe/g Hb)
1.0
2.0
3.0
4.0
5.0
Time (hrs.)

Figure 4

Table 5. --Hematocrit, hemoglobin, red cell count, 24 hour ^{59}Fe incorporation by RBC's, and % reticulocytes of blood samples from hemorrhaged trout. Twenty per cent of the whole blood volume (WBV) was removed between days 1 and 3, and 0.5 ml (5 to 10% WBV) were removed on days 5, 13, 20, and 28 to measure these parameters.

Period	Hematocrit (%)	Hemoglobin (g%)	RBC millions/cmm	μg^{59}Fe/g Hb/24 hr.	Reticulocytes (% of total RBC)
Initial bleeding	38.0 ± 1.6(27)*	8.5 ± 0.4(27)*	1.113 ± 0.045(27)*	0.080 ± 0.008(27)*	1.04
Day 3	30.1 ± 1.7(24)	5.5 ± 0.4(21)	0.830 ± 0.054(24)	0.151 ± 0.025(21)	----
Day 5	22.3 ± 2.3(20)	4.8 ± 0.5(21)	0.620 ± 0.062(21)	0.180 ± 0.052(21)	----
Day 13	13.9 ± 1.1(13)	3.2 ± 0.3(13)	0.478 ± 0.044(14)	0.283 ± 0.044(12)	8.13
Day 20	11.4 ± 1.5(10)	2.2 ± 0.2(10)	0.363 ± 0.049(10)	0.350 ± 0.036(10)	40.44
Day 28	13.4 ± 0.3(9)	2.3 ± 0.1(9)	-----	0.367 ± 0.016(8)	----

* Mean ± S.E. (N)

Figure 5. --Hematocrit, hemoglobin and red cell counts at various bleeding periods in rainbow trout. Ten per cent whole blood volume was removed on day 1 and again on day 3. On days 5, 13, 20, and 28, 0.5 ml of blood were removed (5 to 10% whole blood volume) to measure these blood parameters. See Table 5 for S.E. and N numbers.

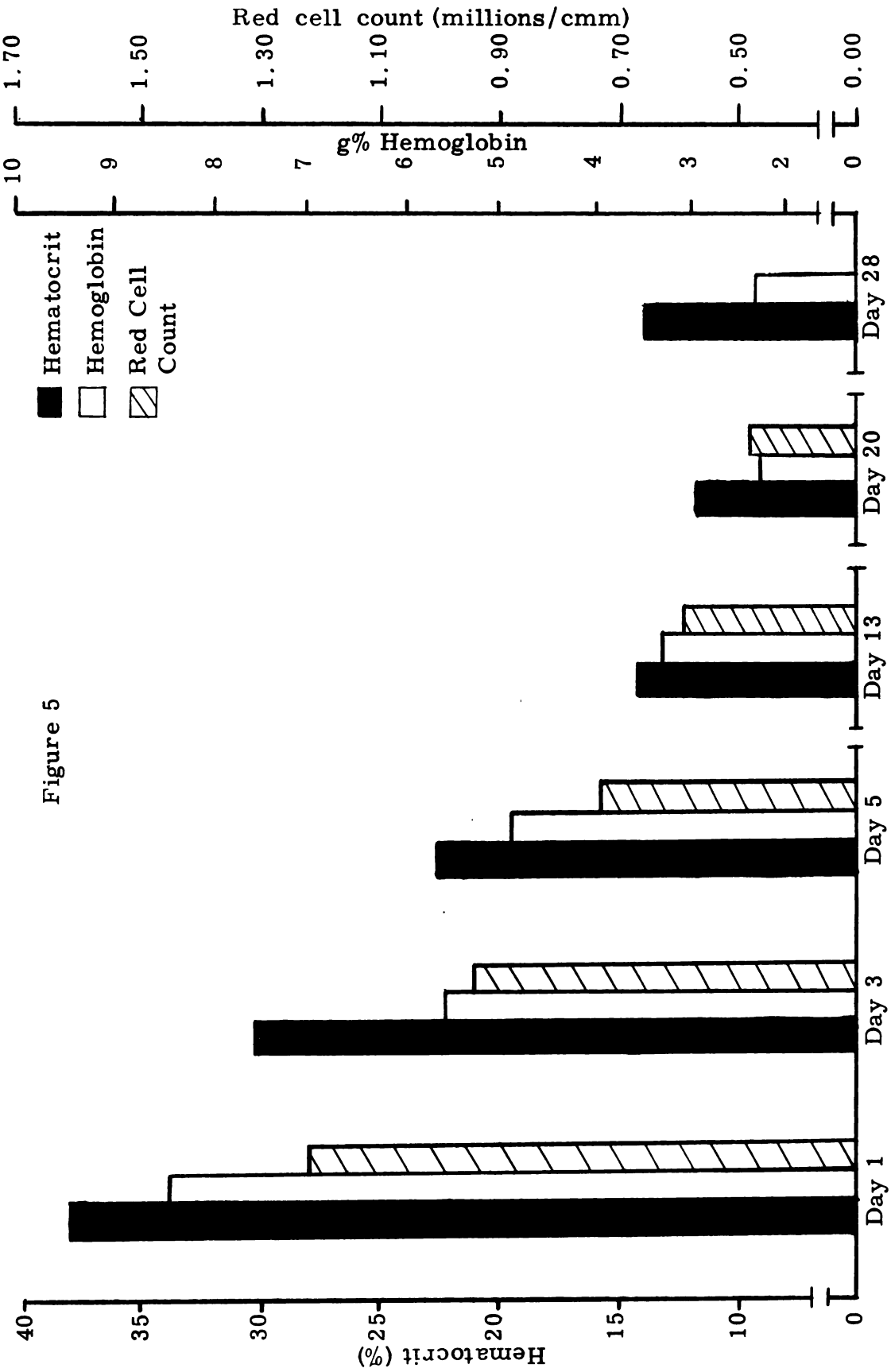
Red cell count (millions/cmm)
1.70
1.50
1.30
1.10
0.90
0.70
0.50
0.00
g% Hemoglobin
10
9
8
7
6
5
4
3
2
0
Hematocrit
Hemoglobin
Red Cell Count
Day 28
Day 20
Day 13
Day 5
Day 3
Day 1
Hematocrit (%)
40
35
30
25
20
15
10
0

Figure 5

The incorporation of ^{59}Fe by RBC's during 24 hours of incubation rose from 0.080 at the initial bleeding to 0.367 μg ^{59}Fe/g Hb 28 days later (Figure 6). A substantial increase in percentage reticulocytes present in the blood occurred between the initial day of hemorrhage, 1.04%, and 20 days later when 40.44% of the red cells were reticulocytes. As illustrated in Figure 7, the increase in μg ^{59}Fe/g Hb was related to the rise in reticulocytes with a correlation coefficient of +0.804.

The results of the Student-Newman-Kuels test for multiple comparison among the mean values of Hct, Hb, RBC, and 24 hour ^{59}Fe incorporation are summarized in Appendix B.

The photomicrographs in Figures 8 to 13 illustrate the increase in reticulocytes in the peripheral circulation. Figure 8 is typical of a control blood sample stained with New Methylene Blue N. There was an abundance of mature erythrocytes in all blood samples taken on the first day of the experiment. The cytoplasm appears shaded with occasional granules of blue-staining ribosomal RNA while the nucleus appears as a dark, compact oval.

The appearance of dark-staining circular immature red cell forms in the blood smears of all hemorrhaged trout was noticeable on day 13 (Figure 9). These reticulocytes appeared as compact masses of dark-staining material. The cytoplasm was filled with

Figure 6. -- ^{59}Fe uptake by trout RBC's in μg ^{59}Fe/g Hb/24 hours. Samples of whole blood (0.2 ml) were incubated with ^{59}Fe for 24 hours at 13° C. The RBC's were washed 3 times with PBS and the amount of activity remaining measured.

