THE ROLE OF THE KIDNEY IN ERYTHROPOIESIS

By

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PART I

INTRODUCTION

The constancy of red blood cell mass in the circulating fluid is an exacting example of homeostasis. Humoral regulation for the insurance of other homeostatic mechanisms is well established. The concept of humoral control of red blood cell production and release is a comparatively new facet in the field of Physiology. It is of general agreement that the degree of erythropoiesis is regulated by the amount of a circulating humoral factor released from a specific tissue(s). This plasma factor has been termed erythropoietin or the erythropoietic stimulating factor (ESF).

Controversy exists concerning the chemical nature, mode of action and site of release and synthesis of erythropoietin. Various workers have proposed specific tissues for the site of production and/or release of this hormone. The kidney has been suggested to be this site and has received experimental support from recent investigations indicating the kidney is the organ of erythropoietin production and release.

It is the purpose of this thesis to investigate the relationship of kidney mass and the degree of erythropoiesis and the erythropoietic response of rats to injections of kidney slice incubates compared to the response induced by injections of known erythropoietic agents.

PART II

HISTORICAL REVIEW OF THE LITERATURE

The following pages relate and briefly discuss the pertinent contributions of various workers which have brought the concept of the erythropoietic stimulating factor or erythropoietin to its present status. After recent revival and confirmation of past experimentation, extensive scientific inquiry is being directed toward this plasma factor, its regulatory stimuli and physiological mode of action, methods of accurate assay, biochemical nature, and its site of release and synthesis.

A. Formulation and Confirmation of the ESF Theory

In 1906 Carnot and Deflandre[14,15] presented the initial observations which suggested that such a factor existed. These early workers injected nine ml of plasma obtained from slightly anemic rabbits into normal rabbits and noted a minor increase in the peripheral red cell mass. Their theory stated that the arterial blood oxygen functions to regulate an intermediate mechanism capable of inducing erythropoiesis. They termed the proposed intermediate factor "hemopoietin", being carried to the bone marrow by the blood stream. During the following two decades other investigators attempted

to repeat these observations. Throughout this period disputatious and contradictory results were presented. Gordon and Dubin in 1934[46] subjected animals to low barometric pressures and induced anemia to increase erythropoiesis as was postulated in the Carnot-Delflandre theory. They were unable to confirm the earlier findings. They stated that their experiments showed conclusively that "hemopoietin" is absent from such serum. Likewise, experimental results obtained by Feenders in 1936[32] showed no similarity to the results found by the original workers. Consequently, the proposed concept for an existing erythropoietic intermediary fell into disrepute.

In 1948 Bonsdorff and Jalavisto[9] revived the concept of humoral control of red cell production. Plasma from patients with circulatory or respiratory insufficiencies or rabbits exposed to low barometric pressure caused a slight increase in the red cell count of normal rabbits. This work created new interest in the proposed erythropoietic intermediary and led to other such investigations which confirmed Carnot and Deflandre's work. Bonsdorff[8] called this plasma factor "erythropoietin", which is more indicative of its effects, since observations of white blood cell and platelet response to this factor show no direct correlation.[23]

Oliva et al. (1949)[103] showed that plasma from human subjects with pernicious anemia caused erythropoietic activity in

normal humans with an accompanying increase in reticulocytes from mean 1.8 to 3.0%. They called this substance "reticulocytogenous factor". These workers maintained that production and reticulocyte entrance into the circulation is regulated by humoral control and that the controlling factor may be considered as a promot r and stimulator of normal erythropoiesis.

An excellent study was conducted by Reissmann in 1950[116] using parabiotic union of white rats to demonstrate humoral regulation of erythropoiesis. Chronic hypoxemia was induced in one partner of the united pair by exposure to an atmosphere of low oxygen content, while the other was kept in a normal atmosphere. Reissmann measured erythropoiesis by estimating the percentage of nucleated red cells in the bone marrow. He noted a statistically significant increase in erythropoiesis in both parabiotic rats and concluded that the stimulus is not partial pressure of oxygen of the red bone marrow per se. He proposed that a humoral factor, initiated by hypoxemia and affecting both members of the parabiotic union, caused an elevation in bone marrow activity.

In 1953 Erslev[23] succeeded in confirming the original work of Carnot and Deflandre. He noted a significant rise in the number of reticulocytes and a definite increase in red blood cell count, hematocrit, and nucleated red cells of the bone marrow in rabbits injected with plasma from rabbits rendered anemic by bleeding.

Plasma from normal donors showed no such response. Erslev concluded anemic plasma contains a factor capable of stimulating red cell production.

B. Erythropoietic Stimulation

The factors which augment the release of erythropoietin are varied, and there exists a degree of controversy as to the true stimulative agents. The most commonly employed parameters to measure erythropoietic response are reticulocyte counts, bone marrow examinations, hematocrit, hemoglobin concentration, red cell counts, and radioactive iron incorporation.

