UPTAKE, DISTRIBUTION, AND INCORPORATION OF ^{59}Fe IN TISSUE AND BLOOD OF RAINBOW TROUT (Salmo gairdneri)

Dissertation for the Degree of Ph. D.

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This is to certify that the

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Richard L. Walker

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Paul O. Fromm

Major professor

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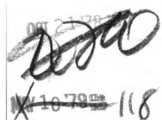

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ABSTRACT

UPTAKE, DISTRIBUTION, AND INCORPORATION OF ^{59}Fe IN TISSUE AND BLOOD OF RAINBOW TROUT (Salmo gairdneri)

By

Richard L. Walker

There is little information in the literature pertaining to iron metabolism and erythrocyte production in fish. This study was designed to evaluate the storage iron facilities in various tissues and to trace the distribution of radioiron in tissues and blood following an intraperitoneal (i.p.) injection of ^{59}Fe. Iron deficiency anemia was induced in an experimental group of rainbow trout in order to measure its effect on red blood cell production and mobilization of storage iron.

Forty percent of the blood volume of 48 experimental rainbow trout was removed in 4 separate bleedings over a seven day period. On the day of the last bleeding these fish and an additional 48 controls were injected, i.p., with ^{59}Fe (1 μCi/100 g). Six control and six experimental fish were killed on days 1, 2, 4, 8, 11, 16, 23, and 30 following the injection. The percent of initial injected ^{59}Fe, total iron concentration, and specific activity were measured in the liver, spleen, head kidney, pyloric caeca, intestine,

and skeletal muscle. Samples of fecal material, bile and urine were also collected for analysis of the ^{59}Fe content. Plasma iron, total iron binding capacity, hematocrit, hemoglobin, and red blood cell ^{59}Fe incorporation were measured.

Most of the ^{59}Fe was absorbed from the peritoneal cavity within 24 hrs. after the i.p. injection. Equilibrium between the plasma ^{59}Fe pool and that of the tissue was established by day 8. Experimental fish RBC ^{59}Fe content increased to 70-80% of the initial injected dose by day 16 compared to 50% in the controls. This was attributed to the difference in reticulocyte count which was 10-12% for the bled and 2-3% for control fish. The rate that iron is incorporated into hemoglobin by immature red cells is much slower (about half) than the rate of RBC ^{59}Fe uptake, thus, iron is temporarily stored in the cytoplasm. The iron for hemoglobin formation was obtained from liver iron stores which dropped from 12% to less than 1% of the initial injected dose by day 16. Total iron concentration in liver decreased from 200 to less than 100 μg Fe/g. The decrease in liver iron may have stimulated iron absorption by the intestine and pyloric caeca. There is evidence for a feedback mechanism mediated by transferrin.

UPTAKE, DISTRIBUTION, AND INCORPORATION OF ^{59}Fe IN TISSUE AND BLOOD OF RAINBOW TROUT (*Salmo gairdneri*)

By

Richard L. Walker

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INTRODUCTION AND LITERATURE REVIEW

There is probably more information known about the metabolism of iron than any other atom or molecule which composes living organisms (Garby and Vuille, 1967). Iron is an essential element involved in the physiological functions of oxygen transport and cellular respiration, and it is present in hemoglobin, myoglobin and cytochromes. From measurements made on human subjects and rats, an overwhelming volume of literature has resulted which deals with iron absorption, the mode of iron transport via the plasma, the utilization and conservation of iron, storage, and excretion. A normal, adult, 70 kg man contains 3-5 g of iron, 55% of which is in the form of hemoglobin, and 10-20% as myoglobin (Moore, 1958). The remainder is stored in the liver, spleen, kidneys and bone marrow, or is in transit within the plasma. Approximately 0.6-1.5 mg of iron is absorbed daily from the diet via the duodenum and upper jejunum, and 0.5-1.0 mg is excreted. Iron is recycled within the body from destroyed red blood cells and returned via the bone marrow as new red cells with very little iron excreted in the urine and feces. Since the details of iron metabolism in lower vertebrates are largely unknown a basic

outline of iron metabolism in mammals (Figure 1) will be used as a model for comparison with the iron cycle as it exists in rainbow trout. The fate of inorganic iron will be traced from ingestion to absorption and eventual incorporation into the hemoglobin of red blood cells, and/or storage as ferritin or hemosiderin in the liver and spleen.

Control of Iron Absorption

Luminal Factors Controlling Iron Absorption

Iron exists in the ferric and ferrous states in ingested food, but it is the ferrous iron which is more easily absorbed (Figure 1). One of the most widely studied and effective iron reducing agents is ascorbic acid. This and other organic acids including succinic, lactic, pyruvic, and citric enhance the absorption of both organic and inorganic iron by acting not only as reducing agents, but also as iron chelators (Van Campen, 1974; Dowdle et al., 1960; Jacobs et al., 1966). Although there is no complete agreement as to the importance of simple sugars such as fructose, lactose, sucrose, and glucose in promoting iron absorption and retention, a majority of the reports speak of the advantages these simple carbohydrates offer. All ten of the essential amino acids are effective in increasing iron uptake.

Figure 1. Flow diagram of iron metabolism in mammals showing the distribution of iron to various pools following absorption from the lumen of the intestine. Ferric ions are attached to transferrin in the plasma after iron crosses the mucosal cell. Transferrin delivers iron to the liver and spleen, the main storage organs, which contain ferritin and hemosiderin, two iron storage proteins. Iron is also transferred to the sites of red blood cell production which are mammalian bone marrow and fish head kidney. The majority of iron is in the form of hemoglobin as Fe^{+2}. Following hemoglobin catabolism by the reticulo-endothelial system, iron is transferred to storage in the liver or spleen, or returned to the sites of red blood cell production. Less than 0.5% of the total iron is lost to the urine or feces.

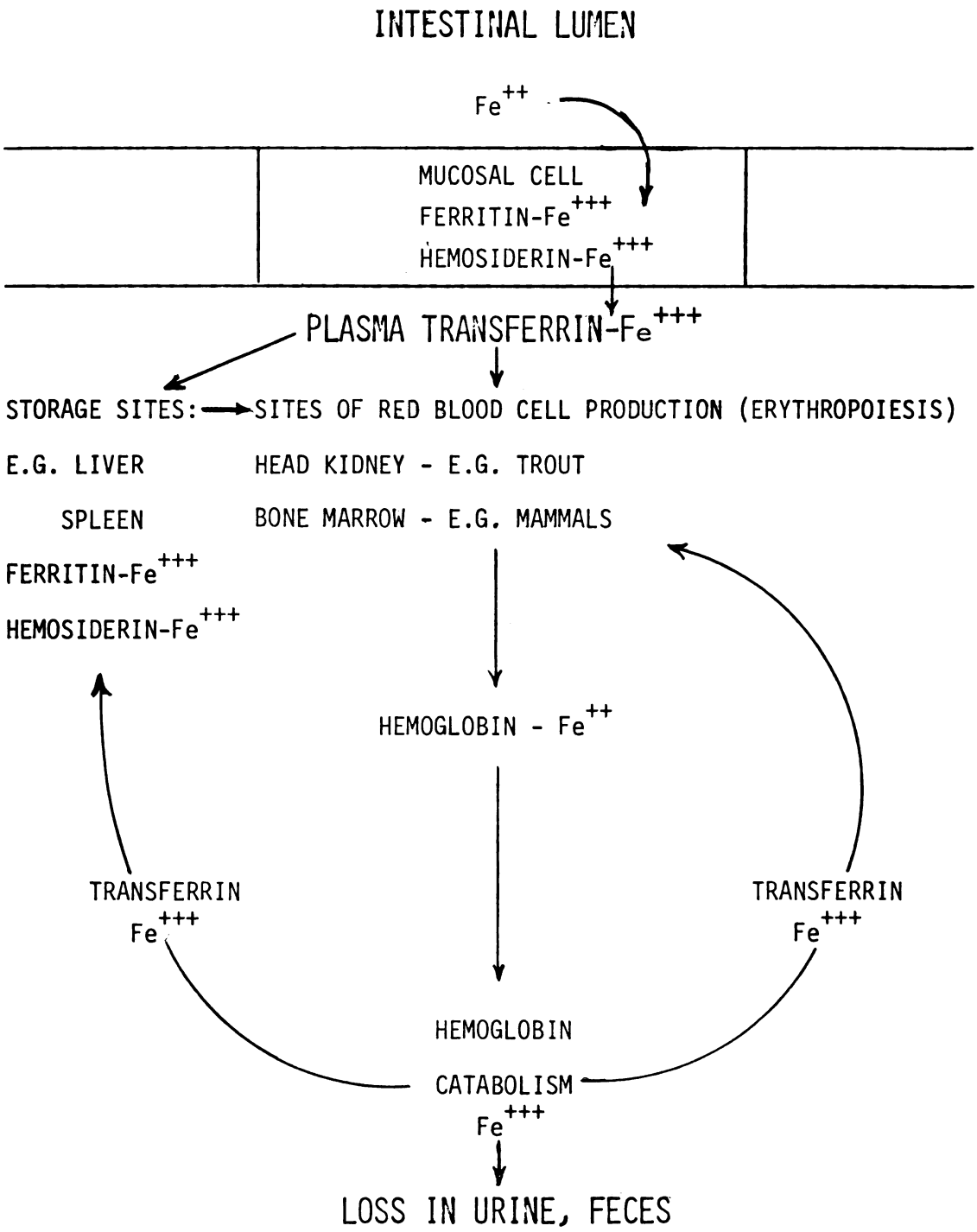
INTESTINAL LUMEN
Fe++
MUCOSAL CELL
FERRITIN-Fe+++
HEMOSIDERIN-Fe+++
PLASMA TRANSFERRIN-Fe+++
STORAGE SITES:
E.G. LIVER
SPLEEN
FERRITIN-Fe+++
HEMOSIDERIN-Fe+++
SITES OF RED BLOOD CELL PRODUCTION (ERYTHROPOIESIS)
HEAD KIDNEY - E.G. TROUT
BONE MARROW - E.G. MAMMALS
HEMOGLOBIN - Fe++
TRANSFERRIN
Fe+++
TRANSFERRIN
Fe+++
HEMOGLOBIN
CATABOLISM
Fe+++
LOSS IN URINE, FECES

Figure 1

Among the factors which depress the absorption of iron from the lumen of the intestine are phosphates and phytates. When complexed with iron these compounds reduce and almost completely block iron absorption. Callender (1964) discusses two other circumstances which result in depressed iron absorption; achlorhydria and secretion of pancreatic juice. Achlorhydria is associated with inadequate HCl production, which leads to incomplete breakdown of foodstuffs and decreased liberation of simple iron salts. Iron remains bound to complex organic molecules which are not absorbed but are lost with the feces. Pancreatic juice may serve more as an intraluminal regulator of excessive iron absorption, and therefore play a beneficial role. The action of pancreatic juice in decreasing iron absorption is not entirely clear, but it is believed that the bicarbonate content of the secretion is the factor which complexes with iron and decreases its availability for absorption (Van Campen, 1974).

Mucosal Cell Iron Absorption

Primary control mechanisms for iron absorption by intestinal mucosal cells are unknown, as are the mechanisms for transfer of information to the control system (Van Campen, 1974). There are several theories regarding mucosal cell iron absorption but there is still lack of agreement as to which is correct.

One of the earliest and most debated theories for control of mucosal iron absorption was the "mucosal block theory" proposed by Hahn et al. (1943) which was later supported by Granick (1946). The theory essentially states that intestinal mucosal cells contain the primary mechanisms for control of iron absorption, and that the absorptive process may be blocked by the saturation of the absorptive mechanism with iron. Granick (1946) maintained that the build-up of ferritin in the mucosal cell blocked iron absorption. The theory that the concentration of ferritin in the mucosal cell is the controlling factor in iron absorption was later supported by Conrad and Crosby (1963) and Crosby (1965), but with some modification of the theory proposed by Granick. They maintained that iron overload in the diet could overwhelm the absorptive mechanism and result in increased iron absorption. But in support of the mucosal block theory, Conrad and Crosby noted that iron deficient animals had a greater absorptive capacity than animals with abnormally high concentrations of iron in the plasma. Crosby (1965) hypothesized that the mucosal cell "ferritin apparatus" is responsive to plasma iron concentration, thus providing a way of relating to the intestinal epithelial cells the body iron status. Radioiron given intravenously (i.v.) to an iron deficient animal does not appear in the villous epithelial cells, and iron given by mouth is readily absorbed. However, radioiron administered i.v. to

iron-loaded patients, with higher than normal plasma iron concentrations, appeared in the intestinal epithelial cells via the "back door" with the result that orally administered radioiron was not absorbed. It is believed that the radioiron in the villous epithelial cells is incorporated into ferritin, thus becoming part of the ferritin apparatus. Crosby further states that ferritin may capture an intracellular iron-carrier molecule which is responsible for movement of iron from the lumen to the serosal surface of the musocal cell. In this manner a small amount of ferritin could block the absorption of a large amount of ingested iron.

The mucosal block theory is rejected by Schade (1972) who found that a decrease in intestinal iron uptake was not correlated with an increase in intestinal ferritin. Schade induced inflammation in a group of rats by giving an intramuscular injection of turpentine which resulted in a reduction in the intestinal absorption of iron. By measuring intestinal ferritin and ^{59}Fe activity following oral administration of radioiron, Schade found that there was little or no decrease in ability of the intestinal epithelial cells to absorb iron in the group of rats with inflammation, but there was a significant decrease in the transfer of iron from the intestine to the body. Although there was an increase in intestinal iron due to the reduction in iron

transport, there was no increase in intestinal ferritin content. Schade attributes any increase in intestinal ferritin, which other researchers have found, to intestinal build-up of iron as a result of disruption of iron transport. In other words, intestinal ferritin concentration is not the primary controlling factor in the transport mechanism, but is secondarily a result of iron build-up. Schade offers no information, nor does he speculate on the nature of the primary controlling factor in iron absorption.

Active Transport and Enzymatic Control

Active transport and enzymatically controlled reactions which are described by Michaelis-Menten kinetics have been hypothesized as having importance in the iron absorption process (Manis and Schacter, 1964; Jacobs et al., 1966). It is now accepted that iron metabolism involves uptake by the mucosal epithelial cell, transport across the mucosal cell, and transport out of the mucosal cell. Manis (1973) presents evidence for an enzymatically controlled reaction for the oxidation of Fe^{+2} to Fe^{+3} within the cell. He proposes that this ferroxidase reaction may be a means of regulating iron absorption since iron must be in the trivalent form before entering the plasma or sequestered by the mucosal cell as part of the trivalent iron pool. The importance of the mucosal cell trivalent iron pool (which may be

ferritin) in the control of iron absorption has already been considered.

Dowdle et al. (1960) demonstrated active transport of ^{59}Fe against a concentration gradient using everted gut sacs. Dependence on oxidative metabolism for generation of phosphate bond energy, and demonstration of saturation kinetics all point to active transport in the absorption of iron by gut segments taken just posterior to the pyloris of rats.

Non-Ferritin Intermediates

Another hypothesis proclaims the importance of non-ferritin iron-protein intermediates in the absorption of iron. Halliday and Powell (1973) discovered the presence of three non-ferritin intermediates while studying the absorption of iron by isolated rat mucosal cells. They described a two-component curve showing an initial, rapid phase of iron uptake in the first two minutes followed by a slower uptake over the next 30 minutes of exposure to medium of ^{59}Fe ferric chloride and ascorbic acid. Following fractionation of the cells and elution, they discovered the three intermediates present during the initial rapid phase of incorporation. These intermediates may be iron-transporting molecules which carry iron across the cell and perhaps to the iron-free protein, apoferritin, the precursor of ferritin. The slower uptake period was dominated by the

presence of ferritin with diminished quantities of the intermediate iron-carrying proteins.

Serosal Absorption of Iron

Iron has been administered intramuscularly (i.m.), intravenously (i.v.), orally, and intraperitoneally (i.p.) with about the same results; namely, appearance of the majority of the iron in red blood cells. In an attempt to measure appearance of iron in intestinal ferritin following i.p. injection of iron-dextran, Thirayothin and Crosby (1962) found iron concentrated in the core of the intestinal villi just above the lamina propria. When iron is administered orally the concentration is greatest at the surface of the intestinal villi. The explanation for the appearance near the lamina propria is the sequestration of iron from the peritoneal cavity by phagocytes which enter the intestinal serosa by diapedesis. The iron engulfed by the phagocytes is incorporated into the villi epithelial cells and the fate of this iron is similar to that absorbed from the lumen of the gut. The iron-laden phagocytes may also cross the epithelial cell and enter the lumen of the gut to be excreted as excess iron (Crosby, 1965).

The majority of iron administered i.p. is absorbed via the lymphatic system and delivered to the venous circulation via the thoracic duct (Thirayothin and Crosby, 1962). Iron entering the plasma is distributed to the body stores

and red blood cells in the same manner as iron administered orally, i.v., and i.m.

Plasma Iron

Iron is moved across the mucosal cell to the plasma pool in accordance with the body iron requirements. The transport into the plasma may involve an active process, but there is a lack of information regarding this step in the iron metabolism pathway. Free, ionic iron (Fe^{+2} and Fe^{+3}) does not exist in the plasma; it is bound to an iron-carrier protein known as transferrin. The process of attachment of iron to the transferrin molecule probably occurs at special receptor sites on the vascular surface of the mucosal cell. There is little information available regarding the mechanics of transfer of iron from the mucosal cell to transferrin, but the process probably involves a redox reaction because mucosal-Fe^{+2} must be oxidized to Fe^{+3} (Figure 1).

The standard plasma iron concentration range in humans is 120 to 146 µg Fe/100 ml (Ramsay, 1958). Average values for fish are 25 µg/100 ml in carp (Field et al., 1943) and 61 µg/100 ml in the tench (Hevesy et al., 1964), both well below the average iron concentration in human plasma. The plasma iron pool of humans represents about 0.1% of the total body iron, and the half-life of iron in plasma is quite short, about 90 minutes (Ramsay, 1958). This figure

was determined by measuring ^{59}Fe removal from the plasma following i.v. injection. Most plasma iron is sequestered by the erythropoietic tissue and incorporated into hemoglobin by immature red blood cells. The daily turnover of plasma iron in humans is about 20-40 mg/day, which is approximately the amount required for daily hemoglobin synthesis. There is a slight diurnal variation in plasma iron concentration, being highest in early morning and lowest during the afternoon. The difference is usually no greater than 15 µg/100 ml.

Transferrin

Structure and Chemical Nature

Transferrin, the beta-globulin protein which serves as the vehicle for iron transport in the plasma, has a molecular weight of about 86,000 and has two separate iron binding sites/molecule, each capable of binding one atom of ferric iron (Fletcher and Huehns, 1968; Bearn and Parker, 1964). The transferrin concentration in human plasma (0.24-0.28 g/100 ml) is such that the number of iron binding sites exceeds the amount of iron in the plasma; the transferrin pool is usually only 30-40% saturated with iron depending on the erythropoietic activity of the bone marrow and the overall iron status of the individual (Laurell, 1951). This figure represents the normal plasma iron content of

120-140 µg Fe/100 ml in humans and 25-60 µg Fe/100 ml in fish. The total iron binding capacity is about 300-360 µg Fe/100 ml plasma, but only under pathological conditions (such as hemochromatosis) will the per cent saturation of the transferrin pool reach 100%. Total iron binding capacity usually does not change even though plasma iron concentration fluctuates diurnally. Abnormally high transferrin levels, resulting in iron binding capacities above 400 µg/100 ml, are usually an indication of chronic iron deficiency (Laurell, 1958). Subnormal transferrin levels (below 260 µg Fe/100 ml) indicate impaired synthesis of plasma protein.

Iron is very tightly bound to transferrin; at physiological pH the equilibrium constants for the two iron binding sites are around 10^{30} (Fletcher and Huehns, 1968). Strong chelating agents such as ethylenediamine tetraacetic acid (EDTA) will not remove iron from transferrin. However, iron is easily removed from the transferrin at receptor sites in immature red blood cells and storage areas in the liver and spleen.

Delivery of Iron to the Reticulocyte

Attachment of iron-loaded transferrin molecules to special receptors on the reticulocyte and the subsequent transport of iron across the cell surface have been thoroughly studied (Jandl et al., 1959; Jandl and Katz,

1963; Katz, 1965). It is known that transferrin molecules saturated with ferric iron have a much greater affinity for the receptor sites on the reticulocyte cell membrane than transferrin lacking one or both atoms of iron (Jandl and Katz, 1963; Fletcher and Huehns, 1968). During transfer of one atom of ferric iron from transferrin to an immature red blood cell, the transferrin molecule remains at the surface of the cell for approximately one minute. Upon loss of one iron atom, the transferrin molecule is quickly replaced by another transferrin molecule carrying the full complement of two atoms of ferric iron. After completing the transfer of iron, transferrin less one atom of iron circulates back to intestinal mucosal cells or to storage sites in the liver and spleen for reloading.