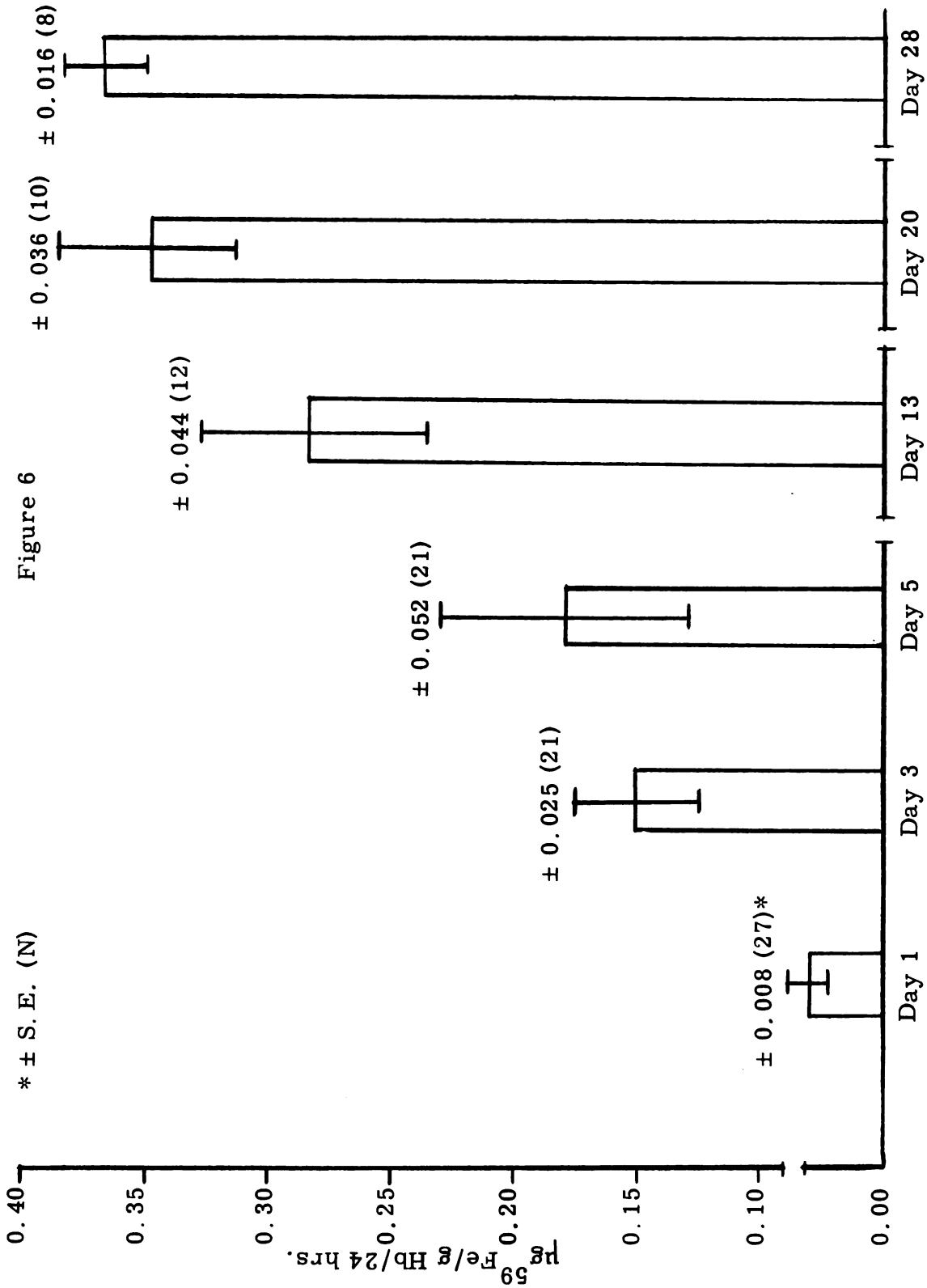
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.00
μg 59Fe/g Hb/24 hrs.
* ± S.E. (N)
± 0.008 (27)*
± 0.025 (21)
± 0.052 (21)
± 0.044 (12)
± 0.036 (10)
± 0.016 (8)
Day 1
Day 3
Day 5
Day 13
Day 20
Day 28

Figure 6

Figure 7. --Relation of radioiron incorporation to per cent reticulocytes among RBC's in rainbow trout. The correlation coefficient was +0.804, indicating that ^{59}Fe incorporation was dependent upon the number of reticulocytes in the blood sample. See Table 5 for S.E. and N numbers.

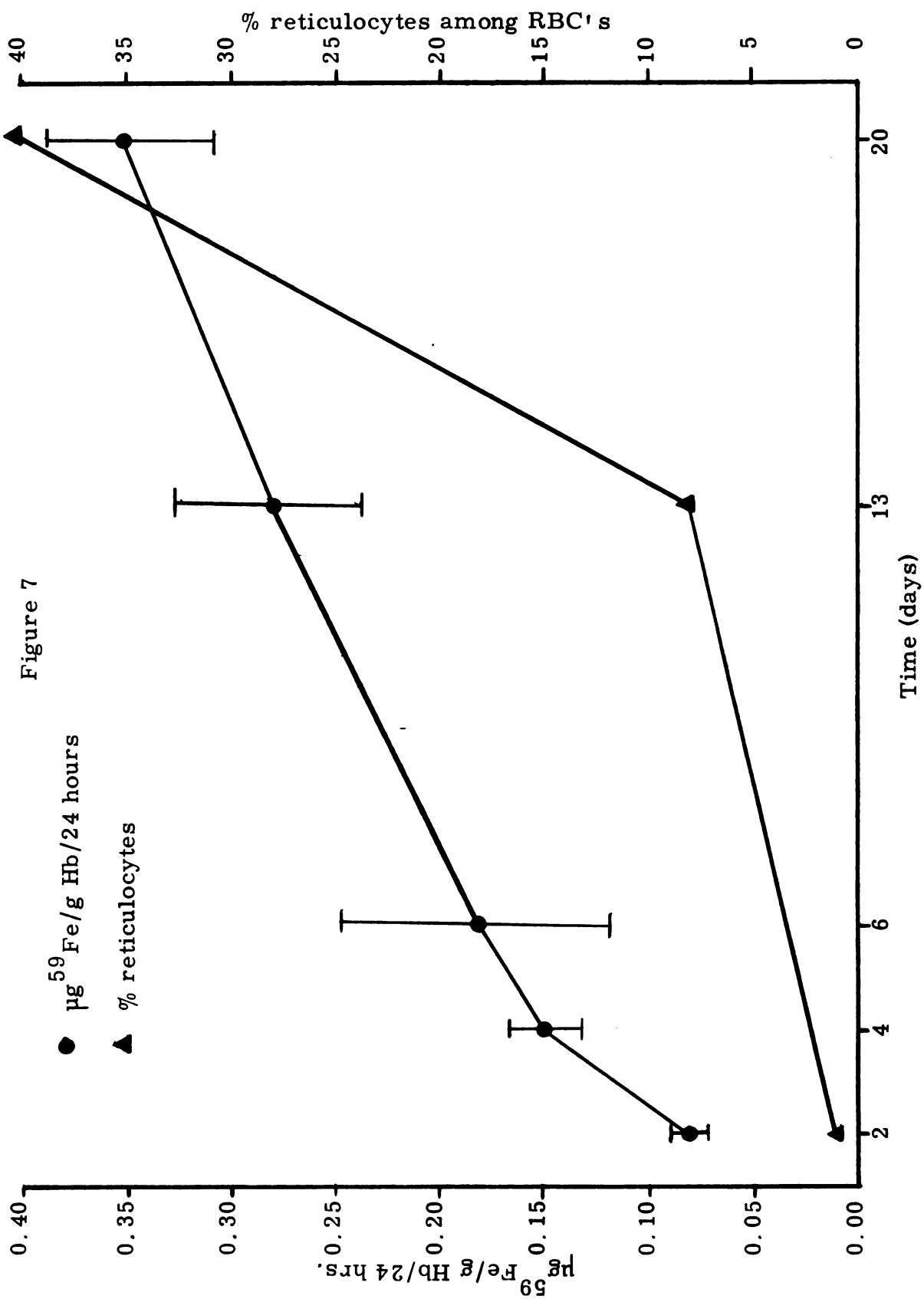
% reticulocytes among RBC's
40
35
30
25
20
15
10
5
0
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
μg 59Fe/g Hb/24 hrs.
2
4
6
13
20
Time (days)
● μg 59Fe/g Hb/24 hours
▲ % reticulocytes

Figure 7

Figure 8. --Control RBC sample stained with New Methylene Blue N (1000X magnification). Mature erythrocytes are abundant in this photomicrograph with distinct nucleus and cytoplasm. The cytoplasm appears shaded with occasional granules of dark-staining ribosomal RNA while the nucleus appears as a dark, compact oval.