Jandl and Katz (1963) were among the first to provide evidence for the presence of transferrin receptors on the surface of reticulocytes. Their experiments involved the use of doubly-labeled transferrin (^{131}I, ^{59}Fe), the metabolic inhibitors cyanide and dinitrophenylphenol, and enzymatic alteration of the cell membrane with trypsin. The ^{131}I label was used to follow attachment of transferrin to the reticulocyte surface, while ^{59}Fe was used to measure reticulocyte iron incorporation. They found that alteration of the cell surface with trypsin prevented attachment of transferrin and, subsequently, blocked iron absorption. Dilute concentrations of the metabolic inhibitors did not

block attachment of transferrin to the cell surface, but they did prevent transfer of iron across the cell membrane. In an excellent summary of these experiments, Katz and Jandl (1964) conclude that transferrin attaches to a specific, allosteric receptor site on the reticulocyte cell surface and that transfer of iron to the cell is an active process. Mature red blood cells do not retain these receptors, therefore they are unable to incorporate iron carried by the transferrin molecule, and contain functional hemoglobin with iron which cannot be exchanged with that of other intracellular or extracellular iron pools.

Fielding et al. (1969) support the findings of Katz and Jandl (1964) and have demonstrated the importance of sulfhydryl radicals in the incorporation of iron from transferrin. The addition of the sulfhydryl inhibitor, p-hydroxymercuribenzoate (PMB) which acts specifically at the cell surface, to a medium containing ^{59}Fe-transferrin and reticulocytes resulted in a significant decrease in reticulocyte iron absorption. They concluded that sulfhydryl groups in the cell membrane are involved in the process of reticulocyte iron uptake, but they were unsure of whether the sulfhydryl groups were involved with attachment of transferrin to specific receptor sites, or with the dissociation of the transferrin-iron complex after adhesion to the cell surface, or both. Later, Edwards and Fielding (1971) reported that dilute concentrations of PMB did not affect transferrin

attachment to the reticulocyte surface, but it was effective in reducing iron uptake by the reticulocyte. They concluded that sulfhydryl groups are active in dissociation of iron from its bond with transferrin after transferrin has attached to the allosteric receptor site.

In summary, transferrin is a beta-globulin of molecular weight 86,000 which serves as the iron-carrier protein in plasma and is responsible for the transport of ferric iron from the intestinal mucosa to iron storage areas in the liver and spleen, and to the erythropoietic tissues. Iron is transferred to immature red blood cells for incorporation into hemoglobin. The transfer process involves attachment of the transferrin molecule to allosteric receptor sites on the reticulocyte surface and the removal of one atom of ferric iron which is transported across the surface of the cell by active means. Sulfhydryl radicals play an important role in the dissociation of iron from transferrin and absorption by the reticulocyte. The whole process is completed within one minute, and the transferrin molecule, minus one iron atom, is replaced by another molecule with two iron atoms. Transferrin reenters the circulation and repeats the cycle with attachment to the receptor sites in the intestine.

Erythropoiesis

Erythropoietic Tissue

In mammals, birds, and reptiles the bone marrow is the main erythropoietic (red blood cell producing) organ. Generally speaking, fish lack sufficient bone marrow for erythrocyte production and, therefore, must rely on some other tissue. The kidney, and particularly the head kidney in rainbow trout (Figure 1), is the main organ or erythropoiesis in teleost fish (Topf, 1953). The renal intertubular tissue contains lymphoid tissue which is the origin of the small lymphoid hemoblasts, the progenetors of the erythrocyte line (Catton, 1957; Klontz et al., 1969). Head kidney tissue appears microscopically similar to red bone marrow, and the spongelike structure of the tissue results in a complex blood flow pattern through the interstices between the tubules (Catton, 1957). New red cells enter the circulation in the head kidney, along with a few immature red cells or reticulocytes. The number of immature red cells in the peripheral circulation varies with the erythropoietic activity and immediate need for additional red cells. The erythropoietic activity is in turn controlled by various external and internal stimuli.

Erythropoietic Stimulants

Stimulants of erythropoiesis include bleeding, hypoxia, injection of phenylhydrazine, and an increase in serum

calcium. Among these, bleeding is the most effective and widely investigated stimulant. In experiments with turtles, Altland and Thompson (1958) found high reticulocyte counts (10-20% of red cell population) which lasted for at least 21 days after a series of bleedings. Hirschfeld and Gordon (1965b) noted an increase in reticulocyte numbers and erythroblast ^{59}Fe incorporation 14 days following removal of 20% of the blood volume of turtles. Rosse et al. (1963) report similar findings in frogs after removal of 30% of the blood volume and also in birds after removal of 25% of the blood volume (Rosse and Waldman, 1966), using thymidine-2-^{14}C incorporation by peripheral immature red blood cells as an indicator of erythropoietic activity. Zanjani et al (1969) used bleeding as a means of stimulating erythropoiesis in fish, and Finch et al. (1959) employed bleeding as the technique for the study of kinetics of erythropoiesis in rabbits. In summary, bleeding has been reported as an effective means of increasing red blood cell production in nearly all vertebrates that rely on hemoglobin or oxygen transport.

Hypoxia, an effective erythropoietic stimulant in homeotherms such as rats and birds, does not appear to affect red cell production in poikilotherms. Altland and Parker (1955) artificially decreased the P_{O_2} of turtle blood, and observed no increase in hematocrit or hemoglobin concentration. Rosse et al. (1963) reported a similar result in frogs exposed to hypoxic conditions, and they speculate that

the increase in erythropoietic activity due to bleeding must be mediated by a mechanism different from the response to hypoxia. The only positive erythropoietic response, increased hemoglobin concentrations and red cell counts, to lowered ambient P_{O_2} was found in goldfish which were acclimated to low oxygen concentrations for several days (Prosser et al., 1957). This resultant increase in oxygen carrying capacity may be a reason why goldfish, and other members of the carp family, are able to live in oxygen-poor water. Since trout require fast-flowing, highly-oxygenated water for survival, it is doubtful that such a response to hypoxia (i.e., a significant increase in hemoglobin concentration) occurs in these teleosts (Dawson, 1933).

Drugs, such as phenylhydrazine (which causes hemolysis of red cells), have been used to artificially stimulate erythropoiesis in several species, including fish. Finch et al. (1959) compared the erythropoietic effects of bleeding and phenylhydrazine injection (i.p.) in rabbits, and found relatively little difference in the erythropoietic response to both stimulants. Tambourin et al. (1973) found phenylhydrazine injections were effective in producing high plasma levels of erythropoietin (see next section) in mice. Smith et al. (1971) report that a single injection of phenylhydrazine (12.5 µg/g body weight) into Chinook salmon produced severe anemia within 10 days, at which time hematocrits and hemoglobin concentrations had been reduced to 1-5% of

normal. Red cell hemolysis was indicated by the appearance of cell-free hemoglobin in the fin rays and by histological changes in liver, spleen, and kidney tissue. However, blood parameters returned to normal levels 95 days after the injection, indicating an increase in erythropoietic activity following the induced anemia.

Serum calcium levels may also affect erythropoiesis. Such is the case in rats as demonstrated by Perris and Whitfield (1971). After calcium chloride or parathyroid hormone injection they found an increase in the mitotic activity of the bone marrow leading to increased reticulocyte production and ^{59}Fe incorporation by immature cells in the peripheral blood. Following parathyroidectomy, there was a decrease in plasma calcium and erythropoiesis, but this situation could be reversed if calcium chloride or parathyroid hormone were administered. In nephrectomized rats, which lack the ability to produce erythropoietin, administration of calcium and/or parathyroid hormone, stimulated mitosis in bone marrow erythropoietic cells without secondarily initiating erythropoietin release. Perris and Whitfield speculate that there may be two mechanisms involved in the control of erythropoiesis in the rat. Calcium may serve in the regulation of non-specific mitosis in the bone marrow cells, while erythropoietin may have a highly specific differentiating action in bone marrow which results in increased erythrocyte production.

Erythropoietic Depressants

The most common depressants of erythropoiesis are starvation and hyperoxia. In turtles starvation lowers plasma iron to as much as 1/3 normal concentration, but otherwise the peripheral hemograms of starved and normal fed turtles are about the same, i.e., hematocrit, hemoglobin and red cell counts are very similar (Hirschfeld and Gordon, 1965b). Starvation for 6 weeks prior to bleeding prevented an increase in erythroblast production and, thus, depressed the erythropoiesis which was seen in normal fed turtles subjected to the same bleeding schedule. Thus, the nutritional status of the animal may exert a considerable effect on erythropoietic activity, and what may appear to be a normal fed animal (according to hematocrit and hemoglobin) may actually be erythropoietically impaired. Zanjani et al. (1969) found that starvation significantly reduced erythropoiesis in fish, but, unlike the turtle, the hemoglobin, hematocrit and red cell counts were also significantly reduced. Even though the effects of starvation appear to depress erythropoiesis in turtles subjected to bleeding, the nutritional status of starved blue gourami (Zanjani et al., 1969) did not seem to hamper increased red blood cell production in response to bleeding. It is obvious from the above discussion that the nutritional status of the experimental animals should be considered in investigations of erythropoiesis.

The inhibition of erythropoiesis by hyperoxia was demonstrated by Fletcher et al. (1973). Using parabiotic rats, they found that induced hyperoxia in one partner by exposure to 95-100% oxygen resulted in depression of normal red blood cell production in the other partner. They attributed the depression to a humoral factor produced in the hyperoxic partner which inhibits erythropoiesis. It has been speculated that erythropoiesis may be controlled by a set of hormones which induce erythropoietic activity during hypoxia and depress erythropoiesis during hyperoxia.

Erythropoietic Stimulating Factor and Renal Erythropoietic Factor

It now appears that erythropoiesis is under the control of a humoral factor, erythropoietin or erythropoietic stimulating factor (ESF), which acts as a stimulant to red blood cell production. ESF is released into the plasma in response to anemia, hypoxia, hemorrhage and other erythropoietic stimuli. It acts at the bone marrow or head kidney to increase both the rate of red blood cell production and their release into the peripheral circulation. Gordon (1959) has written an excellent review of erythropoietin which includes 248 references concerning the origin, physical characteristics, and actions of this hormone. Erythropoietin (a glycoprotein with a molecular weight of about 60,000) is formed in the plasma from a combination of liver globulin and a substance secreted from the kidneys,

renal erythropoietic factor (REF). Zanjani et al. (1967) have shown that REF extracts from kidneys produces ESF when added to dialyzed rat serum and they presented evidence to show that REF is not related to angiotensin or renin. Little is known about the structure or mechanism of action of REF other than it may be activated by renal cyclic-AMP through a protein kinase (Rodgers et al., 1974).

Erythropoietin appears to be species specific. Mammalian ESF will not increase erythropoiesis in birds, reptiles or fish, nor are any ESF fractions from these lower vertebrates effective in mammals. Zanjani et al. (1969) experimented with ovine ESF and found that it had no erythropoietic effects in fish, but anemic fish plasma did stimulate red blood cell production when injected in fish of the same species.

Immature Red Blood Cell Iron Uptake

The number of immature red blood cells in the peripheral circulation is normally quite small (1% red cell population in mammals). But, after subjection to bleeding, hypoxia or any erythropoietic stimulant, there is a substantial increase in the peripheral immature red cell population. This increase initiates mobilization of storage iron for hemoglobin synthesis. The reticuloendothelial (RE) cells in the liver and spleen, which remove old or damaged red blood

cells from circulation, store iron sequestered from the catabolism of hemoglobin. Release of this iron from RE cells is regulated by the erythropoietic activity. Using heat-denatured erythrocyctes containing ^{59}Fe hemoglobin, the movement of iron from the RE cells to the immature red blood cells in the peripheral circulation has been traced (Lipschitz et al., 1971). Following i.p. administration of ^{59}Fe there is a direct correlation between the number of immature red blood cells in the peripheral circulation and the ^{59}Fe activity of the blood (Walsh et al., 1949; Jensen et al., 1953).

As stated previously, iron is delivered to the immature red blood cell directly from transferrin at specific receptor sites on the cell membrane. There may be a second means of iron incorporation by immature red blood cells. For example, in animals with erythropoietically active bone marrow, iron (as ferritin) may be pinocytized by erythroblasts from RE cells in the bone marrow. Whether this is also the case in the fish head kidney is not known. Regardless of the means of obtaining iron, it is stored in the immature red blood cells as ferritin and eventually as ferruginous micelles in mitochondria (Bessis and Breton-Gorius, 1959; Kaplan et al., 1954). During hemoglobin formation the mitochondria release the ferruginous micelles to the cytoplasm and iron is then incorporated into the hemoglobin molecule. The ferritin clusters and iron-laden

mitochondria disappear from the cytoplasm upon completion of hemoglobin formation.

Red Cell Life Span

Hevesy et al. (1964) injected tench i.m. with glycine-2-^{14}C and found the red blood cell life span to be 150-200 days. There is evidence that the red cell life span fluctuates with changing environmental parameters such as atmospheric pressure and temperature, and with the animals' metabolic rate. Mice red blood cells formed during exposure to 0.5 atmospheres had a shorter half-life (39.3 days) than those of control mice (46.9 days) (Abbrecht and Littell, 1972). Although hypoxia due to decreased atmospheric pressure apparently has an effect on mammalian red cell life span, it is doubtful that hypoxic conditions affect poikiloterm red cell longevity since no erythropoietic response to lowered environmental P_{O_2} has been observed in these animals. However, temperature and metabolic rate are both considered factors which influence poikilotherm red cell life span. Altland and Brace (1962) found the red cell life span to be between 600 and 800 days in turtles and 700-1,400 days in toads. The long life span is attributed to the low metabolic rate of these animals. There is evidence that environmental temperature affects red cell life span in frogs (Cline and Waldmann, 1962). At 24-26°C the life span is approximately 200 days, but this value is

increased significantly in frogs exposed to 4°C. Perhaps the poikilothermic frogs are less reliant on hemoglobin for oxygen transport at such low temperatures. The arctic ice fish, for example, does not rely at all on red blood cells and hemoglobin for oxygen transport. This fish lives in water that is just above the freezing point, and under such conditions the metabolic rate is low enough that the amount of oxygen transported in the dissolved state in the plasma is sufficient to meet the oxygen requirements of the tissues. There is currently no information available concerning changes in ambient temperature and metabolic rate as they affect red cells in rainbow trout.

In summary, the main erythropoietic organ in trout appears to be the head kidney. Erythropoiesis is controlled by the plasma ESF concentration which is increased in response to blood loss, or hemolytic anemia. Hypoxia does not appear to be an ESF stimulant in poikilotherms; however, there is some indication that members of the carp family respond to hypoxia with an increase in circulating red blood cells. Starvation, which is an effective erythropoietic depressant in turtles, may not hamper erythropoiesis in fish as evidenced by the increased number of ^{59}Fe incorporating immature red blood cells in the peripheral circulation of the blue gourami following bleeding. However, the nutritional states of the animal should be considered in any study of erythropoiesis.

Iron is mobilized from the storage areas, mainly in the liver and spleen, and transported to immature red blood cells for eventual incorporation into hemoglobin. Prior to hemoglobin formation in immature red cells, iron is stored as ferritin in the cytoplasm and mitochondria. The concentration of ferritin gradually decreases as hemoglobin formation nears completion.

The life span of red blood cells in fish has been measured and is estimated to be at least 150 days. Red cell survival is subject to changes in ambient temperature and the animal's metabolic rate. Although hypoxia appears to result in decreased life span of newly formed red cells in mammals, there is no evidence of shortened red cell survival in poikilotherms subjected to hypoxia.

Storage Iron

Iron is transported to depots from the intestine via transferrin or is sequestered from old and dying red cells. The liver and spleen are the main storage organs which contain the iron storage proteins ferritin and hemosiderin (Figure 1). Other organs and tissues such as the intestine, kidney and skeletal muscle contain trace amounts of ferritin and hemosiderin. Hemoglobin, the main source for storage iron, is sequestered by RE cells and recycled through the storage iron pool before being reutilized (Sanchez-Medal

et al., 1970; Cook et al., 1974). Time in storage and quantity of iron stored vary considerably with the iron status of the animal. Anemia due to blood loss severely reduces the iron storage by stimulating erythropoietic activity. Iron overload (e.g., hemochromatosis) results in a significant increase in the iron content of the spleen and liver. The following discussion is concerned with ferritin and hemosiderin, and the storage of iron in the liver and spleen.

Ferritin and Hemosiderin

Ferritin, the most common form of stored iron, is a water-soluble crystalline protein with a molecular weight of 860,000. It has two components, an iron-free protein, apoferritin, and micelles of a colloidal hydrated iron oxide-phosphate complex (Harrison, 1964). About 17-23% of the dry weight of ferritin is iron. Apoferritin exists in the intestinal mucosal cells and parenchymal and RE cells of the liver and spleen. During absorption and storage, ferric ions are attached to the apoferritin molecule to form the crystalline structure, ferritin. Reduction of the ferric iron of ferritin to the ferrous state by biological reducing agents results in the separation of the iron from apoferritin.

Hemosiderin is composed of protein and non-protein organic constituents of variable nature and quantity

(Harrison, 1964). The structure is ill-defined, but hemosiderin is known to be rich in ferric hydroxide and ferric phosphates. Iron composes about 8 to 45% of the weight of hemosiderin, depending on the nature of organic constituents. It has been suggested that hemosiderin may contain ferritin and apoferritin molecules. Whatever the composition, hemosiderin serves as a secondary iron storage compound, which normally appears in small amounts in liver and spleen.

Liver and Spleen as Iron Storage Organs

The cell types containing high iron concentrations are the RE cells of the liver and spleen, and the liver parenchymal cells. Liver ferritin is chiefly stored in the parenchymal cells (hepatocytes) and some ferritin and hemosiderin is present in the RE cells (Kupffer cells). Parenchymal ferritin is formed almost exclusively from iron delivered by plasma transferrin, while Kupffer cells sequester iron from the catabolism of senescent red blood cells (Cook et al., 1972). Some 93% of liver iron is stored in parenchymal cells and 7% in Kupffer cells (Cook et al., 1974). In hemolytic anemia and other diseases resulting in iron overload, the excess iron is quickly put into storage as hemosiderin in the Kupffer cells (VanWyk et al., 1971). This storage is easily verified in tissue sections of liver in which hemosiderin is discernible as numerous blue

clusters following Perl's reaction for hemosiderin iron (Pearse, 1961). Iron is slowly removed from hemosiderin and placed in storage as ferritin to re-establish the normal ferritin:hemosiderin ratio in the liver.

The spleen, which maintains the largest iron concentration of any organ, is mainly composed of RE cells which contain both ferritin and hemosiderin. The ferritin content is relatively constant but hemosiderin content, like that of the liver Kupffer cells, varies in relation to the iron status of the animal. Hemosiderin has been detected in fish spleens by Grover (1968), and Yu et al. (1971) who noted a direct relation between spleen hemosiderin concentration and erythropoietic activity. After subjecting blue gourami to bleeding Yu et al. (1971) discovered a reduction in the hemosiderin content of the spleen which they attributed to mobilization of storage iron for increased hemoglobin synthesis in immature red blood cells.

Iron Storage in Intestine, Muscle and Kidney

Trace amounts of iron are detectable as ferritin and hemosiderin in the intestinal mucosa, skeletal muscle and kidney. Appearance of iron in these tissues is most noticeable during iron overload following hemolysis, or injection of high concentrations of iron dextran. Intestinal mucosa, which is important in regulation of iron absorption,

is significant in terms of total iron storage under normal conditions.

Summary

Ferritin and hemosiderin are the two iron storage compounds; ferritin being the more prevalent and stable of the two forms. Hemosiderin operates as the temporary iron storage form when excessive amounts of iron overload the body. The liver and spleen are the two main iron storage organs; the spleen having the highest iron concentration. Parenchymal cells in the liver contain ferritin and store almost all the iron found in the liver. The RE or Kupffer cells, of the liver contain some iron as hemosiderin and ferritin sequestered from methemoglobin of lysed red blood cells. The spleen contains many RE cells which destroy old red cells and concentrate iron as hemosiderin and ferritin. Other storage areas in the intestinal mucosa, skeletal muscle and kidney are relatively insignificant.

RESEARCH RATIONALE

This project was originally designed to study the effects of various water-borne pollutants on fish erythrocyte production and iron metabolism. A number of parameters were chosen for investigation, including red blood cell ^{59}Fe incorporation and tissue ^{59}Fe distribution. However, there was very little basic information pertaining to iron metabolism in poikilotherms such as rainbow trout. Therefore, it was necessary to establish a baseline for iron metabolism in fish before using these animals in pollution experiments.

The objectives of the study were:

1. To follow uptake and distribution of intraperitoneally injected ^{59}Fe in blood, plasma and tissues of rainbow trout,
2. To measure the total iron content of various tissues and establish their importance in iron storage, and
3. To evaluate the effects of iron deficiency induced by bleeding on:
   a) red blood cell production,
   b) the utilization of iron in storage, and
   c) the plasma iron and transferrin concentrations.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) weighing 250-300 g were obtained from Midwest Fish Farm Enterprises at Gladwin, Michigan. They were transported to Michigan State University in 80 gallon galvanized metal tanks lined with non-toxic paint. The tanks were contained within insulated boxes fitted with agitators for aeration. At the university fish were held in 120 gallon fiberglass tanks (Frigid Units, Toledo, Ohio) equipped with aeration stones and flowing water supply (2 liters/min) and held at 13 $\pm$ 1°C under 14 hours of light and 10 hours darkness per day. The water was filtered through activated charcoal to remove particulate iron and chlorine.

Experimental Protocol

The fish were divided into an experimental and a control group; each containing 50 animals. Both groups were fed a maintenance diet (100 g feed/10 kg fish) of EWOS salmon pellets (Astra-Ewos, Södertälje, Sweden) three times weekly. The iron content of the food was 300-400 µg Fe/g

food. A total of 40% of the blood volume was removed from each experimental fish in four bleedings (10% blood volume/ bleeding) over a seven day period, allowing 48 hrs between bleedings. On the day of the last bleeding, both experimental and control fish received an intraperitoneal injection of 0.9% saline solution (Appendix A) containing 1 μCi $^{59}FeCl_3$/100 g fish (Amersham-Searle, Chicago, Ill.) and having a specific activity of 5 μCi/μg Fe. Six experimental and six control fish were sacrificed on days 1, 2, 4, 8, 11, 16, 23 and 30 following the ^{59}Fe injection.

Bleeding Technique

Fish were bled from the caudal vein at a point just posterior to the anal fin using a heparinized syringe fitted with a 1½ inch long 21 gauge needle. The needle was inserted through the ventral surface into the hemal arch, and the plunger was pulled back slowly until the desired amount of blood was obtained.

Tissue Sample

After the removal of 4-5 ml of whole blood the fish was killed by a sharp blow to the head and weighed on a Torbal Torsion Balance (Torsion Balance Co., Clifton, N.J.). The following tissue samples were removed (the average

sample weights are in parentheses): liver (0.5 g), spleen, head kidney, and middle third of the kidney (0.3 g), muscle (0.5 g), pyloric caeca (0.5 g), and intestine (0.5 g). After removal of the intestinal contents, the gut was rinsed with 0.9% saline and a 1 cm length of midgut was taken as the intestine sample. The muscle tissue sample was removed from the flank just dorsal to the lateral line. Tissue sample wet weights were measured using a Roller-Smith Balance (Roller-Smith Co., Bethleham, Pa.) and intestinal contents were tare weighed on a Mettler Model B5 Balance (Mettler Instrument Corp., Princeton, N.J.). The tissues and intestinal contents were stored at 0°C in 12 x 75 mm glass culture tubes (Scientific Products, McGraw Park, Ill.) for future total iron and/or ^{59}Fe analyses.

Bile Sample

Before removing the liver, the gall bladder was located and a 1 ml syringe fitted with a 25 gauge needle was used to withdraw as much of the bile as possible. The bile volume was measured and placed in a glass culture tube for measurement of ^{59}Fe activity.

Measuring ^{59}Fe Activity

^{59}Fe activity was determined using a Nuclear Chicago Model 1085 dual-channel gamma counter equipped with a Model 8725 scaler/analyzer and NaI-Tl crystal (Des Plaines, Ill.). In order to select the two ^{59}Fe photopeaks at 1.098 and 1.289 MEV, the base was set at 500 and the window at 250 with a coarse attenuator setting of 8 and fine attenuator at 25 (based on the ^{137}Cs standard calibration at base 610, window 100, coarse attenuator 4, fine attenuator 25). The high voltage settings were 900 coarse, 90 fine and the mode selector switch was on WIDE DIFF. These settings resulted in a detector efficiency of 5-10% of the actual dpm.

To insure consistency in counting efficiency, radioiron standards prepared from the stock $^{59}FeCl_3$ were counted with each group of tissue and blood samples. All samples were counted for 1 min and the activity was corrected for background radiation (10 cpm) and for isotope decay from day zero, the time of the i.p. injection of ^{59}Fe. It was important to maintain constant geometry during counting. Therefore, a standard volume of 1 ml of blood and plasma were counted, and tissue and fecal samples were packed near the bottom of the culture tubes to approximate the same volume.

Hematocrit, Hemoglobin and Reticulocyte Counts

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

Hemoglobin determinations were made spectrophotometrically by the cyanmethemoglobin method (Oser, 1965) using Hycel (Houston, Texas) standards and reagents and a Beckman Model DG-B spectrophotometer (Fullerton, Calif.). A standard curve of the optical density (OD) versus concentration (g%) was plotted from dilution of the prepared standard. Hemoglobin samples were prepared by adding 20 µl of whole blood to 5 ml of cyanmethemoglobin reagent. Hemoglobin concentrations in gram percent were calculated from equation 1:

$$\frac{\text{Concentration (unknown)}}{\text{Concentration (standard)}} = \frac{\text{OD (unknown}}{\text{OD (standard)}}$$

where gram percent and corresponding OD from the standard curve were used for the concentration and OD of the standard.

Reticulocyte (immature red blood cell) counts were made on blood smears prepared by a supra-vital staining technique reported by Lucas and Jamroz (1961). Equal volumes

of fresh whole blood and New Methylene Blue N (in 0.9% saline) were mixed and incubated for 20 min at 13°C. To make a blood smear, a small drop of this mixture was then placed on a clean glass slide and drawn across the surface quickly with the edge of a second glass slide applying very light pressure so as not to rupture the cells. The smears were allowed to air dry, and then were examined using the oil immersion objective of a microscope. Reticulocytes were distinguished from mature red cells by the blue staining RNA present in the cytoplasm. There was some difficulty in distinguishing mature from immature red cells because of the RNA remnants present in some mature cells. Therefore, cells were categorized as to their degree of staining. A section of the slide was selected where the cells were evenly spaced. Using an ocular grid a total of 1,000 cells (immature and mature) were counted. The number of reticulocytes was expressed as a percentage of this population.

Plasma Iron, UIBC and TIBC

Plasma iron was determined colorimetrically by the method of Plaut *et al.* (1972) as described in Appendix B. The results were expressed in µg Fe/100 ml plasma (µg%). Care was taken to prevent hemolysis during the centrifugation of 3-4 ml of whole blood in an IEC Model Cl Clinical

Centrifuge (Needham Heights, Mass.). Plasma from any hemolyzed samples was discarded because of the error introduced by the high concentration of iron in the hemoglobin of lyzed red cells.

The plasma total iron binding capacity (TIBC) was calculated from the plasma iron content and the unsaturated iron binding capacity (UIBC). The method of Kuypers and van Oers (1973) was used for the determination of UIBC of a 0.2 ml sample of plasma (Appendix C). The UIBC assay is based on the incubation of ^{59}Fe-labeled ferric ammonium citrate with the 0.2 ml plasma sample and the incorporation of iron by unsaturated plasma transferrin. Excess iron was removed after incubation by addition of an anion exchange resin strip (Gallard-Schlesinger Chemical, L.I., N.Y.) and the total ^{59}Fe activity remaining in the incubation mixture was used in calculating the UIBC in µg%.

Tissue Total Iron Analysis

Tissue iron was measured colorimetrically after wet-ashing with a sulfuric-nitric acid mixture (Appendix D). The ashing procedure was partially adapted from the Dow Chemical Company (1970) method for "Determination of Mercury in Fish" and from H. Bergman (personal communication). Tissue weighing from 0.1 to 0.5 g (wet weight) was dissolved in 5 parts concentrated sulfuric acid and one part

concentrated nitric acid at 90°C. After digestion for 2-3 hrs in this mixture potassium permanganate was added and the digestion was allowed to continue overnight at room temperature. The digestate was decolorized the following day with hydroxylamine hydrochloride and an aliquot was removed for iron analysis. The colorimetric procedure was the standard bathophenanthroline method for iron reported by Diehl and Smith (1965). This method was sensitive down to 0.04 ppm using the Beckman DB-G spectrophotometer at 540 nm.

Blood Volume

Total blood volume was determined by the ^{51}Cr-RBC method of Conte et al. (1963). Whole blood from a donor fish was incubated with ^{51}Cr (10 μCi/ml blood) at 13°C on a Scientific Industries (Springfield, Mass.) variable speed rotator set at 6 rpm for 24 hrs. The red blood cells were spun down, washed 3 times with phosphate buffered saline (PBS, Appendix A), and rediluted to the desired Hct (30-40%) with PBS. After measuring the specific activity (cpm/ml RBC), the ^{51}Cr-labeled RBCs were injected intracardially using a 27 or 28 gauge needle. The syringe was slowly rinsed twice with the blood of the recipient fish to insure delivery of the total aliquot of labeled blood. After 30

min a blood sample was drawn from the caudal vein and the specific activity was determined (cpm/ml whole blood). Blood volume was calculated by dividing the total injected activity by the specific activity of the recipient blood. Tissue blood volumes (ml/g) were determined by dividing the tissue sample ^{51}Cr activity (cpm/g) by the specific activity of the whole blood (cpm/ml).

Data for calculation of whole blood volume and tissue blood volume were obtained from fish of the same size as used in the iron experiment. The tissue blood volumes were used to correct tissue ^{59}Fe activity and total iron concentration for the blood iron content. It was not possible to determine tissue blood volumes by the ^{51}Cr method in the fish used in the iron experiments because of the time factor and the problem of discriminating ^{51}Cr energy peaks from ^{59}Fe.

Urine Collection

To determine urinary loss of iron fish were catheterized and placed in the apparatus seen in Figure 2. Fish were anesthetized with tricaine methanesulfonate (Finquel®, Ayerst Laboratories, New York), and a PE 90 catheter with flared tip was sutured into the urogenital opening. The fish were then placed in 30 x 6.5 cm glass cylinders sealed with No. 13 rubber stoppers. The catheter was fed through

Figure 2. Urine collection apparatus. Following catheterization of the urogenital opening with PE 90 tubing, trout were placed in four 30cm X 6.5cm. cylindrical glass fish chambers (D) connected to a water reservoir. Water was filtered through glass wool (A) before entering the reservoir (B). A constant head pressure was maintained by an overflow tube (C). Aerated water passed from the reservoir by gravity flow to each fish chamber (D). The catheter was passed through a small hole in the rubber stopper and urine was collected in test tubes (E). After leaving the chamber, water was directed through a fecal trap before passing down the drain (F).

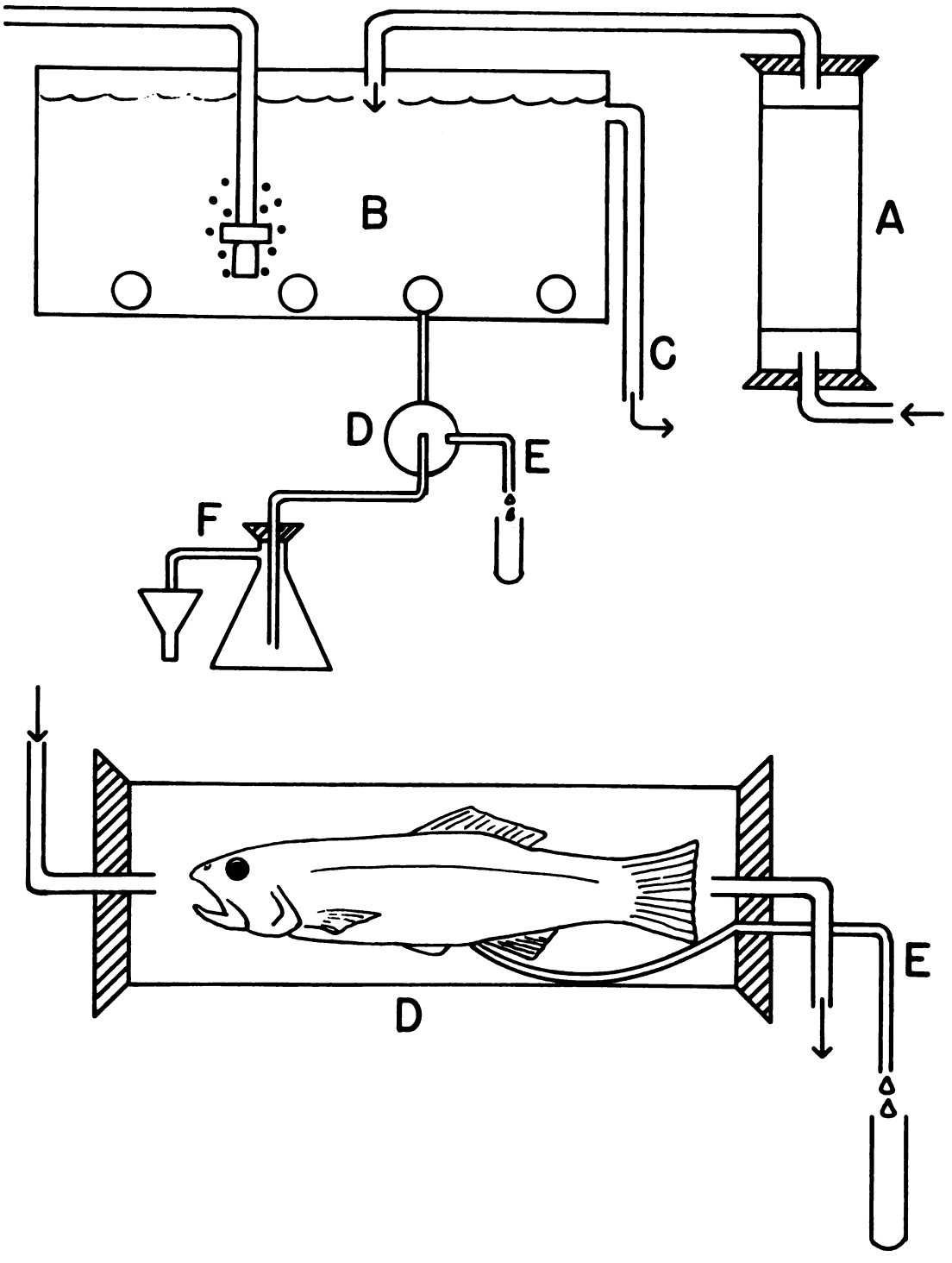

Figure 2

a small hole in the rear rubber stopper and connected to a collecting tube. Each cylinder received a constant flow (150 ml/min) of fresh, highly oxygenated water from a reservoir by gravity flow. Water entering the reservoir at 660-700 ml/min was filtered through a column of glass wool to remove particulate iron. An overflow line was connected near the top of the reservoir to keep the head pressure constant. The outflow tubes from each cylinder were connected to side-arm Erlenmeyer flasks where fecal material was collected for ^{59}Fe analysis before the water passed down the drain. Inflow and outflow oxygen concentrations (mg/l) were checked periodically to make sure the fish received an adequate O_2 supply.

Statistical Analyses

Statistical analyses of the data were done on the M.S.U. Control Data 6500 Computer using a one-way layout analysis of variance for equal or unequal sample size. The means were compared for significant differences by the Student-Newman-Kuels test (Sokal and Rohlf, 1969 a and b) at $\alpha = 0.05$.

RESULTS

General Health of Animals

The trout used in this study were well-fed and appeared quite healthy as indicated by the amount of fecal material in the gut, the concentration of fat in the peritoneal cavity and the hematocrit and hemoglobin concentrations. All but one fish were sexually mature and there were 61 females and 33 males randomly sampled in the study.

Tissue Data Interpretation

Total blood volume of rainbow trout averaged 3.5 ml/100 g fish as determined by dilution of ^{51}Cr-labeled red blood cells. The blood volumes of the various tissues sampled in the study are given in Table 1. Included in this table are the tissue wet weight/body weight ratios for the size trout used in this study. The tissue sample ^{59}Fe content was corrected for tissue blood volume and expressed as percent of the initial injected ^{59}Fe appearing in the total organ or tissue mass. Total iron concentration (corrected for blood) is expressed as µg Fe/g tissue, and specific activity is the concentration of ^{59}Fe in µg/g tissue divided by the total iron content in one gram of

Table 1.--Tissue blood volumes and tissue wet weight/body weight ratios for 250-300 g rainbow trout. Blood volumes were determined from dilution of ^{51}Cr-labeled RBC injected intracardially.

Tissue	Blood Volume (ml whole blood/g)	Tissue Wet Weight (g/100 g body wt.)
Liver	0.150	1.103
Spleen	0.230	0.213
Head Kidney	0.250	0.200
Kidney	0.283	0.620
Pyloric Caeca	0.030	1.273
Muscle	0.006	88.746
Intestine	0.023	0.420

tissue, or:

$$\frac{\mu g\ ^{59}Fe}{\mu g\ \text{Total Iron}}$$

Standard errors are not included in any of the figures due to the resulting cluttered appearance. But the sample means, standard errors and number of observations for tissue total iron, tissue and blood ^{59}Fe content, and tissue specific activity are listed by days in Appendix E.

Hct, Hb and Reticulocytes

The removal of 40% of the blood volume within a 7 day period had a significant effect on the hematocrit, hemoglobin and reticulocyte count of the experimental fish. Bleeding significantly depressed the rad cell volume from

43 to 14% of the blood volume (Table 2) and reduced the hemoglobin concentration from 8.145 to 2.850g%. The first indication of a significant recovery in hematocrit was 16 days following the last bleeding, however, the hemoglobin concentration did not recover until 30 days after bleeding. The difference in recovery of hematocrit and hemoglobin indicates that the red blood cells lost by bleeding are replaced by immature red blood cells which lack complete hemoglobin molecules. The reticulocyte data show an increase in the percent reticulocytes from 2.99 at the last bleeding to 17.64% eleven days later. There is an increase in the per cent reticulocytes to 11.07% on day 8 but this value is not significantly higher than the reticulocyte count on preceding days. The presence of RNA in the cytoplasm, as seen in the supravital stain, is indicative of hemoglobin formation in these cells as they undergo maturation. Recovery from bleeding was complete by day 30 as both hematocrit and hemoglobin were not significantly different from the control and the percent reticulocytes of the bled fish had declined to 5%.

The increase in hematocrit in the control fish between days 1 and 30 was statistically significant. However, there was no concomitant increase in hemoglobin or percent reticulocytes within the 30 day time span.

Table 2.--Hematocrit, hemoglobin, and percent reticulocytes of blood samples from control and experimental fish. Forty percent of the whole blood volume was removed in four separate bleedings from the experimental fish prior to i.p. injection of ^{59}Fe. Included are Mean ± SE(N).

Day	Group	Hematocrit (percent)	Hemoglobin (gram percent)	Reticulocytes (percent)
Bleeding				
1st	Experimental	43.12 ± 1.061(33)	8.145 ± 0.269(33)	
2nd	Experimental	27.48 ± 1.150(29)	6.155 ± 0.260(30)	
3rd	Experimental	25.60 ± 1.158(30)	5.689 ± 0.275(30)	2.740 ± 0.480(10)
4th	Experimental	22.23 ± 0.969(30)		2.991 ± 0.846(10)
Days Post Injection				
1	Experimental	16.33 ± 2.17(6) *	3.479 ± 0.393(6) *	6.980 ± 0.795(5) *
	Control	29.17 ± 2.44(6)	7.067 ± 0.632(6)	4.220 ± 0.753(5)
2	Experimental	14.17 ± 1.92(6) *	2.850 ± 0.396(6) *	
	Control	31.17 ± 2.82(6)	7.050 ± 0.707(6)	
4	Experimental	19.83 ± 1.99(6) *	3.217 ± 0.315(6) *	5.000 ± 1.500(2)
	Control	40.67 ± 3.08(6)	7.717 ± 0.432(6)	2.883 ± 0.573(6)
8	Experimental	26.33 ± 2.29(6) *	4.650 ± 0.479(6) *	11.070 ± 5.456(5)
	Control	40.33 ± 1.52(6)	8.717 ± 0.327(6)	1.733 ± 0.536(6)
11	Experimental	24.67 ± 1.78(6) *	3.917 ± 0.310(6) *	17.640 ± 6.685(5) *
	Control	38.83 ± 3.23(6)	8.267 ± 0.791(6)	2.266 ± 0.689(6)
16	Experimental	31.67 ± 1.02(6)	5.083 ± 0.101(6) *	10.616 ± 2.060(6) *
	Control	34.00 ± 2.29(6)	7.050 ± 0.489(6)	2.840 ± 1.137(5)
23	Experimental	33.50 ± 2.67(6) *	5.917 ± 0.577(6) *	5.067 ± 2.059(6) *
	Control	43.67 ± 2.75(6)	9.167 ± 0.735(6)	0.400 ± 0.184(6)
30	Experimental	35.20 ± 3.81(5)	5.820 ± 0.721(5)	5.000 ± 0.900(2)
	Control	44.00 ± 1.86(6)	7.817 ± 0.411(6)	

*Significant difference at $\alpha = 0.05$.