Figure 9. --RBC sample stained with New Methylene Blue N from a trout 13 days following the removal of 20% of the blood volume.

A. Clump of mature erythrocytes.

B. and C. Reticulocyte in its early stages. The nucleus is circular and fills the whole cell; very little cytoplasm is evident.

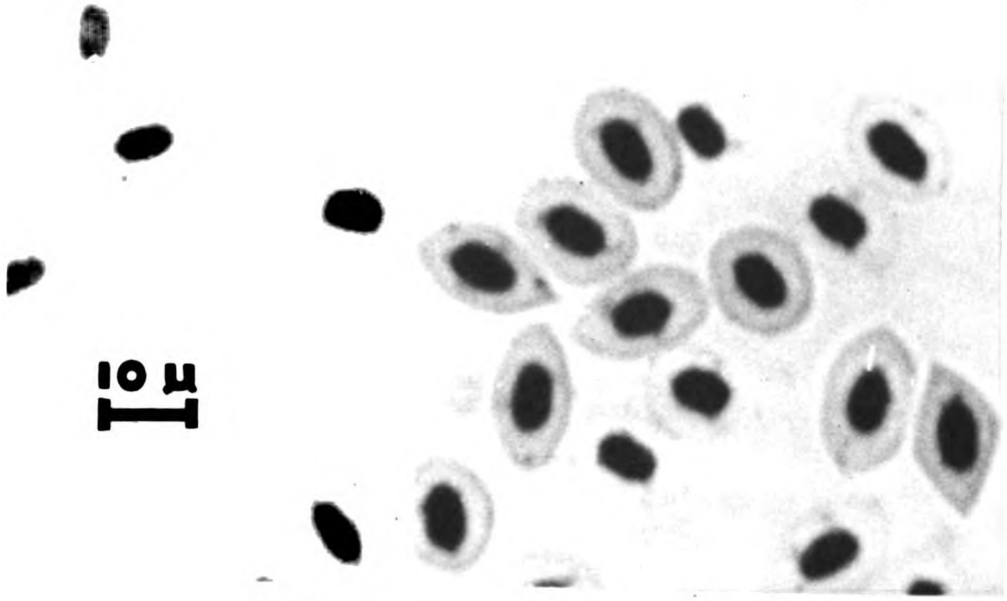

FIGURE 8

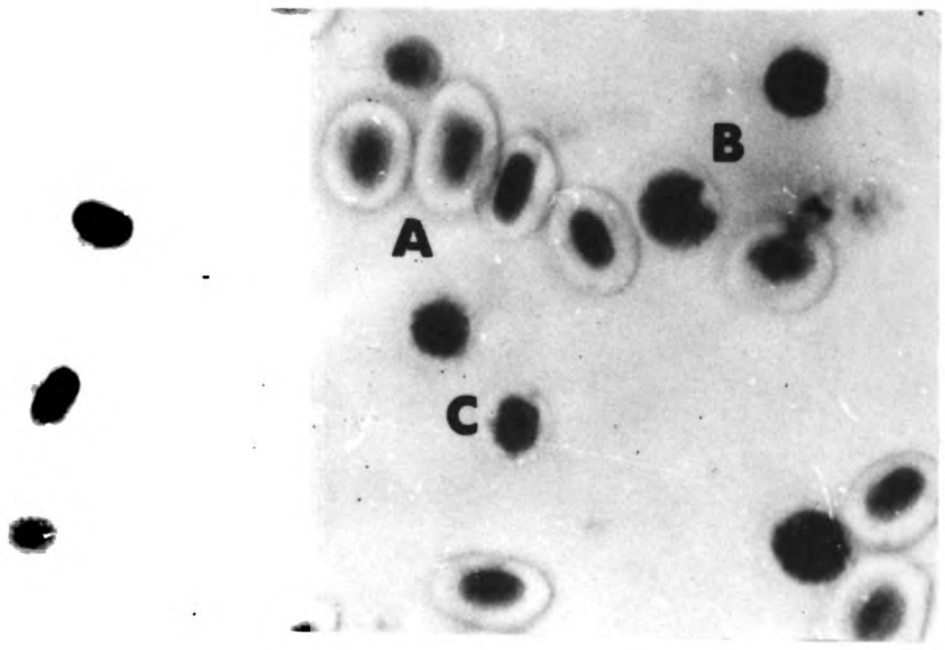

FIGURE 9

Figure 10. --Sample of RBC's stained with New Methylene Blue N from trout 20 days after removal of 20% of their blood volume (1000X magnification).

A. Mature erythrocytes.

B. Reticulocytes with elongate cell shape and cytoplasm filled with dark-staining ribosomal RNA.

Figure 11. --RBC sample stained with New Methylene Blue N from trout 20 days after the removal of 20% of the blood volume.

A. Mature erythrocyte.

B. Reticulocyte with elongate cell shape and cytoplasm filled with dark-staining ribosomal RNA.

C. Older reticulocyte in the transition stage to a mature erythrocyte. The nucleus is compact and the cytoplasm has less dark-staining RNA.

FIGURE 10

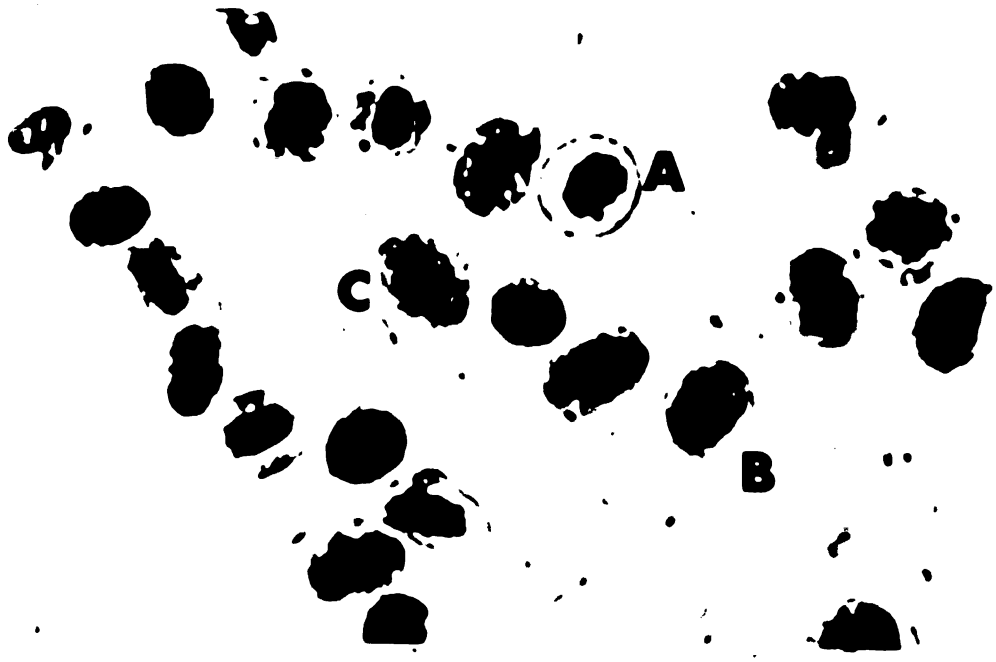

FIGURE 11

Figure 12. --An example of trout mature erythrocytes with elongate cell form, dark-staining nucleus and agranular cytoplasm (2500× magnification).