Comparison of Tissue Specific Activity

Table 3 ranks the tissues and plasma in order from highest specific activity to lowest in experimental and control fish from day 1 to day 30. Head kidney had the highest specific activity of any tissue during the first 4 days following the i.p. injection of ^{59}Fe. Bled fish head kidney was about 4 times greater than the next highest tissue and was exceeded only by the plasma. Control fish head kidney was 1½-2 times greater than the next highest tissue. After 4-8 days the head kidney fell and liver and spleen assumed the position of highest rank in control and bled fish. The pyloric caeca were intermediate in rank, followed by intestine and muscle which had the lowest specific activity. It was interesting to note that even though the plasma specific activity decreased 4-8 fold within 8 days, it was still higher than any tissue throughout the experiment. The only exception was on day 4 when head kidney specific activity was higher than plasma in bled fish.

Plasma

Plasma total iron concentration, percent initial injected dose, and specific activity over the 30 day study period are shown in Figure 3. Plasma total iron concentration averaged 50-60 µg Fe/100 ml plasma. The apparent increase in total iron concentration from day 1 to day 30

Table 3.--Tissue and plasma mean specific activity ranked from highest to lowest on each sampling day of the study. Forty percent of the blood volume was removed from the experimental fish in four separate bleedings prior to i.p. injection of ^{59}Fe.

	$\bar{X}$ Specific Activity x 10^{-5}													
Group	Day 1		Day 2		Day 4		Day 8		Day 11		Day 16		Day 23		Day 30	
Exp.	P	366.00	P	154.00	HK	114.42	P	87.50	P	25.10	P	28.10	P	18.80	P	27.10
	HK	27.32	HK	146.03	P	67.90	HK	10.96	S	8.97	S	6.57	S	2.46	S	3.93
	C	7.81	L	27.44	L	30.57	S	8.15	L	7.78	HK	2.67	L	2.03	L	1.81
	L	6.90	S	22.23	S	21.11	L	7.90	C	7.17	C	2.13	C	0.51	C	0.18
	I	5.39	M	6.01	C	12.72	C	2.24	HK	3.90	L	1.72	HK	0.13	I	0.17
	S	1.85	C	5.06	I	2.68	I	0.40	I	2.07	M	0.09	M	0.07	HK	0.09
	M	0.54	I	2.00	M	0.44	M	0.10	M	0.08	I	0.00	I	0.06	M	0.06
Con.	P	382.00	P	154.00	P	45.10	P	50.80	P	42.50	P	42.70	P	23.20	P	37.70
	HK	52.89	HK	84.98	HK	33.16	L	11.77	L	19.15	L	15.66	L	17.89	L	9.27
	L	18.91	L	57.82	L	20.12	C	8.53	C	9.99	S	5.67	HK	5.37	S	3.61
	C	16.32	S	17.18	C	8.84	HK	8.31	HK	8.19	HK	5.67	C	5.22	HK	3.19
	I	5.55	C	9.28	S	7.23	I	8.30	S	4.18	C	3.56	I	1.86	C	2.81
	S	3.56	I	3.04	I	3.99	S	6.27	I	1.73	I	1.32	S	1.24	I	1.19
	M	0.99	M	1.02	M	0.63	M	0.13	M	0.50	M	0.15	M	0.30	M	0.18

C = Caeca
HK = Head Kidney
I = Intestine
L = Liver
M = Muscle
P = Plasma
S = Spleen

Figure 3.--Percent initial injected ^{59}Fe (A), total iron concentration (B), and specific activity (C) of plasma from experimental and control trout on days 1, 2, 4, 8, 11, 16, 23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to i.p. injection of ^{59}Fe. Each point represents the mean for 6 fish (day 30 exp., n=5). Significant differences ($P < 0.05$) among control and experimental means are listed below. There were no significant differences among the total iron concentration means.

A	Exp.	Day 1 ≠ Days 4, 8, 11, 16, 23, 30
		Day 2 ≠ Days 4, 8, 11, 23
	Con.	Day 1 ≠ Days 2, 4, 8, 11, 16, 23, 30
C	Exp.	Day 1 ≠ Days 2, 4, 8, 11, 16, 23, 30
	Con.	Day 1 ≠ Days 2, 4, 8, 11, 16, 23, 30

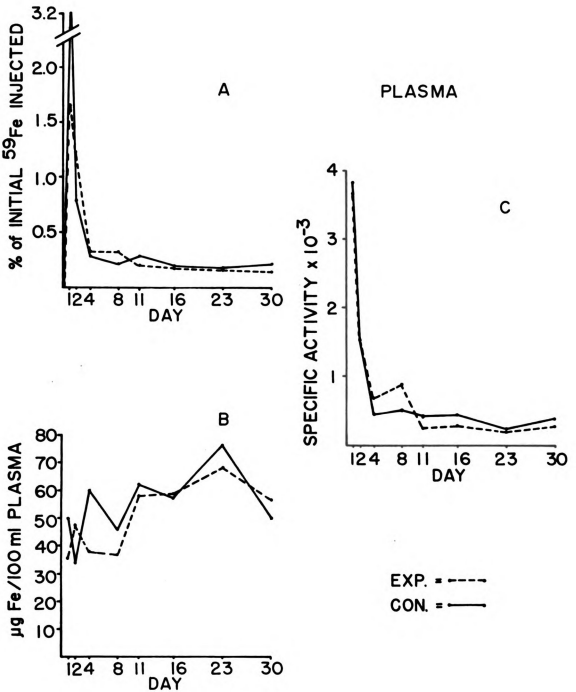
A
% of INITIAL 59Fe INJECTED
3.2
2.0
1.5
1.0
0.5
1 2 4 8 11 16 23 30
DAY
PLASMA
C
SPECIFIC ACTIVITY x 10-3
4
3
2
1
1 2 4 8 11 16 23 30
DAY
B
µg Fe/100 ml PLASMA
80
70
60
50
40
30
20
10
1 2 4 8 11 16 23 30
DAY
EXP. = ----
CON. = ——

Figure 3

was not significant in either the control or experimental fish, nor were there significant differences between these two groups on any of the sample days. In contrast to total iron, the percent of initial injected ^{59}Fe was maximal on day 1 for controls (3.2%) and experimentals (1.7%). For both groups it decreased significantly to 0.3% by day 4 before leveling off at 0.25% from day 8 to day 30. Specific activity decreased 80-90% from day 1 to day 4 and then remained constant from days 4 to 30 in both experimental and control fish. There was no significant difference between experimental and control specific activity or percent of initial injected dose. The half-time for initial clearance of ^{59}Fe from plasma was 24 hrs.

Plasma Iron, UIBC and TIBC

Although plasma iron showed no significant change, UIBC and TIBC did increase around day 16 in both experimental and control groups and remained significantly higher than days 1-11 for the remainder of the study (Table 4). This indicates an increase in transferrin concentration in the plasma of both groups after day 11. Paired comparisons showed no significant differences between mean values of controls and experimentals on any given day. The values for TIBC were unusually high in these fish in comparison to human plasma TIBC (300-400 µg%) and values obtained in

Table 4.--Plasma iron, UIBC and TIBC for experimental and control fish. Forty percent of the whole blood volume was removed from the experimental fish in four separate bleedings prior to i.p. injection of ^{59}Fe. Included are the Mean ± SE(N). There were no significant differences between control and experimental means.

Day	Group	Plasma Iron (µg Fe/100 ml)	UIBC (µg Fe/100 ml)	TIBC (µg Fe/100 ml)
Bleeding				
1st	Experimental	37.9 ± 2.5(10)		
2nd	Experimental	43.3 ± 3.2(6)		
3rd	Experimental	58.4 ±12.7(7)		
4th	Experimental	47.1 ± 7.3(9)		
Days Post Injection				
1	Experimental	34.9 ± 5.6(6)	446.7 ± 24.9(6)	481.6 ± 24.2(6)
	Control	49.6 ± 4.4(5)	398.3 ± 20.9(6)	445.6 ± 24.0(5)
2	Experimental	48.4 ± 8.2(6)	413.0 ± 53.8(5)	458.3 ± 54.0(5)
	Control	33.5 ± 9.0(3)	505.0 ± 65.0(2)	534.5 ± 51.0(2)
4	Experimental	38.1 ± 5.3(6)	449.2 ± 17.6(6)	487.1 ± 18.9(6)
	Control	60.0 ± 8.2(6)	390.0 ± 37.2(6)	450.0 ± 42.2(6)
8	Experimental	36.8 ± 4.7(6)	435.0 ± 29.4(6)	471.8 ± 29.3(6)
	Control	45.8 ± 9.7(6)	475.0 ± 33.1(6)	520.7 ± 30.8(6)
11	Experimental	57.6 ± 6.0(6)	512.5 ± 37.1(6)	570.0 ± 34.2(6)
	Control	62.3 ± 7.1(5)	375.0 ± 52.2(5)	437.4 ± 47.3(5)
16	Experimental	58.2 ± 5.3(5)	654.5 ± 32.4(4)	710.3 ± 33.5(4)
	Control	56.9 ± 8.9(6)	615.5 ± 62.6(6)	672.3 ± 61.5(6)
23	Experimental	68.7 ± 7.3(5)	699.0 ± 37.9(4)	762.7 ± 39.8(4)
	Control	76.9 ±14.3(4)	605.7 ± 27.9(3)	674.4 ± 25.7(3)
30	Experimental	56.5 ±17.6(5)	615.0 ± 73.1(5)	671.6 ± 57.8(5)
	Control	53.5 ± 9.4(6)	569.5 ± 49.0(6)	623.2 ± 40.2(6)

an earlier study (250-300 µg%). Human plasma, which was used as a standard, was within the range for normal human TIBC. Therefore, the high averages for fish plasma TIBC are probably not due to experimental error. Although there was no statistical difference in plasma iron concentration with time, there was a trend toward an increase which coincided with the significant increase in UIBC on days 16-30. This may indicate mobilization of storage iron between days 11 and 30, which would correspond with the decrease in tissue specific activity during the same period.

Head Kidney and Kidney

The head kidney had the highest specific activity of all tissues sampled between days 1 and 4 (Figure 4 and Table 3). This rapid uptake of ^{59}Fe by erythropoietic tissue in the head kidney was followed by an equally rapid reduction (80 to 90%) in radioiron content from day 2 to day 8. The radioiron content of head kidney and kidney tissue was below 2% of the initial injected dose for the remainder of the study. Except for the initial drop in iron concentration in the experimental head kidneys seen on days 2 and 4 the total iron content did not change significantly during the 30 day period. There were no significant differences between head kidney total iron concentration in controls and experimental animals nor were there differences in

Figure 4.--Percent initial ^{59}Fe injected (A), total iron concentration (B) and specific activity (C) of the head kidney of experimental and control trout on days 1, 2, 4, 8, 11, 16, 23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed over a seven day period prior to injection of ^{59}Fe. Tissue total iron concentration, and ^{59}Fe activity were corrected for tissue blood volume. Each point represents the mean for 6 fish (Day 30 exp., n=5). Significant differences ($P < 0.05$) among control or experimental means are listed below.

A	Exp.	Day 1 ≠ Days 2,4,8,11,16,23,30
		Day 2 ≠ Days 1,8,11,16,23,30
		Day 4 ≠ Days 1,8,11,16,23,30
	Con.	Day 1 ≠ Days 8,11,16,23,30
		Day 2 ≠ Days 8,11,16,23,30
		Day 4 ≠ Days 8,11,16,23,30
B	Exp.	Day 1 ≠ Days 2,4,16,23
C	Exp.	Day 1 ≠ Days 2,4,11,16,23,30
		Day 2 ≠ Days 1,4,8,11,16,23,30
		Day 4 ≠ Days 1,2,8,11,16,23,30
	Con.	Day 1 ≠ Days 8,11,16,23,30
		Day 2 ≠ Days 4,8,11,16,23,30

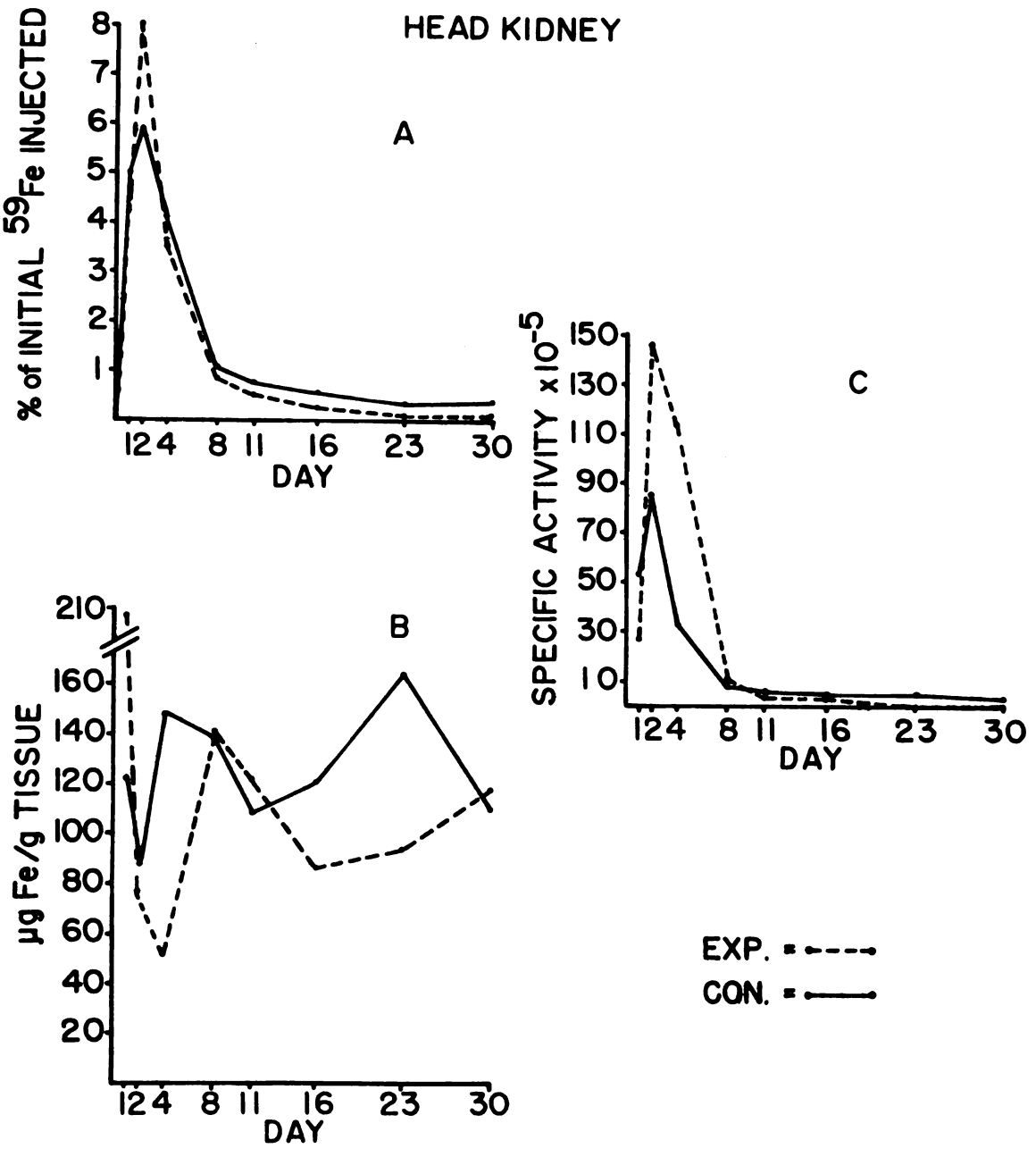
HEAD KIDNEY
A
% of INITIAL ^{59}Fe INJECTED
8
7
6
5
4
3
2
1
1 2 4 8 11 16 23 30
DAY
B
µg Fe/g TISSUE
210
160
140
120
100
80
60
40
20
1 2 4 8 11 16 23 30
DAY
C
SPECIFIC ACTIVITY x10^{-5}
150
130
110
90
70
50
30
10
1 2 4 8 11 16 23 30
DAY
EXP.
CON.

Figure 4

percent of initial injected dose or specific activity. Total iron concentration and specific activity were not measured in the remaining kidney tissue.

Liver

As seen in Figure 5 there are some significant differences between experimental and control fish liver ^{59}Fe content, total iron concentration and specific activity. As early as day 11 the percent of initial injected ^{59}Fe had decreased significantly in the experimental fish liver and by day 23 the total iron concentration of the experimental liver was lower than the control (Figure 5B). The specific activities for the experimental group were significantly different from control values at days 16-30. This indicates the importance of the liver as an iron storage depot, a reserve which was substantially depleted when erythropoiesis increased. The high specific activity and percent initial dose relative to other tissue such as caeca, intestine and muscle is an indication that absorbed iron is initially placed in storage in the liver as ferritin or hemosiderin. Total iron, percent initial dose, and specific activity remained fairly constant from days 1-30 in control fish.

Figure 5.--Percent initial ^{59}Fe injected (A), total iron concentration (B) and specific activity (C) of the livers of experimental and control trout on days 1,2,4,8,11,16,23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to the injection of ^{59}Fe. Tissue total iron concentration and ^{59}Fe activity were corrected for the tissue blood volume. Each point represents the mean for 6 fish (Day 30 exp., n=5). In paired comparisons of experimental and control means, significant differences were found at $P < 0.05$ (*). Significant differences ($P < 0.05$) among experimental means are listed below. Control means were not significantly different.