Figure 13. --RBC sample stained with New Methylene Blue N from a trout 20 days after removal of 20% of the blood volume.

A. Mature erythrocytes.

B. Early reticulocytes.

C. Late reticulocyte.

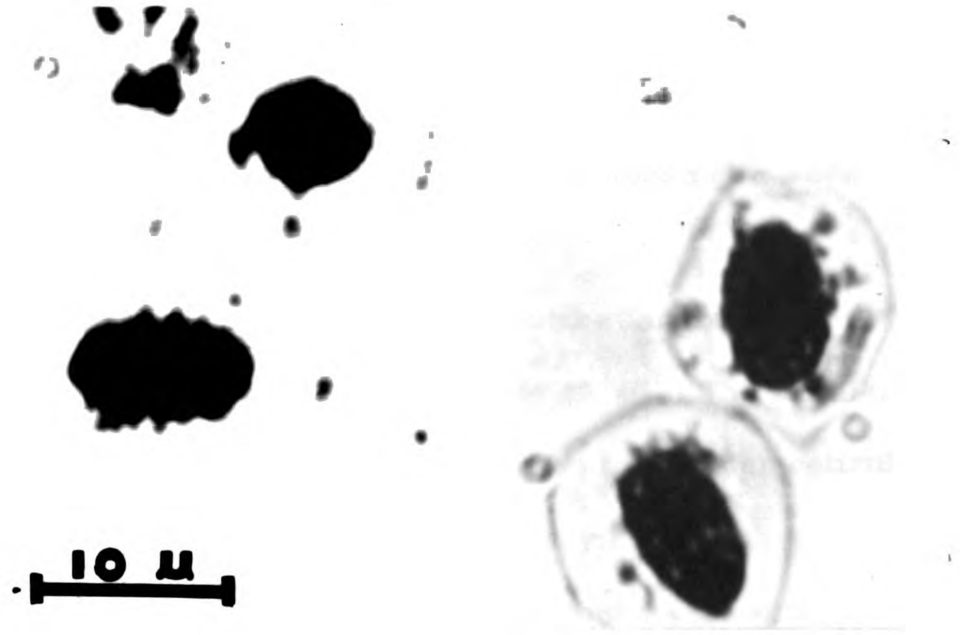

FIGURE 12

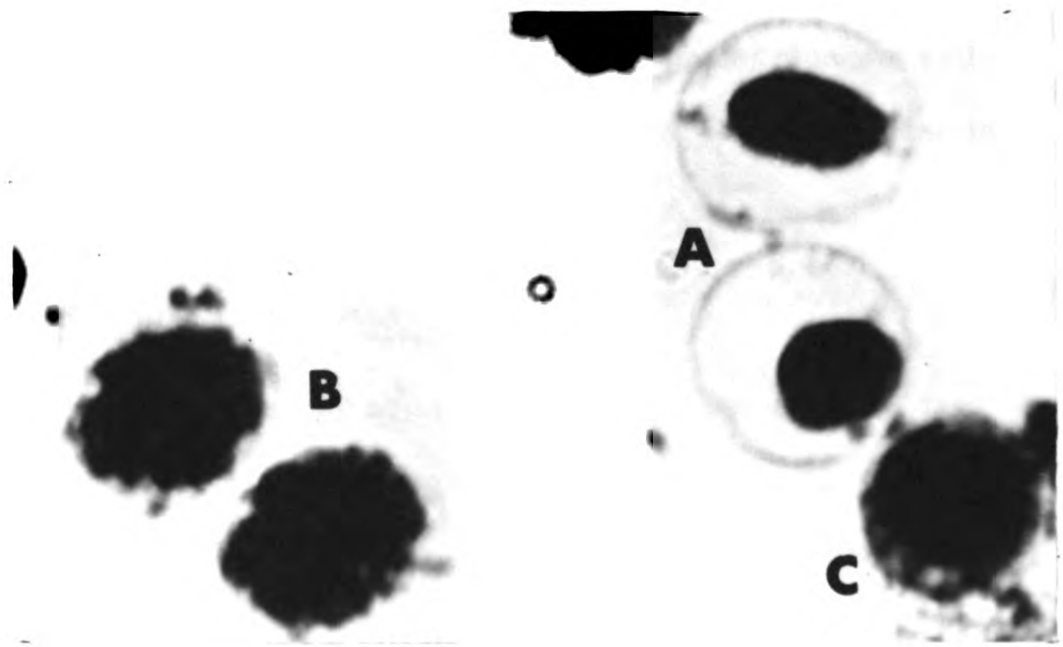

FIGURE 13

blue-staining ribosomal RNA which accounted for the abundance of granular material. Eight per cent of the red blood cells were reticulocytes.

By day 20 the concentration of immature forms present in the peripheral circulation had increased to 40.4%. The smaller, elongate cells of Figures 10 and 11 are believed to be older reticulocytes just beginning the transition to mature erythrocytes.

Figure 12 is an example of mature erythrocytes with elongate cell form, dark-staining nucleus, and agranular cytoplasm. The immature forms in Figure 13 illustrate the scant cytoplasm of the early reticulocyte with circular cell membrane, granular cytoplasm and a nucleus which composes most of the cell. The late stage of reticulocytosis was noted by the presence of elongate cell forms, filled with dark-staining ribosomal RNA of the endoplasmic reticulum.

Bled vs. Non-bled Uptake Study

In the uptake study using blood pooled from 4 fish 28 days after initial bleeding, RBC's of bled fish incorporated approximately 30 times more activity as RBC's from control trout, based on the y-intercept values of component I (Table 6 and Figure 14). The initial rate of uptake was $1\frac{1}{2}$ times greater for the bled trout with a $T\frac{1}{2}$ of 1.8 hours compared to a 2.9 $T\frac{1}{2}$ for control trout.

Table 6. --Half time and y-intercept of components I and II for ^{59}Fe uptake by bled and control trout RBC's. Twenty per cent of the whole blood volume was removed from fish in the bled group on day 1 of the experiment.

Sample	Component	$T_{\frac{1}{2}}$ (hours)	y-intercept ($\mu g\ ^{59}Fe$/g Hb)
Control	I	2.97	0.0051
Control	II	18.84	0.0044
Bled	I	1.83	0.1547
Bled	II	23.42	0.1442

Figure 14. --Semi-log plot of components I and II of ^{59}Fe uptake rates for control and bled trout. Bled trout were hemorrhaged 10% whole blood volume on day 1 and on day 3, and 0.5 ml of blood was removed on day 5, 13, 20, and 28. The equation for component I and II for the bled group was

$$C_T = 0.1547\, e^{-\frac{.693}{1.83} t_I} + 0.1442\, e^{-\frac{.693}{23.42} t_{II}}$$

The equation for the control group was

$$C_T = 0.0051\, e^{-\frac{.693}{2.97} t_I} + 0.0044\, e^{-\frac{.693}{18.84} t_{II}}$$

The y-intercept values indicate that RBC's from bled trout incorporated 30 times as much activity as those from control trout after 24 hours of incubation at 13° C. See Appendix B.

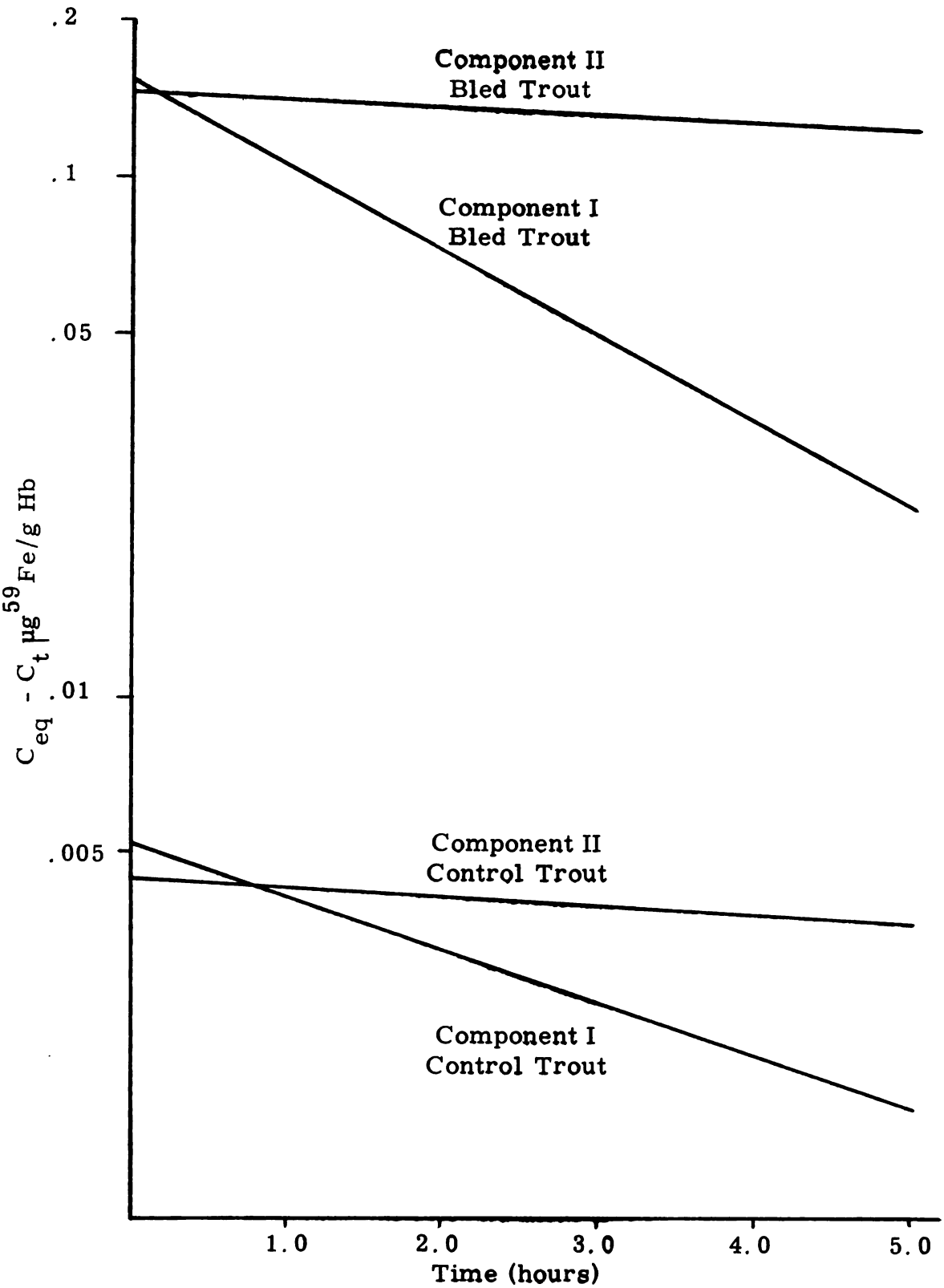
.2
.1
.05
.01
.005
Component II
Bled Trout
Component I
Bled Trout
Component II
Control Trout
Component I
Control Trout
$C_{eq} - C_t$ μg ^{59}Fe/g Hb
1.0
2.0
3.0
4.0
5.0
Time (hours)

Figure 14

DISCUSSION

Dialysis

The results of the dialysis experiments coincide with those of Walsh (1949) who reported that immature human red cells were capable of assimilating iron and synthesizing heme in vitro and Shemin, London, and Rittenberg (1950) who reported similar findings with nucleated red cells from ducks. The fact that iron was tightly bound to whole trout blood is supported by Jandl et al. (1959), who found that radioiron was absorbed onto the membranes of red cells and was not easily removed even with the use of EDTA, an effective chelator. Fletcher and Huehns (1968) attributed the tight binding to the presence of transferrin in the plasma of human blood. The presence of an iron-binding protein in teleost plasma was reported by Utter, Ames, and Hodgins (1970) in an electrophoretic study of transferrin in coho salmon plasma.

Uptake Studies

Evidence from the uptake studies indicates that the iron-binding protein plays a significant role in iron incorporation, since

trout red cells suspended in plasma incorporated 1.5 times as much ^{59}Fe as those suspended in PBS. This agrees with Jensen et al. (1953), who found that duck red cells suspended in plasma took up 1.5 times more iron than cells suspended in 0.15M NaCl. Jandl et al. (1959) made similar observations on heme which had been isolated from human reticulocytes suspended in plasma and saline. They attributed the difference between plasma and saline to be the fact that immature red cells irreversibly incorporate iron only from transferrin.

The two-component uptake curves for fish RBC's were the result of two processes occurring during iron incorporation. Component I is believed to represent the binding of ^{59}Fe to the stroma or red cell membrane. This appeared to be a fairly rapid process involving the transport of iron from transferrin to a specific site of attachment on the membrane. Component II may represent the iron incorporation by the storage areas on the mitochondria or into the heme (Schemin et al., 1950). This may involve an iron receptor in the cytoplasm which could transport the iron to either site in a relatively slow process. A two-component equilibrium curve was noted by Jensen et al. (1953), who reported the rate of iron incorporation to be more rapid within the first 6 hours of incubation and then decreased as equilibrium was approached at 24 hours.

Data reported here indicate that the presence of reticulocytes and other immature forms in the peripheral circulation is responsible for the increased amount and rate of iron uptake. Walsh et al. (1949) demonstrated this to be true for human blood where samples containing less than 1% reticulocytes took up no measurable quantity of radioiron compared to the amount of ^{59}Fe uptake in blood from patients with pernicious anemia. The results of the dialysis experiment with blood from control and bled trout indicated a possible correlation between the effects of bleeding and radioiron incorporation. Observations made during the long-term reticulocyte study confirmed this to be true. An excellent correlation has been noted between the amount of iron uptake and the number of reticulocytes present in the peripheral circulation of ducks (Jensen et al., 1953). Jandl et al. (1959) report similar findings in experiments with samples of peripheral blood of humans.

Effects of Hemorrhage

That hemorrhage is an excellent erythropoietic stimulus in trout is supported by data obtained in the bleeding experiment. Removal of between 20 and 30% of the blood volume over a 5-day period and subsequent weekly removal of 0.5 ml (5-10%) of blood resulted in drastic changes in Hct, Hb, RBC count, per cent

reticulocytes and ^{59}Fe uptake. The mean values for these parameters on the initial day of bleeding were generally higher than those reported by Schiffman and Fromm (1959); however, this may be due to seasonal variation. The sharp fall in all three parameters 5 days after the initial bleeding was in direct contrast to the results of Zanjani et al. (1969) in their study of the effects of bleeding on erythropoiesis in the gourami. They reported that red cell regeneration after removal of 25 to 30% of the whole blood volume occurred rapidly and, as early as 5 days following bleeding, considerably greater values were obtained for Hct, Hb, and RBC count in bled as compared to non-bled fish. Such a rapid recovery did not occur in rainbow trout.

A significant increase in circulating immature red cell forms was noted in rainbow trout after the initial removal of 20 to 30% of the whole blood volume. In experiments with turtles, Altland and Thompson (1958) first noticed a marked increase in reticulocytes 17 days after the initial bleeding and reported that high reticulocyte counts continued for at least 21 days and as long as 50 days in some instances. In another study with turtles, Hirschfeld and Gordon (1965) did not find significant differences in reticulocyte counts of control and bled turtles until after 2 weeks of "repeated bleedings." Thus the increase in reticulocytes appearing

in the peripheral circulation is a delayed, long-term response in turtles. This was also true for rainbow trout, since the high reticulocyte count on day 20 indicated that the increased release of immature forms into the circulation was still in progress.