A Exp. Day 2 ≠ Days 16,23,30

B Exp. Day 1 ≠ Days 2,4,8,11,16,23,30

C Exp. Day 2 ≠ Days 8,11,16,23,30
Day 4 ≠ Days 1,8,11,16,23,30

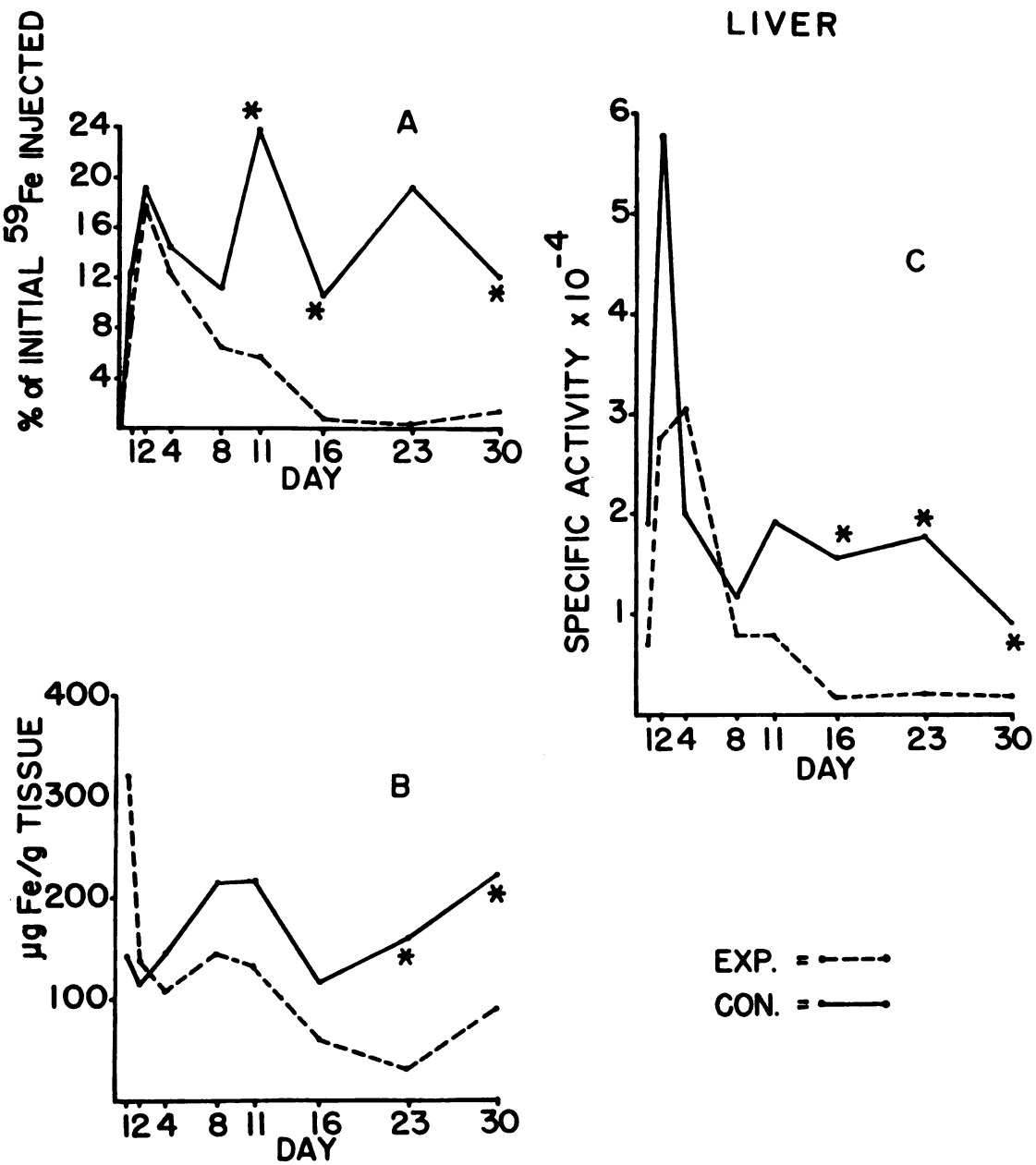
LIVER
% of INITIAL 59Fe INJECTED
A
24
20
16
12
8
4
1 2 4 8 11 16 23 30
DAY
SPECIFIC ACTIVITY x 10-4
C
6
5
4
3
2
1
1 2 4 8 11 16 23 30
DAY
µg Fe/g TISSUE
B
400
300
200
100
1 2 4 8 11 16 23 30
DAY
EXP. =
CON. =

Figure 5

Spleen

There was no significant differences between values for control and experimental spleens in terms of total iron content, ^{59}Fe incorporation, or specific activity. A sharp increase in percent of initial dose of ^{59}Fe was noted (Figure 6A) in the spleen from day 1 ($\simeq$1.75%) to day 2 ($\simeq$4.5%) followed by a gradual decrease in activity to day 23 (0.5%). The rise in activity from day 23 (0.5%) to day 30 (1.25%) is not significant but may be indicative of a general increase in splenic activity after day 23. Specific activity showed similar changes with time (Figure 6B) as percent of initial dose. Total iron concentration ranged from 900 µg Fe/g spleen to as low as 225 µg Fe/g and averaged 300-400 µg Fe/g for experimental and control spleens. There were no significant differences in spleen total iron content among controls and experimentals due to the large variances in the data.

Pyloric Caeca

Following a peak in ^{59}Fe concentration in the experimental fish at day 4 post injection, there is a reduction in radioiron in the pyloric caeca to 0.5% of the initial injected activity by day 8. Less than 0.2% of the activity remained at 23 days following ^{59}Fe injection (Figure 7A).

Figure 6.--Percent of initial ^{59}Fe injected (A), total iron concentration (B) and specific activity (C) of the spleens of experimental and control trout on days 1,2,4,8,11,16,23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to injection of ^{59}Fe. Tissue total iron concentration and ^{59}Fe activity were corrected for the tissue blood volume. Each point represents the mean for 6 fish (Day 30 exp., n=5). Significant differences ($P < 0.05$) among control or experimental means are listed below.

A	Exp.	Day 2 ≠ Days 1,4,8,11,16,23,30
		Day 4 ≠ Days 2,23
C	Exp.	Day 2 ≠ Days 1,11,16,23,30
		Day 4 ≠ Days 1,11,16,23,30
	Con.	Day 2 ≠ Days 1,4,8,11,16,23,30

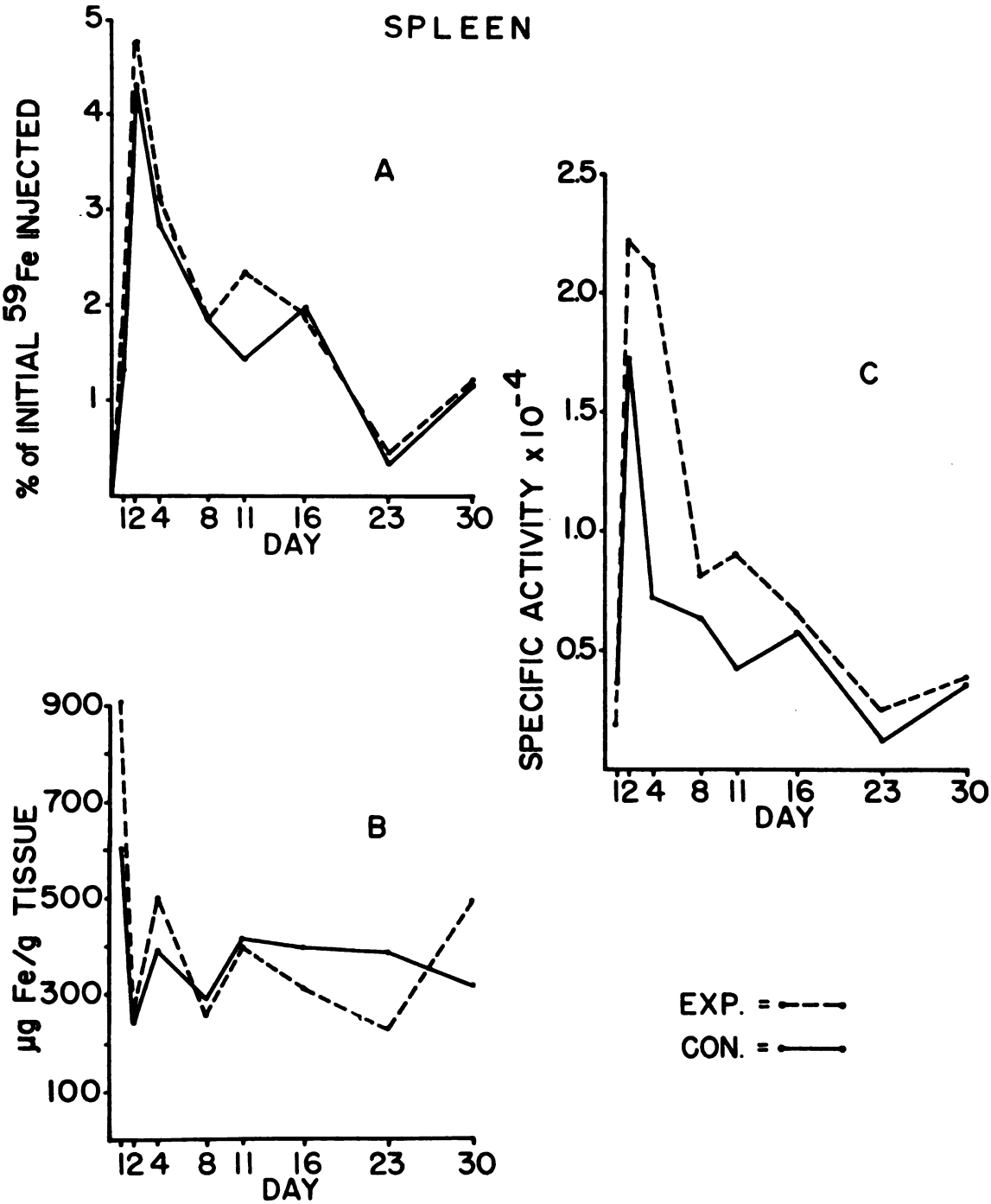
SPLEEN
A
% of INITIAL 59Fe INJECTED
5
4
3
2
1
1 2 4 8 11 16 23 30
DAY
B
µg Fe/g TISSUE
900
700
500
300
100
1 2 4 8 11 16 23 30
DAY
C
SPECIFIC ACTIVITY x 10-4
2.5
2.0
1.5
1.0
0.5
1 2 4 8 11 16 23 30
DAY
EXP. =
CON. =

Figure 6

Figure 7.--Percent initial ^{59}Fe injected (A), total iron concentration (B) and specific activity (C) of the pyloric caeca of experimental and control trout on days 1,2,4,8,11,16,23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to the injection of ^{59}Fe. Tissue total iron concentration and ^{59}Fe activity were corrected for the tissue blood volume. Each point represents the mean for 6 fish (Day 30 exp. n=5). In paired comparisons of experimental and control means, significant differences were found at $P < 0.05$ (*). Significant differences ($P < 0.05$) among control or experimental means are listed below.

A Exp. Day 4 $\neq$ Days 8,23,30

B Exp. Day 2 $\neq$ Days 4,8,11,16

C Con. Day 4 $\neq$ Days 16,23,30

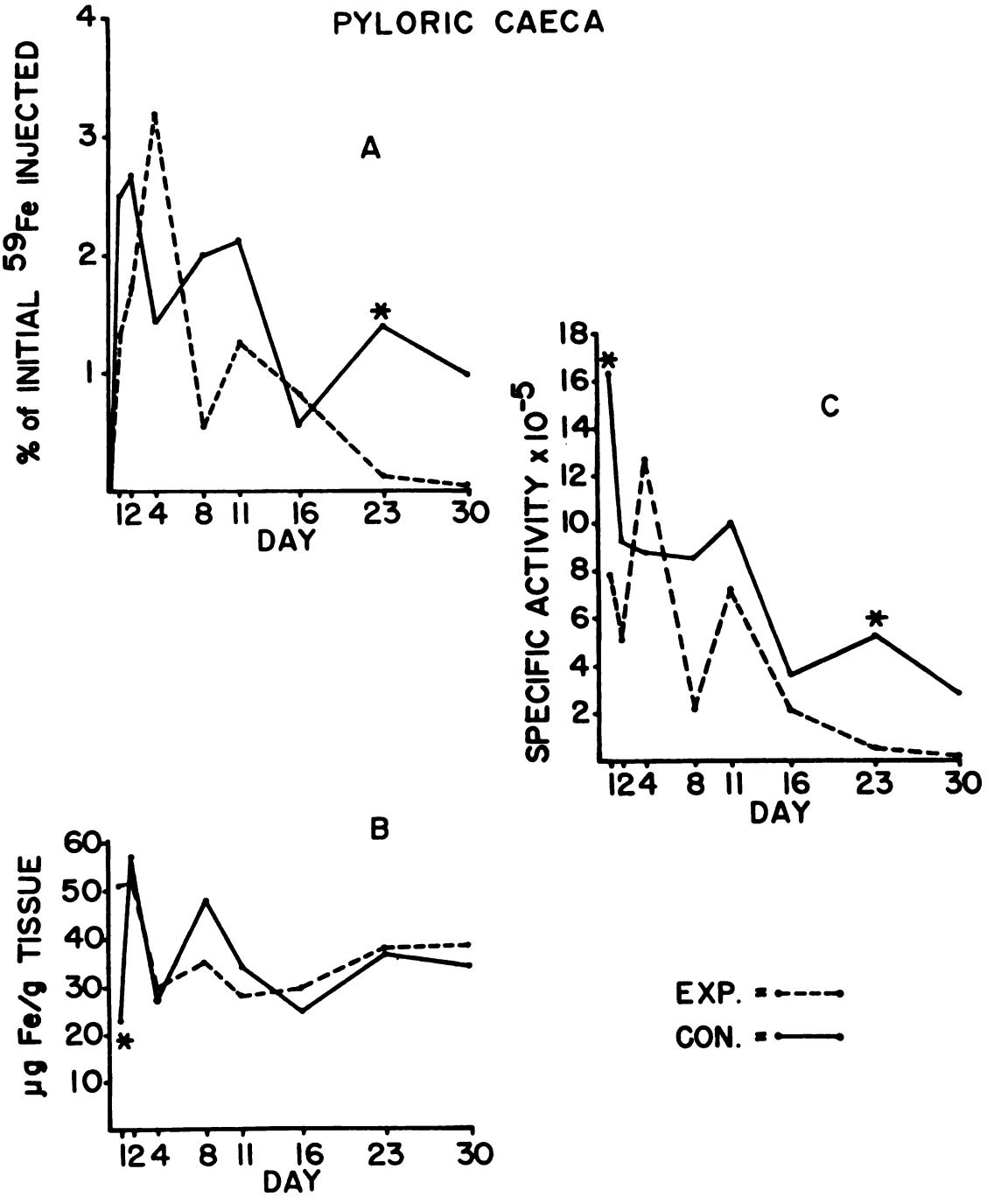
PYLORIC CAECA
A
% of INITIAL 59Fe INJECTED
4
3
2
1
1 2 4 8 11 16 23 30
DAY
B
µg Fe/g TISSUE
60
50
40
30
20
10
1 2 4 8 11 16 23 30
DAY
C
SPECIFIC ACTIVITY x10-5
18
16
14
12
10
8
6
4
2
1 2 4 8 11 16 23 30
DAY
EXP.
CON.

Figure 7

This decrease differs from that shown by pyloric caeca tissue from controls whose content ranged from 2.67 to 0.57% of the initial injected activity. Due to variability there was no significant difference in control caecal activity from day 1 through day 30.

Fluctuation in the total iron concentration (µg Fe/g tissue) of the control caeca was not statistically significant. The average concentration was about 35 µg Fe/g caecum. Except for the high iron content of 52 µg Fe/g caecum on day 2, the experimental fish caeca did not differ in iron concentration (30-35 µg/g) from day 1 to day 30, nor were the daily averages statistically different from the controls.

The specific activity of the control caeca is significantly higher than the experimental caeca at day 23. This is a reflection of the significant loss of ^{59}Fe from the caeca of experimental fish by day 23 which was not accompanied by change in the total iron content. The significant difference in the control multiple comparison is due to low total iron concentration and a high ^{59}Fe activity on day 1. Comparison of the other control means shows no significance and generally reflects the lack of difference among the ^{59}Fe activity and total iron content.

Intestine

The variation in intestine percent ^{59}Fe incorporation, total iron content and specific activity are shown in Figure 8. Control specific activity decreases significantly from day 1 to day 30 and reflects the decrease in the ^{59}Fe content of this organ from 0.5 to 0.1% of the initial injected activity. The experimental group intestinal ^{59}Fe activity was lower than the controls at day 23 and the specific activity was lower than the controls at day 30. Total iron content averaged 30-40 µg Fe/g intestine for experimental and control from days 2 to 30 but the variance in individual sampling periods was considerable as illustrated on day 1 where the range was 245 to 16 µg Fe/g intestine. As with the caecum this variance in total intestinal iron had a considerable effect on the statistical significance of the specific activity when comparing daily averages within the control and experimental groups and between the two groups at various sampling periods. Overall it appears that the specific activity and ^{59}Fe content of the experimental groups was lower than the control, especially from days 16-30. It appears that the caecum and intestine have the same pattern of ^{59}Fe incorporation following i.p. administration of this isotope.

Figure 8.--Percent initial ^{59}Fe injected (A), total iron concentration (B) and specific activity (C) of the intestine of experimental and control trout on days 1,2,4,8,11,16,23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to injection of ^{59}Fe. Tissue total iron concentration and ^{59}Fe activity were corrected for the tissue blood volume. Each point represents the mean for 6 fish (Day 30 exp., n=5). In paired comparisons of experimental and control means, significant differences were found at $P < 0.05$ (*). Significant differences ($P < 0.05$) among control means are listed below. The experimental means were not significantly different.

C Con. Day 1 $\neq$ Days 2,4,8,11,16,23,30

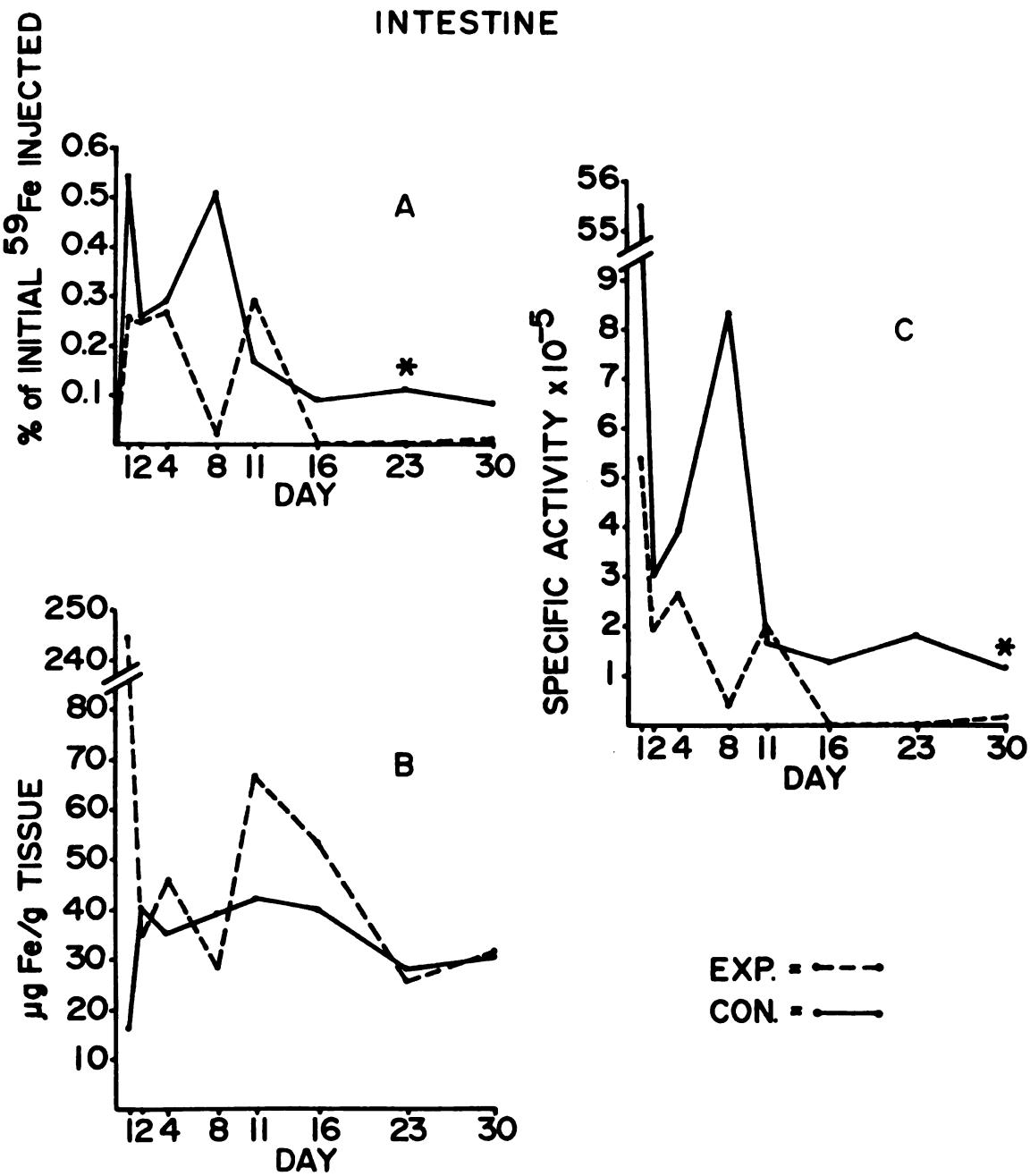
INTESTINE
% of INITIAL 59Fe INJECTED
0.6
0.5
0.4
0.3
0.2
0.1
A
*
1 2 4 8 11 16 23 30
DAY
µg Fe/g TISSUE
250
240
80
70
60
50
40
30
20
10
B
1 2 4 8 11 16 23 30
DAY
SPECIFIC ACTIVITY x 10-5
56
55
9
8
7
6
5
4
3
2
1
C
*
1 2 4 8 11 16 23 30
DAY
EXP.
CON.