Hemorrhage resulted in a significant increase in ^{59}Fe incorporation by trout red cells within 5 days of the initial bleeding. Hirschfeld and Gordon (1965) found a significant increase in the levels of ^{59}Fe-labeled turtle erythroblasts at 6 days following initial hemorrhage; and Zanjani et al. (1969) report considerably greater values in radioiron incorporation by gourami RBC's as early as 5 days following bleeding. The increase in ^{59}Fe uptake was noted before the appearance of trout reticulocytes, although the two are related. The more accurate parameter used to measure erythropoiesis is believed to be ^{59}Fe incorporation, due in part to the difficulty in distinguishing reticulocytes from mature red cells, both of which stain with New Methylene Blue N. Hirschfeld and Gordon (1965) refer to the inaccuracy of reticulocyte counts and indicate that better measurements of erythropoietic activity can be made with the use of radioiron and autoradiographs.

From the results observed in the experiments previously described, it is concluded that hemorrhage has a direct influence upon the mechanism for re-establishing red cell volume in rainbow

trout. The decrease in red cell volume, volume % O_2, and perhaps blood pressure probably triggers the production and output of a hormone such as ESF which is distributed via the circulation to target tissues for the stimulation and increase of red cell production. These erythrocyte production and storage centers include the kidney and spleen (Duthie, 1939). With the increase in the production of polychromatocytes, as outlined by Klontz et al. (1963), the ESF titers decrease due possibly to destruction by an enzyme or antibody, or due to a feedback inhibition mechanism to the sites of ESF production, the kidney and liver. Red cell precursors enter the peripheral circulation and undergo maturation (Andrew, 1965; and Jordon, 1938). They receive the iron necessary to complete the molecules of hemoglobin from transferrin present in the plasma. After hemoglobinization, or maturation, the red cells are able to carry oxygen to the cells of the body in exchange for carbon dioxide. Upon destruction of the red cell, hemoglobin is broken down and iron is released to storage pools or to transferrin. The process from initial stress to the output of immature red cell forms into the circulation appears to be variable, but in rainbow trout it may be the matter of 5 days to a week before the response can be detected.

Application to Fisheries and Environmental Studies

Results of the hemorrhage experiments in this study indicate that erythropoiesis and the appearance of red cells in the peripheral circulation of fish can be measured accurately by RBC ^{59}Fe incorporation and can be easily supplemented with reticulocyte and RBC counts, hematocrit and hemoglobin determinations. Determination of these parameters should have merit in measuring the erythropoietic effect of environmental pollution and in studies of the general health of hatchery fish.

In a study of the effects of heavy metals on fish, information on histological changes in the erythropoietic tissues could be supplemented with determinations of the number of immature erythrocytes present in the circulating blood by the methods outlined above. Pollutants such as heavy metal ions may affect the oxygen carrying capacity of blood by blocking heme synthesis or binding to transferrin, eventually resulting in tissue hypoxia. As an initial response to oxygen deficiency, RBC storage areas in the spleen may be emptied, causing an increase in the number of circulating immature forms, which could be detected histologically and by ^{59}Fe uptake studies. Release of cells from the storage areas may be insufficient compensation and, as a result, after a period

of time values for circulating reticulocytes and RBC ^{59}Fe uptake may decrease. The erythropoietic centers would then be stimulated and again this response could be checked histologically and any new production and release of RBC should be reflected in increased ^{59}Fe uptake by RBC's. If the sites of red cell production are destroyed by heavy metals, the number of circulating immature RBC's would gradually decrease following spleen RBC depletion, as would RBC ^{59}Fe uptake.

A decrease in oxygen-carrying capacity due to substitution of heavy metal ions for iron combined with destruction of erythropoiesis centers would eventually cause death. Release of immature red cells from storage areas would represent a transient compensation for tissue hypoxia.

Procedures for Monitoring Immature RBC Numbers in Fish

Monitoring of the effects of pollutants or disease on the physiology of fish blood can be done by the procedures outlined here. Hematological measurements are efficient, accurate and require little expensive equipment. Blood samples may be taken at any monitoring station and transported to a laboratory for the following analyses:

1. Hematocrit, Hemoglobin and RBC counts. Repeated checks of these basic hematological parameters will give an indication of the overall well-being of the experimental animals.
2. Determination of RBC ^{59}Fe incorporation. Data for groups of fish could be obtained by making determinations on a pooled sample of blood, whereas, using only 0.2 ml of blood from individuals provides the opportunity of studying this parameter in individual fish over a period of time. This determination is relatively simple but does require use of a gamma ray detector.
3. Reticulocyte counts. Although difficulty was encountered in distinguishing reticulocytes from mature cells using New Methylene Blue N, measurements of immature RBC forms can be a valuable parameter in stress physiology along with information on RBC ^{59}Fe uptake. Reticulocyte determinations are tedious, but modification of the staining technique could eliminate some of the drudgery. Hirschfeld and Gordon (1965) state that the benzidine-Wright staining technique for hemoglobin is a more sensitive method for measuring immature forms in the circulation than the supravital technique using New Methylene Blue N. Lucas and Jamroz (1961) have published an atlas of avian

hematology which contains several color illustrations of developing nucleated red cells from their origin to maturation using the benzidine-Wright and supravital stains. This reference may be valuable in identification of immature fish RBC's and it contains outlines of the various staining procedures.

4. Autoradiographic techniques have also been suggested by Hirschfeld and Gordon (1965) as a sensitive measure of immature red cells in circulating blood. Smears of radiolabeled RBC's can be prepared, dipped in Kodak NT 33 nuclear track emulsion, exposed at 4° C for 10-14 days and developed in Kodak D-19.

SUMMARY AND CONCLUSIONS

1. Red cells from control trout were capable of incorporating 30 to 36% of the ^{59}Fe present in whole blood and red cells from bled fish bound as much as 57% of the available radioiron over a 48-hour period.

2. Trout erythrocytes incorporated 1.5 times more radioiron when suspended in plasma than when suspended in PBS.

3. An iron-binding protein functionally similar to human transferrin is present in the plasma and plays a significant role in iron incorporation.

4. There were two rate constants for iron incorporation by trout erythrocytes. It is suggested that the initial fast uptake, with a half-time of 3 hours, represents the transfer of iron to the red cell membrane and the slower uptake, with a $T\frac{1}{2}$ of 19 hours, represents the incorporation of iron into heme or some intracellular storage site.

5. The average ^{59}Fe uptake by control trout red cells was 0.005 μg/g Hb between time zero and equilibrium (48 hours).

6. Red blood cells from bled trout incorporated 30 times more activity than control trout red cells between time zero and equilibrium (48 hours).

7. Normal trout red cells incorporated 6 to 7 times the amount of activity taken up by dog red cells for the same time period.

8. Hemorrhage of 20 to 30% of the total blood volume caused a sharp decrease in hematocrit, g% hemoglobin and red cell count. Iron incorporation increased from 0.080 to 0.367 μg ^{59}Fe/g Hb between the initial bleeding and day 28. There was a direct correlation (Corr. Coef. = 0.804) between per cent reticulocytes and ^{59}Fe incorporation by the red cells.

9. It appears that ^{59}Fe is a very accurate and useful tool for measuring erythropoiesis in trout and exceeds the accuracy of reticulocyte counts as an indicator of hematological stress, since it is difficult to distinguish immature from mature red cells using New Methylene Blue N stain.