Figure 8

Muscle

The fate of ^{59}Fe in muscle is shown in Figure 9. There was a considerable decrease in ^{59}Fe content and specific activity in the muscle of experimental and control fish. Clearance of radioiron was essentially complete by day 8. Differences in specific activity or percent of the initial injected dose between experimental and control means were not significant. Total iron content remained 20-35 µg Fe/g muscle in control fish, but varied from 143 to 12 µg Fe/g muscle in the experimental fish. However, there was essentially no statistical difference in total iron content between experimental and control groups nor among the sample means. Although the total iron content of muscle (µg/g) was similar to that of intestine and pyloric caeca, the specific activity of the muscle was 19 times lower. Muscle contained a higher percent of the initial dose which is attributed to the fact that the total muscle mass constitutes some 88% of the body weight.

Bile Volume

Bile volume in the gall bladder varied significantly from day 1 to day 30. The plot in Figure 10A displays the rhythmic change in volume which is significantly lower on days 8 and 16 in both experimental and control fish.

Figure 9.--Percent initial ^{59}Fe injected (A), total iron concentration (B) and specific activity (C) of the muscle of experimental and control trout on days 1,2,4,8,11,16,23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to the injection of ^{59}Fe. Tissue total iron concentration and ^{59}Fe activity were corrected for the tissue blood volume. Each point represents the mean for 6 fish (Day 30 exp., n=5). Significant differences ($P < 0.05$) among control or experimental means are listed below.

A	Exp.	Day 1 ≠ Days 8,11,16,23,30
	Con.	Day 1 ≠ Days 8,16,23,30
		Day 2 ≠ Days 8,16,23,30
B	Exp.	Day 1 ≠ Days 2,4,8,11,16,23,30
C	Exp.	Day 2 ≠ Days 1,4,8,11,16,23,30
	Con.	Day 1 ≠ Days 16,30
		Day 2 ≠ Days 8,16,23,30

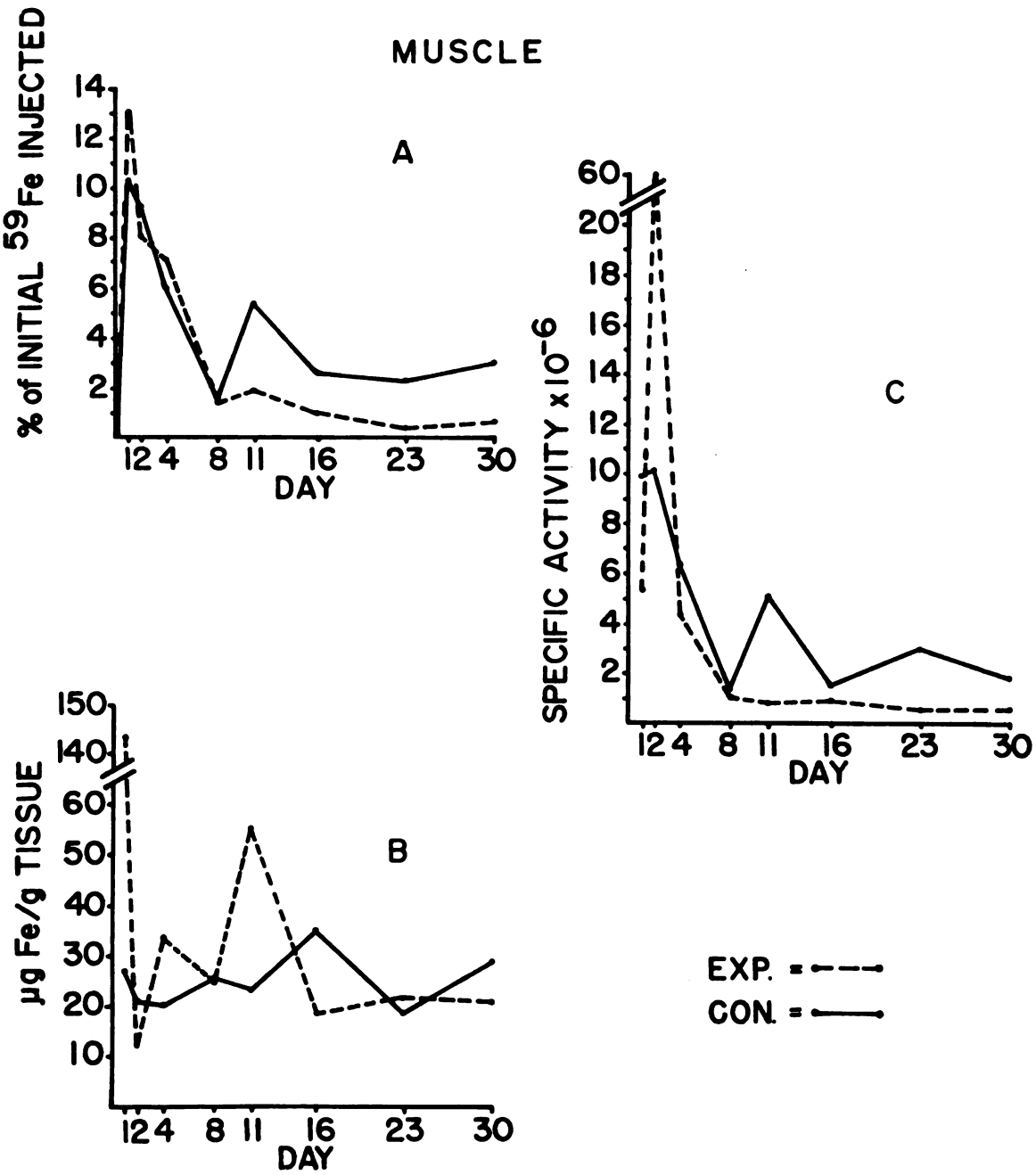
MUSCLE
A
% of INITIAL 59Fe INJECTED
14
12
10
8
6
4
2
1 2 4 8 11 16 23 30
DAY
B
µg Fe/g TISSUE
150
140
60
50
40
30
20
10
1 2 4 8 11 16 23 30
DAY
C
SPECIFIC ACTIVITY x10-6
60
20
18
16
14
12
10
8
6
4
2
1 2 4 8 11 16 23 30
DAY
EXP.
CON.

Figure 9

Percent Initial Dose in Bile

The percent initial injected ^{59}Fe appearing in bile is shown in Figure 10B. There was a general decline in activity from day 1 to day 11 in controls and to day 16 in experimentals followed by an increase in activity to day 30. Although there were no statistical differences in activity among the sample days for control or experimental fish bile, the apparent differences in activity were intriguing. The percent initial injected activity was never above 0.01%, but the increase from 0.001% (days 11 and 16) to 0.004 and 0.0065% on day 30 may indicate loss of some iron to the bile after destruction and desquamation of ^{59}Fe-labeled red blood cells on days 23 and 30.

Intestinal Contents and Urine

After an initial peak in ^{59}Fe content in the feces at 3 to 4% of the initial injected dose on day 1, the activity rapidly drops to less than 0.02% by day 2 (Figure 10C). From day 2 to day 30 the ^{59}Fe content remains below 0.02-0.01% in both experimental and control fish. Intestinal contents from three fish on day 1 were extremely radioactive (10,000 cpm) which accounts for the relatively high percentage of initial dose seen in the intestinal contents on that day. The initial peak may be due to accidental

Figure 10.--Bile volume (A), and percent of initial injected ^{59}Fe in 1 ml. bile (B) and in fecal material (C) from control and experimental trout on days 1,2,4,8,11,16,23 and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of the experimental fish was removed in 4 separate bleedings over a 7 day period prior to injection of ^{59}Fe. Each point represents the mean for 6 fish (Day 30 exp., n=5). Bile volume in experimental fish is significantly different from control fish on day 2 ($P < 0.05$ = *). Significant differences ($P < 0.05$) among control or experimental means are listed below.

A	Exp.	Day 8 ≠ Days 2,4,23,30
		Day 16≠ Days 2,4,23
	Con.	Day 4 ≠ Days 1,2,8,16
		Day 11≠ Days 1,2,8,16
		Day 23≠ Days 1,2,8,16
		Day 20≠ Days 1,2,8,16

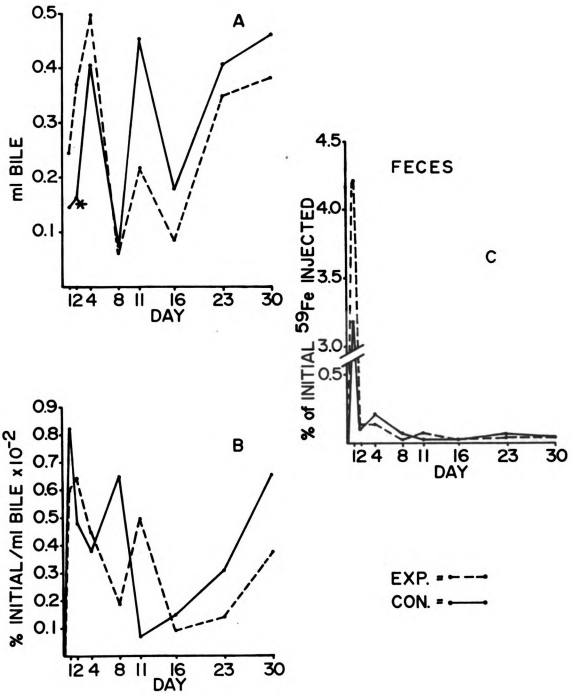
A
ml BILE
0.5
0.4
0.3
0.2
0.1
1 2 4 8 11 16 23 30
DAY
B
% INITIAL/ml BILE x10-2
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1 2 4 8 11 16 23 30
DAY
C
FECES
% of INITIAL 59Fe INJECTED
4.5
4.0
3.5
3.0
0.5
1 2 4 8 11 16 23 30
DAY
EXP.
CON.

Figure 10

injection of the ^{59}Fe saline solution directly into the lower intestine.

The radioactivity of urine within 4 days of the injection of ^{59}Fe was hardly above background. Therefore it was concluded that insignificant amounts of iron (less than 0.1%) were excreted via the urine.

Whole Blood and Whole Animal ^{59}Fe Activity

Figure 11A&B illustrates the percent of initial injected dose incorporated by the blood and that recovered in the whole animal. Radioiron incorporated by the total blood volume was essentially that taken up by the red cells since the plasma contained less than 1% of the injected dose by day 4. Peak activity was reached by day 16 when blood from experimental fish contained an average of 80% of the initial injected activity and the controls averaged 55%. This difference was significant and the controls remained significantly lower on day 23. The activity appeared to decrease from maximum in both groups but this drop was not significant. The increase in percent reticulocytes corresponds to the increase in ^{59}Fe activity in the experimental fish blood. As stated previously, the reticulocyte count is significantly higher in bled fish than in the controls on days 11, 16 and 23.

Figure 11.--Percent initial injected ^{59}Fe in whole blood (A), and in the whole animal (B) for control and experimental trout on days 1, 2, 4, 8, 11, 16, 23, and 30 following an i.p. injection of ^{59}Fe. Percent reticulocytes (-Δ-) are also shown in A for control and experimental fish. Forty percent of the blood volume was removed from experimental fish in 4 separate bleedings over a 7 day period prior to injection of ^{59}Fe. Each point represents the mean for 6 fish (day 30 exp., n=5). In paired comparisons of experimental and control means significant differences were found at $P < 0.05$ (*). Significant differences ($P < 0.05$) among control and experimental means are listed below.

A (percent of initial ^{59}Fe injection)

Exp.	Day 1 ≠ Days 8,11,16,30
	Day 2 ≠ Days 11,16
Con.	Day 1 ≠ Days 8,11,16,23,30
	Day 2 ≠ Days 8,11,16,23
	Day 4 = Day 16

(percent reticulocytes)

Con.	Day 1 ≠ Day 23

B

Con.	Day 1 ≠ Days 11,16

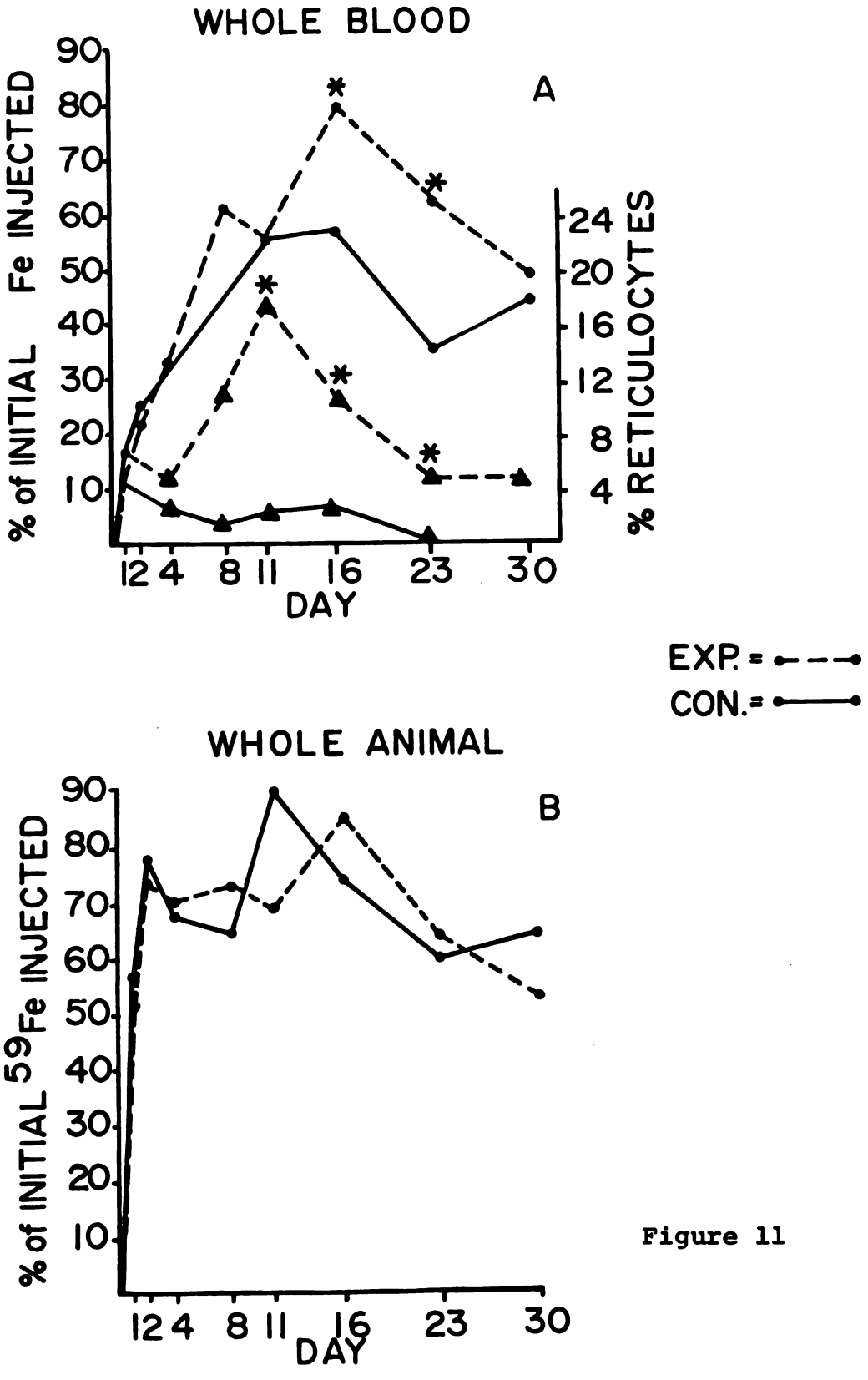
WHOLE BLOOD
A
% of INITIAL Fe INJECTED
% RETICULOCYTES
DAY
EXP. =
CON. =
WHOLE ANIMAL
B
% of INITIAL 59Fe INJECTED
DAY

Figure 11

The percent activity in the whole animal, which is the sum of the activity in all tissues sampled and in the total blood volume, reached equilibrium at 70-80% of the injected dose in both groups by day 2. This indicates that the absorption of ^{59}Fe injected i.p. was complete within 2 days after the injection, and almost all of the activity was accounted for in tissues sampled in this study. The majority of the activity appeared in the red blood cell volume. There was no significant change in total body activity from day 2 to 30 in either experimental or control fish.

DISCUSSION

The main objective of this study was to follow the metabolism of radio-labeled iron in fish for a 30 day period following i.p. injection. Previous studies (Hevesy et al., 1964; Hirschfeld and Gordon, 1965a,b; Meints and Carver, 1972) have indicated that i.p. injection of ^{59}Fe was an appropriate and effective means of administering iron to lower vertebrates. In the study reported here, ^{59}Fe was readily absorbed from the peritoneal cavity and 70-90% of the injected dose was recovered in various iron pools. Absorption may have been via the lymph system which, in mammals, removes extracellular fluid from the peritoneal cavity and delivers it to the venous circulation. Although there is little information pertaining to the lymphatic system in teleosts, it does exist in eels. In this species fluid collected by the lateral cutaneous and hemal lymphatic vessels is delivered to a caudal lymph heart which pumps it into the caudal vein (Randall, 1970). Thirayothin and Crosby (1962) speculate that lymphatic absorption is one of two pathways for the assimilation of iron in rats following i.p. injection. The second is via absorption of iron-laden phagocytes across the intestinal serosa leading to the

appearance of iron in the lamina propria, and, eventually, in the venous circulation of the intestines. Regardless of the mode of absorption, i.p. injection of ^{59}Fe proved to be an efficient and simple way of introducing this tracer into the body iron pools of rainbow trout.

The appearance of ^{59}Fe in plasma was very rapid. By the time the first sample of plasma was drawn for analysis 1 day after the i.p. injection, the majority of the initial injected dose had been absorbed from the peritoneal cavity and cleared from the plasma (Figures 3 and 12). The clearance of ^{59}Fe from fish plasma is mathematically described by a logarithmetic function which may be divided into the three components (Figure 12) perhaps comparable to those characterized for human plasma by Pollycove (1964) and Finch et al. (1965). The first component represents the initial clearance of ^{59}Fe-labeled transferrin from the plasma into the tissue extravascular spaces which occurs within 24 hrs of administration. The second component represents the return of ^{59}Fe-transferrin to the plasma pool, a process which is responsible for the change in the slope of the clearance curve between days 2 and 16 following injection. The removal of ^{59}Fe from the plasma by the erythropoietic and iron storage sites is a gradual process which is represented by the third or equilibrium component of plasma iron clearance between days 16 and 30.

Figure 12.--Clearance of ^{59}Fe from the plasma of trout following an i.p. injection of this isotope. The observed clearance is the culmination of three independently occurring processes described by the equation:

$$C_t = C_1^0 e^{-k_1 t} + C_2^0 e^{-k_2 t} + C_3^0 e^{-k_3 t}$$

where

C_t = concentration of ^{59}Fe in the plasma at time t

C_1^0, C_2^0, C_3^0 = time zero specific activity of components 1, 2, and 3

k_1, k_2, k_3 = rate constants for components 1, 2, and 3

e = base of the natural logarithms

t = time in days

The half times for clearance are:

$$T_{\frac{1}{2}} = \frac{.693}{k_1} = \frac{.693}{.693} = 1 \text{ day for } C_1$$

$$T_{\frac{1}{2}} = \frac{.693}{k_2} = \frac{.693}{.231} = 3 \text{ days for } C_2$$

$$T_{\frac{1}{2}} = \frac{.693}{k_3} = \frac{.693}{.0059} = 116.7 \text{ days for } C_3$$

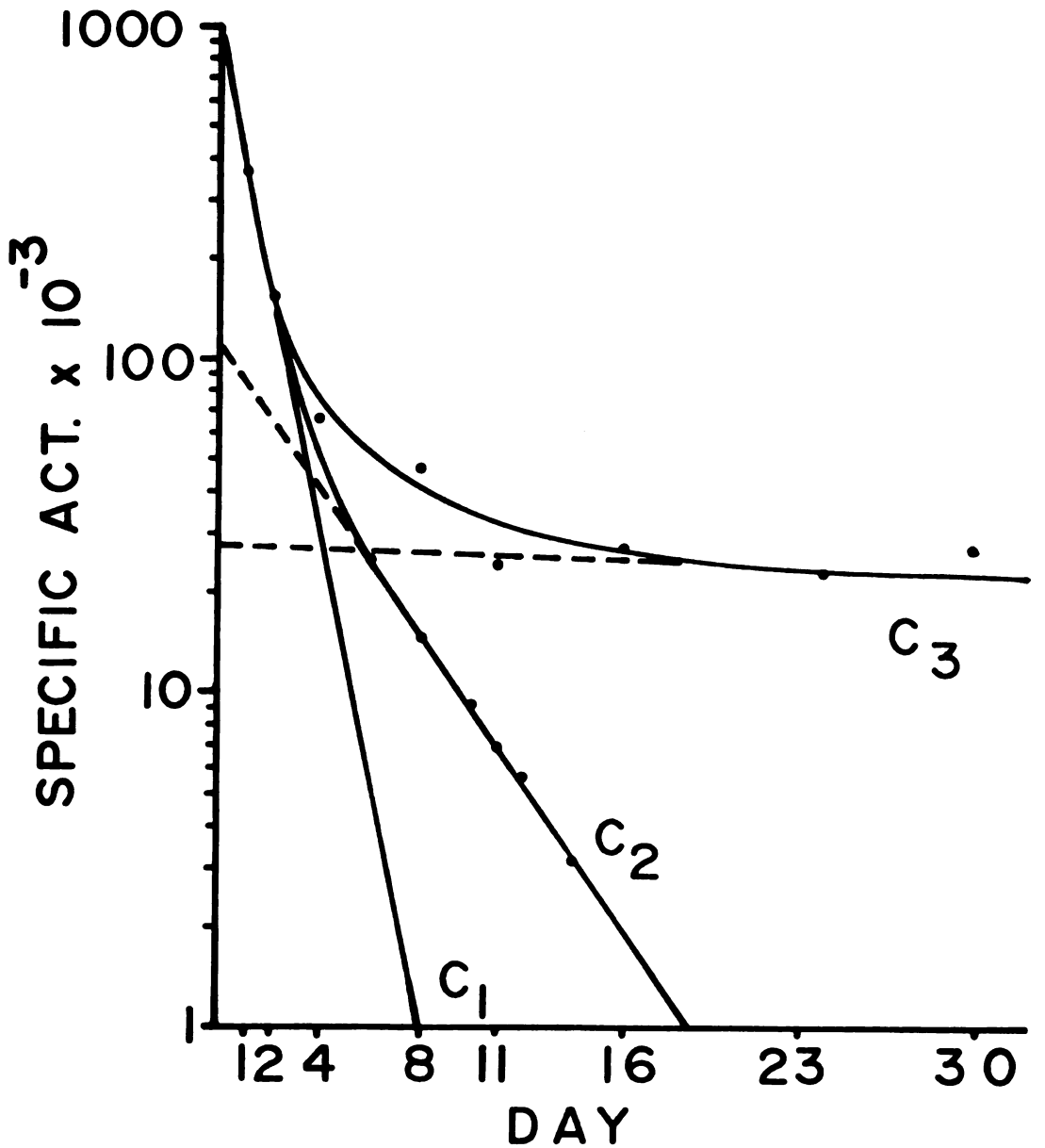

Figure 12

Movement of ^{59}Fe into the extravascular spaces of various tissues is evident from the initial ^{59}Fe peaks in tissue specific activity seen two days after i.p. injection. Clearance of ^{59}Fe from muscle was rapid, indicating return of most all of the ^{59}Fe-laden transferrin to the plasma after circulation through the extravascular spaces. In contrast, the gradual decline in specific activity of tissues such as liver, spleen and caecum may be indicative of binding of ^{59}Fe to receptor sites for storage in these tissues.

The rapid appearance of ^{59}Fe in the head kidney and the high specific activity of this tissue indicate that much of the plasma ^{59}Fe is removed by the erythropoietic tissue. Pollycove (1964) reported that as much as 90% of the iron leaving the plasma enters the "erythropoietic labile pool" and less than 5% enters the iron storage pool in humans. In this light, it was not surprising to see a high concentration of ^{59}Fe in the head kidney on day 2 following the i.p. injection. The percent of initial dose in the remaining kidney was about the same as the head kidney, but the concentration of ^{59}Fe (cpm/g) was 1.5 to 2 times greater in the head kidney where the majority of the erythropoietic tissue is centered. The activity in the remaining kidney may be attributed to the existence of some erythropoietic tissue in the middle third of the kidney (Klontz et al., 1965).

The rapid decrease in head kidney ^{59}Fe activity was probably caused by an increase in erythrocyte radioiron incorporation (Figure 11A). Similar findings were reported by Hevesy et al. (1964) for the tench and Hirschfeld and Gordon (1965a) for turtles. There was also an excellent correlation between the erythrocyte iron incorporation and the number of reticulocytes present in the peripheral circulation. Walsh et al. (1949) and Jensen et al. (1953) were among the first to recognize this correlation in other species. The output of immature erythrocytes by the head kidney of the bled fish was not significantly higher than the controls until 11 days after bleeding. This difference in reticulocyte population after day 11 was undoubtedly responsible for the significant difference noted between control and experimental red blood cell ^{59}Fe incorporation on days 16 and 23.

Even though the increased reticulocyte population was responsible for increased RBC ^{59}Fe uptake in the bled fish, the RBC iron was not immediately incorporated into heme. This conclusion is supported by the fact that the hematocrit of bled fish recovered by day 16 but the hemoglobin concentration did not return to normal until day 30. Smith et al. (1971) reported similar results with salmon following injection of phenylhydrazine, a hemolytic agent. Phenylhydrazine-induced anemia was more severe, reducing hematocrit and hemoglobin concentrations by 95%. Recovery required 95 days,

at which time hematocrit had returned to normal, but hemoglobin concentration was still below normal. Heme production, as reported by Hevesy et al. (1964), is much slower than ^{59}Fe incorporation. In their study on tench, they found that ^{59}Fe-heme did not approach equilibrium until 20 days post ^{59}Fe injection, unlike total erythrocyte radioiron content which approached equilibrium at day 10. Iron was incorporated into the erythrocyte faster than it could be utilized for heme synthesis. This suggests that non-heme iron is stored in the cytoplasm of immature fish erythrocytes prior to heme formation. A similar situation in immature human red cells was reported by Kaplan et al. (1954) who discovered the presence of stainable non-hemoglobin iron in the cytoplasm. They attributed the presence of this non-heme iron to a normal phase of intracellular storage during the process of hemoglobin synthesis. Bessis and Breton-Gorius (1959) have identified the storage form as ferritin and ferruginous micelles in human erythroblasts. A non-heme iron pool has also been demonstrated in avian erythrocytes (Jensen et al., 1953). It is possible that a similar situation exists in fish red blood cells. Iron enters the red cell and is stored as ferritin until it is placed into the heme moiety.

It is also conceivable that not all the iron stored in the red cell is converted to heme as the cell matures.

There may be a small fraction of non-heme iron which is exchangeable with iron in the plasma. Hevesy et al. (1964) noted a decrease in total erythrocyte ^{59}Fe content with time which they attributed to such an exchange. Although non-heme ^{59}Fe was replaced by unlabeled plasma iron, the heme-^{59}Fe activity did not decrease during the same period. Jensen et al. (1953) noted that when iron is incorporated into the heme moiety it is irreversibly bound, therefore no exchange can take place with either the cytoplasmic non-heme iron or with plasma iron. Any decrease in the total hemoglobin-^{59}Fe content of the red blood cell is attributed to red cell death. Therefore, in this study the decrease noted in ^{59}Fe activity of the red blood cells after day 16 must be attributed to exchange of erythrocyte non-heme iron with unlabeled plasma iron. Jensen et al. (1953) found that mature avian erythrocytes were also capable of non-heme iron exchange. Therefore, the exchange seen in fish erythrocytes is probably not limited to young red cells and may partially account for the uptake of ^{59}Fe by red blood cells from the control fish.

In summary, the incorporation of ^{59}Fe by red blood cells probably represents two physiological processes occurring simultaneously; i.e., the incorporation of iron for hemoglobin formation by immature cells and the exchange of iron between red cell cytoplasmic stores and the plasma.

The latter is not restricted to immature or recently mature erythrocytes, but may also take place across the cell membrane of older red blood cells.

Liver, spleen and pyloric caeca all exhibited a peak in specific activity between day 2 and day 4 post injection followed by a sharp decrease by day 8. This may represent the initial circulation of ^{59}Fe through the extravascular spaces and its reentry into the plasma pool. In fact, the cyclic reappearance and disappearance of ^{59}Fe in liver and caecum at 6-7 day intervals may be the result of movement of iron into and out of the interstitial fluid of these organs.

The liver of control fish was the only tissue in which the percent of original dose remained above 12% throughout the entire 30 day period of the experiment. The liver was the major organ for ^{59}Fe storage and was the only tissue in which there were significant differences between control and experimental ^{59}Fe content, specific activity, and total iron concentration. Control liver specific activity was highest of any tissue after day 8 (Table 3), however, this was not the case for the experimental group, in which liver specific activity fell below that of the spleen. The significant decrease in total iron concentration, etc., coincided with the increase in blood ^{59}Fe content for experimental fish above that of controls. This indicates that in bled fish more iron was mobilized from the liver iron stores for incorporation

into the immature red cells. No other tissue displayed such a unique response to the increase in demand for iron.

Following the apparent flux of ^{59}Fe into and out of the splenic extravascular fluid between day 1 and 4, the ^{59}Fe approached equilibrium with the splenic iron storage pool between days 4 and 16. The percent initial dose appeared to decrease on day 23, however, there was no significant difference between experimental and control specific activity or total iron concentration. Splenic iron stores were not significantly reduced due to bleeding. In contrast, Yu et al. (1971) report a decrease in splenic hemosiderin bodies within 24-48 hrs following bleeding, indicating the mobilization of splenic iron for erythropoiesis. They report that spleens of bled fish contained significantly less hemosiderin than controls. A decrease in splenic iron content of the magnitude noted by Yu et al. (1971) was not evident in the present study. Attempts were made to section the spleens and stain for hemosiderin, but there was considerable interference with the specificity of the stain due to the presence of ceroid, an unsaturated lipid. Wood and Yasutake (1956) and Grover (1968) report similar problems with the histochemical stain for splenic hemosiderin in rainbow trout and bluegills.

The pyloric caeca and intestinal ^{59}Fe content, like that of the spleen, approached equilibrium between days 4 and 11. There was considerable variance in the gut activity

during the study which may have been due in part to faulty i.p. injection. However, the difference in control and experimental ^{59}Fe content on day 23 may indicate an increased rate of removal of iron from storage sites in bled fish. Neither caecal tissue nor intestine ever contained more than 3% of the initial dose and the total iron concentration was 5-10 times less than the iron concentration in the liver and spleen. Therefore, intestine and caeca are relatively unimportant in terms of iron storage. The fact that ^{59}Fe content decreases in the intestine and caeca in response to bleeding may not have been significant in terms of supplying red blood cells with iron, but was significant in that it may have favored an increase in iron absorption by the gut. This decrease in ^{59}Fe content coincides with the initial decrease in experimental liver total iron concentration. In this experiment, it is logical that a significant decrease in iron in a major storage organ such as the liver might trigger an increase in rate of intestinal absorption of iron to replace that removed by bleeding.

The mucosal block theory proposed by Granick (1946) states that there is an inverse correlation between the mucosal cell iron content and the amount of iron absorbed by the intestine. Thus, when mucosal cell iron content is low, there is an increase in intestinal iron absorption from the lumen of the gut. Whether or not the results reported here support the mucosal block theory is questionable. Even though

radioiron content decreased in the intestine and caeca of bled (iron-depleted) fish, the total iron content did not change significantly. Also, intestinal iron absorption was not measured during the study, therefore there is no way of knowing if the decrease in radioiron content of the intestine and caeca had any effect on iron absorption. Except for the initial peak in radioactivity in the intestine and caeca due to extravascular iron flux, one would expect to see an immediate decrease in ^{59}Fe activity in these tissues from bled animals. According to Crosby (1965) and Van Campen (1974) there should be essentially no radioiron in the mucosal cells after day 2. Although the percent initial dose in either tissue was below 1% by day 8, the radioiron content was not significantly depleted until 16-23 days after the i.p. injection of ^{59}Fe.

Since these animals were fed every other day, the iron content of the food (300-400 µg Fe/g) and the rate of iron absorption may have been enough to fulfill the demand for iron in bled fish. There was no apparent decrease in total iron content of any tissue within 16 days of the last bleeding. Therefore, the absorptive mechanism may have responded to the initial bleeding with an increase in iron absorption 7 days prior to the i.p. injection of ^{59}Fe. The increased absorption may have been enough to supply the head kidney with iron for immature red cells. There may have been no

need for a further increase in the absorptive process in the gut until there was significant mobilization of liver iron. Apparently the mobilization of intestinal and caecal iron was necessary only after liver iron stores were substantially depleted (<100 µg Fe/g liver). This suggests a feedback mechanism between liver iron content and the control of iron absorption.

Although there is no proof, mediation of the feedback mechanism may be via the transferrin concentration in the plasma. Transferrin is a beta-1 globulin which is probably formed in the liver where virtually all α and β globulin synthesis occurs (Davidsohn and Henry, 1969). One might suspect that a decrease in liver iron concentration might trigger a mechanism responsible for increasing plasma transferrin concentration. Hevesy et al. (1964) report normal fish liver iron concentration to be 425 ± 192 µg Fe/g liver with a mean TIBC of 250 µg%. In the present study, control liver iron concentration never exceeded 250 µg Fe/g. However, TIBC was abnormally high (>400 µg%) throughout the study. When the liver iron concentration of bled fish decreased below 100 µg/g there appeared to be a corresponding increase in TIBC (above 700 µg%). The TIBC for controls increased during the same period but not to the same extent. The differences between control and experimental TIBC are not clear-cut, thus existence of a functional correlation between liver iron concentration and TIBC is

pure speculation. If the relationship is real, then the increase in TIBC in response to decreased liver iron may trigger the intestinal absorptive mechanism by removing the mucosal block. This would allow increased absorption of iron from the lumen of the gut.

The role of muscle, with the lowest specific activity and iron concentration, was insignificant in the overall metabolism of iron in rainbow trout. The initial ^{59}Fe flux reflects movement into and out of the extravascular fluid of muscle with no evidence of storage or utilization of ^{59}Fe in myoglobin formation. The slight differences between control and experimental ^{59}Fe content on days 11 and 23 were also insignificant.

The loss of ^{59}Fe in the urine, feces and bile was negligible. Only 4 or 5 fish out of 96 lost significant amounts of ^{59}Fe to the feces and this was probably due to accidental injection directly into the lower gut. In fact, 70-90% of the initial dose could be accounted for in the tissues of experimental and control fish 11 days after the i.p. injection. There was a slight but insignificant decrease in whole animal ^{59}Fe content after day 16. This decrease corresponds to an increase in bile radioactivity during the same period. The increase in percent initial dose in the bile after day 16 is statistically insignificant, but the upward trend may signify the appearance of ^{59}Fe from denatured red blood cells. Normally, nearly all of the iron

from catabolized hemoglobin enters the reticuloendothelial cells of the spleen as described (for fish) by Yu et al. (1971) and Smith et al. (1971). However, a small amount of iron may appear with bilirubin in the bile.

Future Research

Several interesting questions have been raised as a result of this study. The answers to these questions require the investigation of additional variables in the study of iron metabolism. Isolation of hemoglobin, transferrin, and RBC non-heme iron by electrophoretic techniques would have improved this study. Analytical determination of ferritin and histological investigation of hemosiderin bodies should have been included to substantiate some of the conclusions and speculations. However, measurement of these parameters was not realistic in a study of this magnitude. Now that there is some basic information established about the metabolism of iron in trout, the finer points can be investigated. The fate of iron in red blood cells should be studied to elucidate the possible presence of iron storage proteins in the cytoplasm and to determine the rate of incorporation of this iron into heme. This would require fractionation of the cell and isolation of the heme and non-heme fractions.

The relationship between transferrin concentration in the plasma and liver iron content should be studied. One might induce iron deficiency through severe bleeding over one or two weeks, remove the liver, determine the transferrin production *in vitro*, and compare this to production by suitable control liver samples.

Another important aspect is intestinal absorption of iron. Interference with the absorptive process will certainly affect iron metabolism on a chronic scale. An in-depth study of absorptive rates and the absorptive mechanism should be conducted under normal and iron deficient conditions. This might involve cannulation and perfusion of major intestinal arteries and veins to investigate the effects of supra and subnormal concentrations of plasma iron and transferrin on the rate of iron absorption across the mucosa.

The existence of an erythropoietic stimulating factor in fish is now under investigation. Zanjani *et al*. (1969) reported the presence of a factor in the plasma of anemic fish which initiates increased red blood cell production in recipient fish. This factor has been isolated from human urine, and there are hemagglutination and bioassay procedures for determining human plasma ESF concentration. However, fish ESF has not been isolated. The characterization of and assay for fish ESF would be an interesting and

valuable area of research. With the development of an assay procedure, one could measure changes in fish plasma ESF levels in response to various stimuli known to increase erythropoiesis in mammals, such as decreased blood P_{O_2} and hemolytic anemia. Prosser et al. (1957) found that goldfish could be acclimated to an environmental oxygen content as low as 2 ml O_2/liter of water and they increased their hemoglobin concentration. It would be interesting to measure the plasma ESF titer in goldfish adapted to low oxygen and compare them with rainbow trout after exposure to low environmental dissolved oxygen. Trout are much more sensitive to hypoxia than goldfish or carp; perhaps due to a lack of erythropoietic response (in trout) to low environmental O_2 concentration. The ineffectiveness of hypoxia to stimulate erythropoiesis has been reported in turtles (Altland and Parker, 1955; Altland and Thompson, 1958) and in frogs (Rosse et al., 1963).

Red blood cell ^{59}Fe incorporation appears to be the most sensitive parameter for the study of the effects of environmental pollutants on fish iron metabolism and erythrocyte production. Zanjani et al. (1969), Hevesy et al. (1964) and Walker (1972) have shown that fish red blood cells can be successfully incubated in vitro at room temperature. The rate and amount of ^{59}Fe uptake by RBCs can easily be determined. It is possible to show differences in ^{59}Fe incorporation which are directly related to the percentage

of immature RBCs present in the circulating blood. One problem encountered in in vitro studies is the maintenance of the iron concentration of the medium used in all incubations. One way to circumvent the problem is to prepare a medium of hydroxymethyl aminomethane (TRIS) buffered physiological saline containing a known iron concentration and a specific amount of transferrin which approximates the concentration normally found in fish plasma (0.2-0.3 g transferrin/100 ml). The specific activity of this medium is important in the calculation of iron incorporation by the red blood cells.

No one parameter by itself is significant in terms of the physiological response of an animal to stress, therefore, hemoglobin, hematocrit and reticulocyte counts should be monitored in addition to RBC ^{59}Fe incorporation. Plasma iron concentration and TIBC are also important indicators of iron metabolism and ferrokinetics of fish.

CONCLUSIONS

In this study, the uptake and distribution of ^{59}Fe to various pools was monitored in control and iron deficient rainbow trout following i.p. injection of the tracer. Total iron concentration and specific activity of tissues and organs were measured over a 30 day post injection period.

1. Within 24 hours most of the radioiron was absorbed from the peritoneal cavity, probably via the lymphatic system.
2. Upon entering the plasma, radioiron is assimilated by transferrin. ^{59}Fe-labeled transferrin probably enters the extravascular fluid of tissues, circulates, and reenters the plasma. During this process some ^{59}Fe is released from the transferrin and becomes incorporated into tissue iron storage sites.
3. The erythropoietic response to bleeding is slow since it was 11 days after the last bleeding before reticulocyte counts were significantly higher in bled fish than in the controls.
4. The incorporation of ^{59}Fe by red blood cells probably represents two physiological processes occurring simultaneously; the incorporation of iron for the

purpose of hemoglobin formation and the exchange of iron between cytoplasmic stores and the plasma as shown by Hevesy et al. (1964).

5. The liver, spleen and head kidney are the major iron storage organs. Intestine, pyloric caeca and muscle are insignificant in terms of iron storage.
6. The liver iron pool is the primary source for iron utilized in hemoglobin formation.
7. There may be a feedback mechanism between liver iron stores and the mucosal cells of the intestine. When liver iron concentration decreases intestinal absorption of iron increases. The mediator of this system may be transferrin.
8. The spleen contains the highest total iron concentration but turnover of the splenic iron stores appears quite slow in contrast to liver iron stores.
9. Red blood cell ^{59}Fe incorporation, per cent reticulocytes, hematocrit and hemoglobin are the best indicators of erythropoiesis. Liver iron content and plasma transferrin concentration are the primary indicators of iron metabolism.

APPENDICES

APPENDIX A

PHYSIOLOGICAL SALINES

0.9 g% Saline

9.0 g Sodium chloride diluted to 1 liter
(280-290 m0sm)

Phosphate Buffered Saline (PBS)

One liter of a 280 m0sm PBS solution was prepared as follows:

Sodium chloride	7.82	g
Potassium chloride	0.30	g
Magnesium sulfate	0.14	g
Potassium phosphate monobasic	0.46	g
Sodium phosphate dibasic	2.02	g
Dilute with distilled water to make	1000	ml

APPENDIX B

PLASMA IRON DETERMINATION

Method of

Plaut et al. 1972

REAGENTS

Protein Precipitating Reagent:

Trichloroacetic acid (TCA)	25.0 g
Distilled water	250 ml
Concentrated HCl	4.17g
Thiourea	1.67g
Dilute with distilled water to make	500 ml

Store at room temperature.