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APPENDICES

APPENDIX A

SOLUTIONS AND STAINS

Hendrick's Blood Diluting Solution
(Hesser, 1960)

Sodium sulfate	10.0 gm
Sodium chloride	2.5 gm
Sodium citrate	1.5 gm
Glacial acetic acid	50.0 ml
Distilled water to make	500.0 ml

Phosphate Buffered Saline Solution (PBS)

One liter of a 280 mOsm PBS solution was prepared as follows.

Sodium chloride	7.83 gm
Potassium chloride	0.30 gm
Magnesium sulfate	0.14 gm
Potassium phosphate monobasic	0.46 gm
Sodium phosphate dibasic	2.02 gm
Distilled water to make	1000 ml

New Methylene Blue N Stain for Reticulocytes
(Wintrobe, 1961)

New Methylene Blue N	0.5 gm
Potassium oxalate	1.6 gm
Distilled water	100 ml

APPENDIX B

EQUATIONS AND CALCULATIONS

Calculation of $\mu g^{59}Fe/g$ Hb

The amount of radioactivity (μc) added to the total blood sample was converted to μg Fe using the specific activity ($\mu c/\mu g$) measured for the stock solution of $^{59}FeCl_3$. Equation 4 was used to calculate the μg of ^{59}Fe in each aliquot of blood before washing.

$$\frac{\text{total } \mu g\ ^{59}Fe \text{ added to total blood sample}}{\text{number of aliquots removed}} = \frac{\mu g\ ^{59}Fe}{\text{aliquot}} \qquad (4)$$

Counts/minute before wash were converted to cpm/$\mu g^{59}Fe$ by equation 5,

$$\frac{\text{cpm/aliquot before wash}}{\mu g\ ^{59}Fe/\text{aliquot}} = \frac{\text{cpm before wash}}{\mu g\ ^{59}Fe} \qquad (5)$$

and the counts/minute after wash were converted to $\mu g^{59}Fe$ incorporated by

$$\frac{\text{cpm after wash}}{\text{cpm before wash}/\mu g\ ^{59}Fe} = \mu g\ ^{59}Fe \text{ incorporated} \qquad (6)$$

The number of $\mu g^{59}Fe$/g Hb was calculated from the $\mu g^{59}Fe$ incorporated and the average g% hemoglobin measured for the pooled blood sample as shown in equations 7 and 8.

$$\text{g Hb/100 ml blood} \times \text{ml blood/aliquot} = \text{g Hb/aliquot} \tag{7}$$

$$\frac{\mu g^{59}\text{Fe incorporated}}{\text{g Hb/aliquot of blood}} = \mu g\ ^{59}\text{Fe/g Hg} \tag{8}$$

Mathematical Analysis of Uptake Equilibrium Curve

In all the uptake studies the concentration of ^{59}Fe within the red cells was plotted on arithmetic paper as $\mu g^{59}Fe$/g Hb on the ordinate and time on the abscissa. The equilibrium curve which resulted from such a plot demonstrated a rapid increase in iron incorporation within the first 4 hours of incubation which leveled off into a rather slow uptake (Figure 15). A point near the tail-end of the curve (at the time of last sample) was labeled the equilibrium concentration, C_{eq}. The concentration, $\mu g^{59}Fe$/g Hb, at each sample period was subtracted from the equilibrium concentration, and the difference, $C_{eq} - C_t$, was plotted on semi-log paper with time on the abscissa (Figure 16). The parabolic shape of the plot indicated that there were two components in the uptake curve: one component represented the initial uptake period from time zero to

Figure 15. --Example of an ^{59}Fe uptake equilibrium curve. C_{eq} represents the RBC ^{59}Fe concentration at equilibrium.

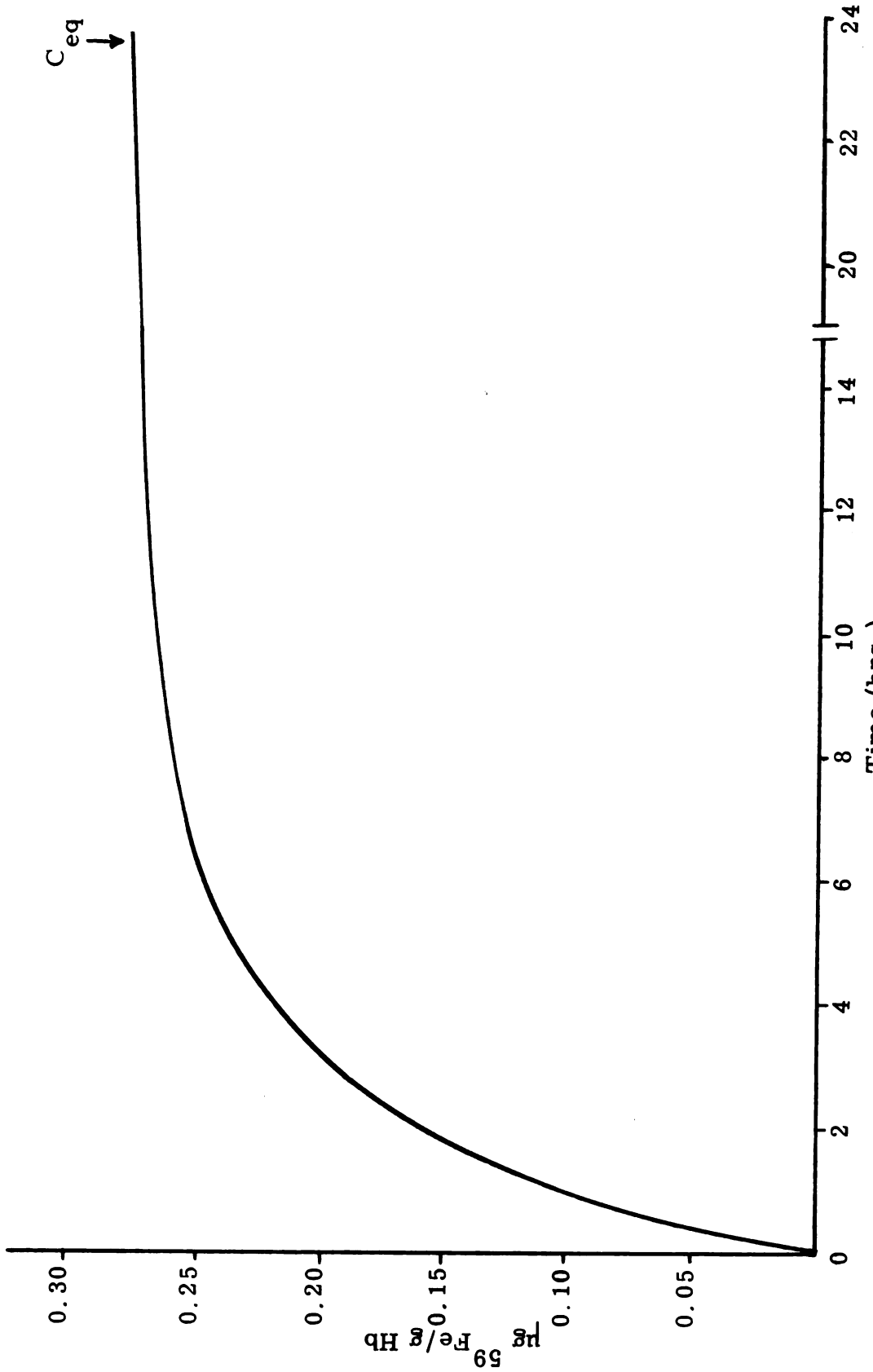
C_{eq}
0.30
0.25
0.20
0.15
0.10
0.05
$\mu g\ ^{59}Fe/g$ Hb
0
2
4
6
8
10
12
14
20
22
24
Time (hrs.)

Figure 15

Figure 16. -- Example of calculation and semi-log plot of components I and II of a two-component uptake curve.