Color Reagent:

Sodium acetate	35 g
3-(2-pyridyl)-5,6,diphenyl-1,2,4 triazene (Ferrozine®, Hach Chemical, Ames, Iowa)	200 mg
Dilute with distilled water to make	100 ml

Standard (stock): 1000 µg/ml

Use pure (99%) iron wire (Baker Chemical Co., Phillipsburg, N.J.)
Polish with sand paper or emery cloth
Weigh out 1 g and dissolve in 5 ml of concentrated HCl
Dilute to 1 liter with iron free distilled water

Working Standard:

From this stock solution of 1 g/L make a set of standards above and below the expected range of plasma iron concentration:

10 µg/100 ml
50 µg/100 ml
100 µg/100 ml
250 µg/100 ml

Procedure:

Use iron free glassware in every step. This is done by soaking in 5 N HCl overnight and rinsing with iron free water.

1. Add 3 ml of the protein precipitating reagent to 1 ml of plasma, working standards, and blank (iron free water).

2. Mix tubes thoroughly and allow to stand for 10 min.

3. Centrifuge for 10 min at 3,000-6,000 rpm.

4. Transfer all of the supernatant to a separate tube and discard the precipitate.

5. Centrifuge for 5 min at 3,000-6,000 rpm or until the supernatant is completely clear of any remaining white precipitate.

6. Transfer 3 ml of supernatant to another tube, add 0.6 ml of the color reagent and mix.

7. Zero the spectrophotometer and adjust the blank to read 100% T at 560 nm. Read the standards and the unknown plasma samples. From a plot of the standard concentrations vs. the optical density, calculate the unknown concentrations in µg Fe/100 ml plasma.

APPENDIX C

UNSATURATED IRON BINDING CAPACITY (UIBC)

Method of

Kuypers and vanOers (1973)

REAGENTS

Ferric-Isotope Solution:
(Ferric ammonium citrate)

1. Dissolve 414.3 mg of ferric ammonium sulfate·12 H_2O in each ml of 0.1 N HCl. Pipet 1 ml of this solution into a centrifuge tube and add 30-40 µCi $^{59}FeCl_3$.

2. Add 0.5 ml of concentrated ammonium hydroxide to precipitate the iron. Centrifuge and wash the precipitate with 5% ammonium hydroxide.

3. Dissolve precipitate in 2 ml of iron free water containing 100 mg of citric acid. Dilute to 90 ml with 0.05 M tris buffer (2-amino-2-(hydroxymethyl)-1,3-propanediol) and adjust the pH to 7.0 with 5% ammonium hydroxide. Dilute to 100 ml with iron free water.

4. Store at 4°C.

5. Determine the iron concentration by the bathophenanthroline method (Appendix D).

Anion Exchange Resin:

A 22 x 24 cm sheet of Neptonanion exchange resin was obtained from Gallard-Schlesinger Chemical Co., New York (Product No. S 43000). The sheet was cut into 35 x 7 mm strips which were stored at room temperature in 0.5 M TRIS (pH 7.5). Do not refrigerate.

Glassware:

1. All glassware was made iron free (Appendix B) before use.

2. 12 x 75 mm glass culture tubes used in the incubation tubes were coated with Siliclad ® (Clay-Adams, Inc., New York).

Procedure:

1. Pipet 0.2 ml of plasma or 0.2 ml of 0.9 g% saline blank and 0.2 ml of the ferric-isotope solution into a culture tube and mix continuously for 2 hrs on the Variable Speed Rotator at room temperature.

2. Add a resin strip and 2 ml of 0.05 M Tris buffer (pH 7.5) and continue the incubation for 6 hrs to absorb excess ferric-isotope solution.

3. Determine ^{59}Fe activity in the culture tube, and then remove the resin strip and recount the tube.

4. The UIBC is computed from the following equation:

   A = ^{59}Fe activity incorporated by 0.2 ml plasma = cpm in plasma culture tube after removing resin strip minus the cpm in the blank tube after removing resin strip.

   B = ferric-isotope solution specific activity = cpm/µg Fe

   C = µg Fe incorporated by 0.2 ml plasma = $\frac{A}{B}$

   UIBC = C x 500 = µg Fe/100 ml plasma

APPENDIX D

TISSUE IRON ANALYSIS

Adopted from the Methods of
Dow Chemical (1970),
Harold Bergman (personal communication)
and Diehl and Smith (1965)

Tissue Digestion Procedure:

1. All glassware used was iron free (Appendix B).

2. Tare weight tissues in Pyrex brand micro-Kjeldahl flasks (A. H. Thomas Co., Philadelphia, Pa.)

3. Add 5 ml of concentrated HCl and 1 ml of concentrated HNO_3 to the tissue and to five 1 ml iron standards and a distilled H_2O blank.

4. Place the flasks in a shaking water bath at 90°C and digest tissues for 2-3 hrs.

5. Remove the flasks from the water bath and, after cooling, add 15 ml of 6% potassium permanganate. Allow the tissue oxidation to continue overnight at room temperature.

6. The following morning, decolorize excess permanganate by the drop by drop addition of 10% hydroxylamine hydrochloride.

7. Any samples which are not clear or which contain undigested tissue are discarded. This occasionally is evident in large samples of intestine with high lipid contents.

IRON DETERMINATION

(Diehl and Smith, 1965)

REAGENTS

Color Reagent:

Sodium salt of 4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (Bathophenanthroline, sulfonated sodium salt)

1.5 mM solution

Reducing Agent:

Ascorbic acid 1 M solution

Buffer:

Sodium acetate 4.5 M solution

1. Filter the 4.5 M sodium acetate solution through No. 1 (7 cm) Whatman filter paper using a Büchner funnel.

2. Add the chelating resin, Chelex 100 (BioRad Labs, Richmond, Calif.) to the filtrate and mix for 2-3 hrs. Allow the resin to settle, carefully decant the sodium acetate solution and filter to remove any resin beads.

PROCEDURE

1. Dilute the digestates to 40 ml in the micro-Kjeldahl flasks with iron free water.

2. Using iron free glassware, transfer 4 ml of digestate to a 25 ml volumetric flask.

3. Add 3 ml of 1 M ascorbic acid, 5 ml of the color reagent, and 6 ml of the 4.5 M sodium acetate buffer to each 25 ml volumetric flask in the order as given.

4. Dilute to volume with iron free water, mix and allow 30 min for color development.

5. After zeroing the spectrophotometer, adjust the blank to read 100% T at 540 nm. Unknowns and standards are read as percent T and converted to O.D.

6. Iron concentrations are expressed in µg Fe/g tissue.

APPENDIX E

Table 5.--Mean, standard error, and number of observations [(X ± SE(N)] of percent initial injected ^{59}Fe, total iron concentration, and specific activity of tissue, plasma, and whole blood of control and bled fish on days 1,2,4,8,11,16,23, and 30 following i.p. injection of ^{59}Fe. Forty percent of the blood volume of experimental fish was removed in four separate bleedings over a seven day period prior to the i.p. injection.

Day Post Injection	Group	Percent in Whole Blood	Percent in RBC	Hct (Percent)	Hb (g percent)	Percent in Plasma	Bile percent/ ml x 10^{-2}
1	Exp.	13.48± 3.64(6)	7.58±1.85(6)	16± 2(6)	3.47±0.39(6)	1.72±0.55(6)	0.60±0.33(5)
	Con.	16.84± 3.51(6)	4.78±1.02(6)	29± 2(6)	7.07±0.63(6)	3.23±0.95(6)	0.82±0.27(6)
2	Exp.	22.30± 4.23(6)	18.47±4.73(6)	14± 2(6)	2.85±0.40(6)	1.19±0.33(6)	0.64±0.11(6)
	Con.	25.45± 2.11(6)	9.67±1.09(4)	31± 3(6)	7.05±0.71(6)	0.77±0.18(4)	0.48±0.21(6)
4	Exp.	32.60± 6.58(6)	15.88±3.02(6)	20± 2(6)	3.22±0.32(6)	0.34±0.08(6)	0.44±0.10(6)
	Con.	32.39± 5.47(6)	8.20±1.27(6)	41± 3(6)	7.72±0.43(6)	0.29±0.06(6)	0.38±0.16(6)
8	Exp.	61.25±12.60(6)	24.97±4.97(6)	26± 2(6)	4.65±0.48(6)	0.33±0.11(6)	0.19±0.09(5)
	Con.	44.69±10.40(6)	11.12±2.53(6)	40± 2(6)	8.72±0.33(6)	0.22±0.04(6)	0.64±0.20(4)
11	Exp.	56.25±10.30(6)	26.60±5.09(6)	25± 2(6)	3.92±0.31(6)	0.19±0.03(6)	0.50±0.18(6)
	Con.	55.16± 3.32(6)	17.13±2.67(6)	39± 3(6)	8.27±0.70(6)	0.27±0.04(6)	0.07±0.05(6)
16	Exp.	80.41± 4.56(6)	21.78±1.95(6)	32± 1(6)	5.08±0.10(6)	0.19±0.02(4)	0.09±0.06(5)
	Con.	57.16± 5.19(6)	16.99±2.15(6)	34± 2(6)	7.05±0.49(6)	0.20±0.05(6)	0.15±0.11(6)
23	Exp.	62.92± 5.19(6)	20.16±2.18(6)	34± 3(6)	5.92±0.58(6)	0.15±0.02(6)	0.14±0.04(6)
	Con.	36.25± 9.38(6)	9.93±4.00(5)	44± 3(6)	9.17±0.74(6)	0.17±0.03(5)	0.31±0.11(6)
30	Exp.	49.72± 9.26(5)	15.17±3.92(5)	35± 4(5)	5.82±0.72(5)	0.10±0.03(5)	0.38±0.30(5)
	Con.	45.22± 4.78(6)	11.18±1.54(6)	44± 2(6)	7.82±0.41(6)	0.18±0.02(6)	0.67±0.23(6)

continued

Table 5--continued

Days Post Injection	Group	Bile Volume (ml)	Spec. Act. x 10^{-5} Plasma	Percent in Liver	µg Fe/g Liver	Spec. Act. x 10^{-5} Liver	Percent in Kidney
1	Exp.	0.24±0.07(6)	366±101(6)	8.84±2.60(6)	320±59(6)	6.90± 2.81(6)	8.60±2.11(6)
	Con.	0.15±0.02(6)	382± 92(5)	12.34±3.06(6)	142±43(6)	18.91± 6.00(6)	8.12±1.68(6)
2	Exp.	0.37±0.06(6)	154± 30(6)	15.70±4.47(6)	138±35(6)	27.44± 8.45(6)	12.50±0.92(6)
	Con.	0.16±0.03(6)	154± 43(3)	19.09±2.14(6)	116±36(6)	57.82±28.51(5)	10.90±1.81(6)
4	Exp.	0.50±0.06(6)	68± 15(6)	12.53±3.85(6)	108±25(6)	30.57±11.71(6)	7.61±1.63(6)
	Con.	0.41±0.07(6)	45± 7(6)	14.40±3.28(6)	145±45(6)	20.13± 5.13(6)	5.77±1.39(6)
8	Exp.	0.07±0.02(6)	87± 41(6)	6.36±2.26(6)	145±21(6)	7.90± 2.91(6)	0.96±0.23(6)
	Con.	0.08±0.05(6)	51± 9(6)	11.10±2.36(6)	215±53(6)	11.77± 3.68(6)	1.93±0.57(6)
11	Exp.	0.22±0.07(6)	25± 5(6)	5.71±3.66(6)	131±28(6)	7.78± 4.80)6)	0.77±0.62(6)
	Con.	0.46±0.09(6)	43± 12(5)	23.70±2.53(6)	217±19(6)	19.15± 0.97(6)	1.83±0.57(6)
16	Exp.	0.09±0.02(6)	28± 2(4)	0.83±0.51(6)	61±11(6)	1.72± 0.90(6)	0.26±0.24(6)
	Con.	0.14±0.04(6)	43± 21(6)	10.67±2.52(6)	118±26(6)	15.66± 2.61(6)	0.66±0.24(6)
23	Exp.	0.35±0.10(6)	19± 3(5)	0.30±0.29(6)	33± 4(6)	2.03± 2.03(6)	0.00±0.00(6)
	Con.	0.41±0.06(6)	23± 6(4)	19.08±8.59(6)	162±32(6)	17.89± 5.18(6)	0.85±0.61(6)
30	Exp.	0.38±0.11(5)	27± 12(5)	1.43±0.88(5)	92±21(5)	1.81± 1.13(5)	0.48±0.48(5)
	Con.	0.46±0.04(6)	38± 8(6)	12.14±2.79(6)	224±31(6)	9.27± 1.18(6)	1.54±0.53(6)

continued

Table 5.--continued

Days Post Injection	Group	Percent in Spleen	µg Fe/g Spleen	Spec. Act. x 10^{-5} Spleen	Percent in Head Kidney	µg Fe/g H. Kidney	Spec. Act. x 10^{-5} Kidney
1	Exp.	1.75±0.49(6)	911±177(6)	1.85±0.58(6)	4.50±1.09(6)	208±36(6)	27.32± 9.10(6)
	Con.	1.34±0.35(6)	602±252(6)	3.56±1.28(6)	5.04±0.96(6)	122±22(6)	52.89±20.86(6)
2	Exp.	4.75±0.69(6)	264± 51(6)	22.23±6.55(6)	7.98±1.11(6)	77±25(5)	146.03±30.70(5)
	Con.	4.33±1.89(6)	245± 73(6)	17.18±4.11(6)	5.93±1.24(6)	88±23(6)	84.98±28.58(4)
4	Exp.	3.13±0.78(6)	499±363(6)	21.11±6.19(6)	3.51±0.71(6)	52±13(4)	114.42±62.23(4)
	Con.	2.82±0.59(6)	391±117(6)	7.23±1.20(6)	4.12±0.85(6)	148±39(6)	33.16± 9.31(6)
8	Exp.	1.81±0.63(6)	255± 51(6)	8.15±2.76(6)	0.89±0.19(6)	141±62(4)	10.96± 3.25(4)
	Con.	1.85±0.47(6)	290± 36(6)	6.27±1.83(6)	1.09±0.24(6)	138±30(6)	8.31± 1.39(6)
11	Exp.	2.34±0.63(6)	398±101(6)	8.97±4.24(6)	0.53±0.32(6)	121±22(6)	3.90± 2.02(6)
	Con.	1.46±0.14(6)	415±108(6)	4.18±1.05(6)	0.77±0.16(6)	108±15(6)	8.19± 2.32(6)
16	Exp.	1.92±0.30(6)	311± 78(6)	6.57±1.25(6)	0.25±0.13(6)	86± 6(6)	2.67± 1.38(6)
	Con.	1.97±0.45(6)	395± 81(6)	5.67±1.57(6)	0.56±0.22(6)	120±18(6)	5.67± 2.52(6)
23	Exp.	0.47±0.14(6)	228± 93(6)	2.46±1.02(6)	0.01±0.01(6)	94± 9(6)	0.13± 0.13(6)
	Con.	0.34±0.09(6)	398±195(6)	1.24±0.41(6)	0.30±0.21(6)	164±57(6)	5.37± 4.39(6)
30	Exp.	1.19±0.37(5)	494±142(5)	3.93±1.59(5)	0.01±0.01(5)	118±21(5)	0.09± 0.07(5)
	Con.	1.17±0.58(6)	320± 41(6)	3.61±1.62(6)	0.40±0.15(6)	110±11(6)	3.19± 1.07(6)

continued

Table 5.—continued

Days Post Injection	Group	Percent in Caeca	µg Fe/g Caeca	Spec. Act. x 10^{-5} Caeca	Percent in Muscle	µg Fe/g Muscle	Spec. Act. x 10^{-5} Muscle
1	Exp.	1.32±0.46(6)	50± 3(2)	7.81±1.50(2)	13.05±3.28(6)	143±43(6)	0.54±0.27(6)
	Con.	2.48±0.44(6)	22± 4(4)	16.32±1.08(4)	10.42±2.16(6)	27±13(3)	0.99±0.46(3)
2	Exp.	1.72±0.46(6)	52± 3(6)	5.06±1.34(6)	8.22±1.67(6)	12± 6(3)	6.01±4.63(3)
	Con.	2.67±0.45(6)	57±17(4)	9.28±3.16(4)	9.30±1.58(6)	21± 4(6)	1.02±0.11(6)
4	Exp.	3.18±1.24(6)	30± 3(5)	12.72±6.11(5)	7.16±3.04(6)	34± 8(6)	0.44±0.12(6)
	Con.	1.44±0.27(6)	27± 1(5)	8.84±1.24(5)	6.04±1.92(6)	20± 2(6)	0.62±0.20(6)
8	Exp.	0.52±0.10(6)	35± 6(6)	2.24±0.42(6)	1.37±0.97(6)	25± 4(6)	0.10±0.08(6)
	Con.	1.97±0.70(6)	48± 9(6)	8.53±3.34(6)	1.63±0.86(6)	26± 3(6)	0.13±0.07(6)
11	Exp.	1.25±0.74(6)	28± 2(6)	7.17±4.05(6)	1.86±1.18(5)	56±15(6)	0.08±0.05(5)
	Con.	2.12±0.61(6)	33± 2(6)	9.99±2.93(6)	5.38±1.82(6)	23± 4(6)	0.50±0.19(6)
16	Exp.	0.81±0.38(6)	29± 3(4)	2.13±0.89(4)	0.95±0.55(6)	19± 2(6)	0.09±0.05(6)
	Con.	0.56±0.15(6)	25± 4(4)	3.56±1.51(4)	2.57±1.42(6)	35± 5(6)	0.15±0.08(6)
23	Exp.	0.12±0.11(6)	38± 4(5)	0.51±0.48(6)	0.36±0.36(6)	22± 4(5)	0.06±0.06(6)
	Con.	1.38±0.43(6)	37± 4(6)	5.22±1.13(6)	2.26±1.39(6)	18± 2(6)	0.30±0.19(6)
30	Exp.	0.06±0.04(5)	38± 6(5)	0.18±0.12(5)	0.61±0.61(5)	21± 2(5)	0.06±0.06(5)
	Con.	0.99±0.47(6)	35± 5(4)	2.81±1.19(4)	3.01±1.53(6)	29± 6(6)	0.18±0.06(6)

continued

Table 5.--continued

Days Post Injection	Group	Percent in Intestine	µg Fe/g Intestine	Spec. Act. x 10^{-5} Intestine	Percent in Feces
1	Exp.	0.26±0.06(6)	244±111(6)	5.39± 3.77(6)	4.21±2.62(6)
	Con.	0.54±0.10(6)	16± 8(4)	55.52±38.91(4)	3.19±3.10(6)
2	Exp.	0.25±0.08(6)	35± 8(2)	2.00± 1.61(2)	0.14±0.07(6)
	Con.	0.25±0.04(6)	40± 5(6)	3.04± 0.38(6)	0.11±0.01(6)
4	Exp.	0.27±0.14(6)	46± 12(5)	2.69± 1.07(5)	0.14±0.07(6)
	Con.	0.29±0.09(6)	35± 4(6)	3.99± 1.20(6)	0.21±0.07(6)
8	Exp.	0.02±0.01(6)	29± 3(6)	0.40± 0.22(6)	0.03±0.01(6)
	Con.	0.51±0.27(6)	40± 15(6)	8.30± 6.12(6)	0.07±0.01(6)
11	Exp.	0.29±0.12(6)	67± 9(6)	2.07± 1.03(6)	0.08±0.03(6)
	Con.	0.17±0.05(6)	42± 9(6)	1.73± 0.48(6)	0.03±0.01(6)
16	Exp.	0.00±0.00(6)	53± 26(6)	0.00± 0.00(6)	0.03±0.01(6)
	Con.	0.09±0.05(6)	40± 7(6)	1.31± 0.64(6)	0.03±0.002(6)
23	Exp.	0.00±0.00(6)	26± 1(6)	0.07± 0.07(6)	0.04±0.01(6)
	Con.	0.10±0.04(6)	28± 3(6)	1.86± 0.69(6)	0.07±0.01(6)
30	Exp.	0.01±0.01(5)	31± 4(5)	0.17± 0.12(5)	0.04±0.01(5)
	Con.	0.08±0.03(6)	31± 2(6)	1.19± 0.31(6)	0.04±0.02(6)

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