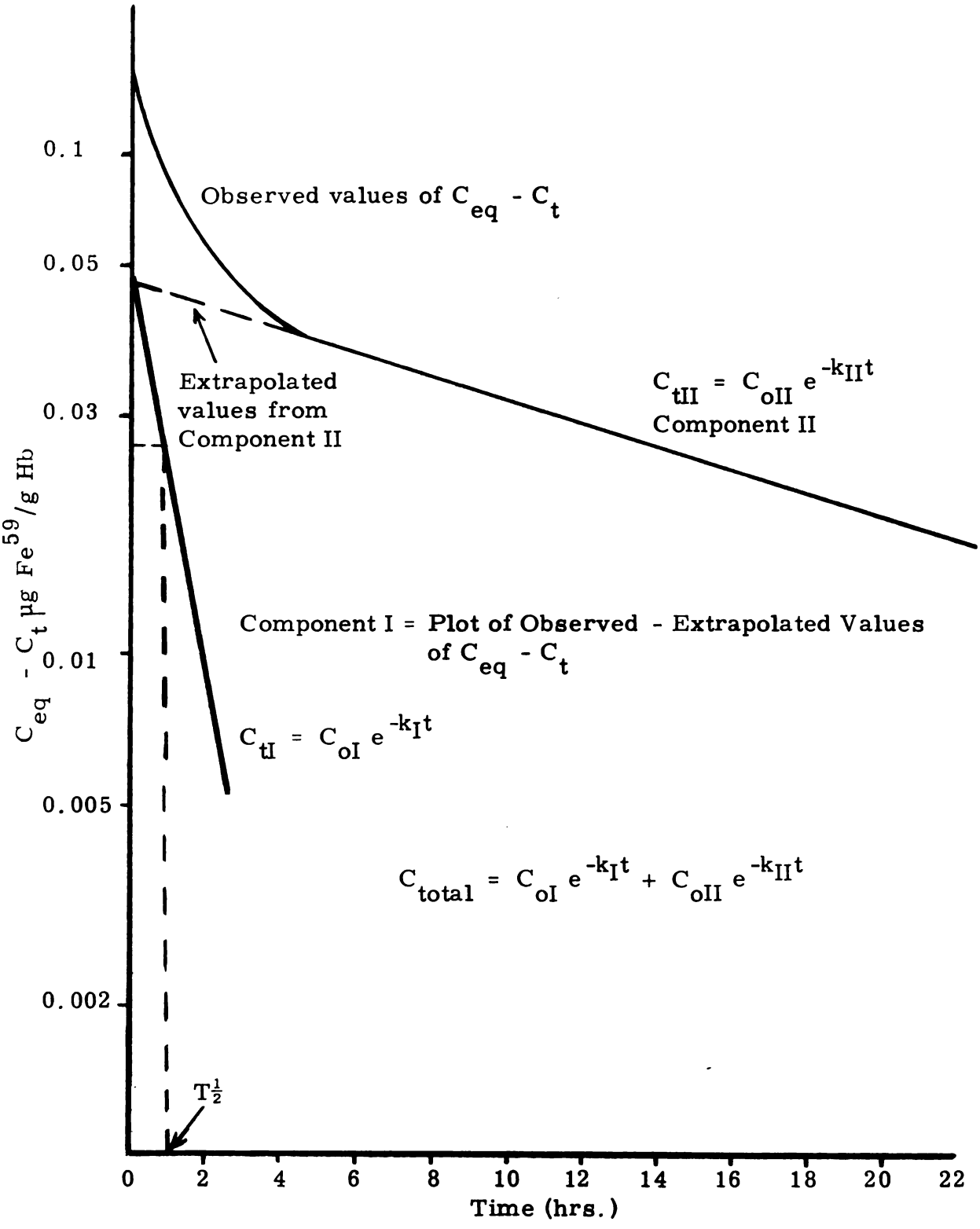
0.1
0.05
0.03
0.01
0.005
0.002
$C_{eq} - C_t$ μg Fe^{59}/g Hb
Observed values of $C_{eq} - C_t$
Extrapolated values from Component II
$C_{tII} = C_{oII} e^{-k_{II}t}$
Component II
Component I = Plot of Observed - Extrapolated Values of $C_{eq} - C_t$
$C_{tI} = C_{oI} e^{-k_I t}$
$C_{total} = C_{oI} e^{-k_I t} + C_{oII} e^{-k_{II} t}$
$T\frac{1}{2}$
0
2
4
6
8
10
12
14
16
18
20
22
Time (hrs.)

Figure 16

about 4 hours, and the second component represented the uptake between 4 hours and equilibrium. A linear regression analysis was made of the second component with the use of an Olivetti, Underwood Programma 101 computer, and the $C_{eq} - C_t$ values were extrapolated to time zero. The y-axis values obtained from the extrapolation of Component II were subtracted from the observed values of Component I for the corresponding time, t. A linear regression analysis of the difference was computed and the slope, y-intercept, and half-time of the difference were used to plot Component I (equation 9).

$$C_t = C_o\, e^{-kt} \tag{9}$$

Where: C_t = concentration at time t

C_o = concentration at time zero (y-intercept)

e = base of natural logarithm

k = rate constant = $0.693/T_{\frac{1}{2}}$ = slope

t = time in hours

$T_{\frac{1}{2}}$ = half-time or time necessary to reach $\frac{1}{2}$ the concentration at any time t

Components I and II were expressed by equations 10 and 11.

$$C_{tI} = C_{oI}\, e^{-k_I t} \tag{10}$$

$$C_{tII} = C_{oII}\, e^{-k_{II}t} \tag{11}$$

Equation 12 is an expression of the total activity incorporation in the two-component analysis and represents the activity acquired by two separate uptake processes of the red cell.

$$C_{total} = C_{oI}\, e^{-k_I t} + C_{oII}\, e^{-k_{II}t} \tag{12}$$

Student-Newman-Kuels Test for Comparison of Multiple Means Among Unequal Sample Sizes (Sokal and Rohlf, 1969a and b)

The observed range value must be higher than the LSR test statistic to be significantly different at the $\alpha = 0.05$ level. All least significant range (LSR) values are significantly different except those marked with an asterisk.

Hematocrit

LSR Test Statistic

Day	1	3	5	13	20
1	---				
3	4.455	---			
5	5.616	4.808	---		
13	7.056	6.555	5.568	---	
20	8.224	7.867	7.373	6.680	---

Observed Range Values

Day	1	3	5	13	20
1	---				
3	7.891	---			
5	15.792	7.901	---		
13	24.114	16.223	8.322	---	
20	26.268	18.796	10.895	2.273*	---

Hemoglobin

LSR Test Statistic

Day	1	3	5	13	20
1	---				
3	1.086	---			
5	1.302	1.142	---		
13	1.752	1.651	1.378	---	
20	1.927	1.871	1.704	1.617	---

Observed Range Values

Day	1	3	5	13	20
1	---				
3	3.183	---			
5	3.842	0.659*	---		
13	5.279	2.096	1.437	---	
20	6.503	3.220	2.661	1.224*	---

Red Blood Cell Count

LSR Test Statistic

Day	1	3	5	13	20
1	---				
3	0.133	---			
5	0.165	0.141	---		
13	0.205	0.190	1.163	---	
20	0.245	0.234	0.218	0.196	---

Observed Range Values

Day	1	3	5	13	20
1	---				
3	0.283	---			
5	0.493	0.210	---		
13	0.635	0.352	0.142*	---	
20	0.750	0.467	0.257	0.115*	---

$\mu g\ ^{59}Fe/g$ Hb

LSR Test Statistic

Day	1	3	5	13	20	28
1	---					
3	0.080	---				
5	0.096	0.085	---			
13	0.126	0.120	0.100	---		
20	0.143	0.140	0.127	0.118	---	
28	0.156	0.154	0.145	0.146	0.127	---

Observed Range Values

Day	1	3	5	13	20	28
1	---					
3	0.071*	---				
5	0.100	0.029*	---			
13	0.203	0.132	0.103	---		
20	0.270	0.199	0.170	0.067*	---	
28	0.287	0.216	0.187	0.084	0.017*	---