1. Anoxia

Anoxia, insufficient oxygen in the blood to meet the physiological requirements of the body, has long been believed to operate directly in regulation of red cell production. It is generally accepted that oxygen content of arterial blood regulates erythropoiesis. The most commonly held postulations for this regulation are arterial blood oxygen control of red blood cells by direct or indirect means, determining the amount of oxygen received to the marrow tissue, or controlling oxygen saturation and tension of bone marrow.[23] Grant and Root[50] reported oxygen saturation and tension of bone marrow blood to be normal in dogs which have been

chronically or acutely bled but showing elevated erythropoietic activity. Clinical studies of anemic patients reveal no reduction in bone marrow pO_2, but an actual arrest of erythropoiesis was observed in their bone marrow when subjected to low oxygen tension. These observations strongly imply that oxygen tension does not directly affect bone marrow but that some intermediate mechanism exists.[153]

According to Grant and Root[51] true erythropoietic stimulation is achieved experimentally by three conditions: anoxic anoxia, anemic anoxia, and excessive cobalt therapy. The authors reported that other non-related means have at present failed to satisfy all criteria of true stimulation. Anoxic stimulation more nearly fulfills all aspects to meet this criteria. Grant and Root elaborate on proposed means by which these factors produce stimulation.

As early as 1893 anoxia was believed to regulate erythroid activity. Miescher[90] suggested a relative degree of anoxia exists at all times in the bone marrow to insure a constancy of red blood cells and to initiate their release.

In 1915 Dallwig *et al.*[22] postulated that through reduction in oxygen tension of inspired air (hypoxia) red bone marrow is stimulated with an accompanying increase of erythrocyte activity and hemoglobin levels in rabbits, dogs, and white rats. Low barometric pressure or decreased oxygen tension are suggested means of

achieving depressed oxygen levels in inspired air. Rosin et al. (1948)[121] reported that bone marrow in vitro studies conducted in their laboratory did not agree with the proposed mechanism of Dallwig and co-workers.

Astola and colleagues[1] used variable oxygen pressures on developing chick embryos to observe hemoglobin synthesis. They found only the area vasculosa responded to low oxygen pressure. The workers reached no conclusions concerning the compatibility of the erythropoietin which exists in the embryo and is released in response to oxygen depression and the erythropoietin of mammals released after hypoxic stimulation.

Investigations were conducted by Berk et al. (1948)[2] attempting to correlate the percentage oxygen saturation in the sternal marrow with various levels of erythropoietic stimulation. The experimental subjects were convalescent controls, anemic patients, and patients with polycythemia vera. They found these patients possessed normal oxygen tensions and saturations. However, in the polycythemic cases the percent oxygen saturation was reduced. Other clinical observations concerning oxygen tensions as an erythropoietic stimulative agent include the work of Stohlman et al. (1954)[131] and Pannacciuli et al. (1959).[107] The former investigators concluded that patients developed polycythemia secondary to hypoxia arising from the entrance of unoxygenated blood into the systemic circulation.

The latter workers showed that transfusions from subjects submitted to hypoxia elicit erythropoiesis in normal subjects.

The hypothesis that a humoral factor is released in response to hypoxic anoxia has been supported by other investigations. It has been observed that hypoxic plasma markedly accelerates red cell production in the normal subject.[52,130] Fried et al.[38] showed that an atmosphere high in oxygen retards or decreases the rate of erythropoiesis in experimental rats. Grant[49] demonstrated that lactating mother rats and mice exposed to pressures of 300-400 mm Hg induced an elevated hematocrit, hemoglobin, red cell count, and oxygen capacities in their suckling young kept in normal atmosphere. Those litters nursed by normal mothers (at sea level pressure) showed no such response. In 1957 Erslev[25] postulated that an intermediary existed between a precise oxygen sensitive tissue and the bone marrow. He noted a significant reticulocytosis in rabbits subjected to a 50% hemoglobin reduction (anemic anoxia) and 50% oxygen tension reduction of respired air (hypoxic anoxia). In a later work Erslev[27] proposed that erythropoietin functions to maintain the body's oxygen requirement and hemoglobin balance. Thomas[139] found, through in vitro bone marrow studies, no level of oxygen tension which was capable of stimulating heme synthesis. Complete anoxia was observed to stop synthesis of heme. In a later investigation Thomas and Lochte (1958)[140] found heme synthesis or oxygen consumption were not

increased through vitamin B_{12} treatment on in vitro pernicious anemia bone marrow. The workers did not postulate on possible mechanisms.

An important high altitude study was conducted by Hurtado, Merino, and Delgado in 1945.[62] The workers observed the influence of anoxemia of high altitudes on erythropoietic activity. They noted exposure to depressed barometric pressure caused a polycythemic response in most cases. Accompanying this response, erythropoietic hyperactivity and increased amounts of serum bilibrubin were reported. Hurtado et al. proposed that the hemopoietic responses, resulting in polycythemia, are related to the degree, duration, and continuity of the anoxic stimulus achieved in climatic conditions inducing low arterial oxygen saturation. More recent studies of erythropoiesis and high altitudes were reported by Reynafarje in 1957[117] and 1959.[118] Reynafarje reported hyperplastic bone marrow conditions were associated with reticulocytosis and red cell iron incorporation increases in native residents of high altitudes. Both Hurtado et al. and Reynafarje noted that when these persons were brought down to sea level they gradually achieved blood characteristics similar to those found in persons who have always lived at sea level environments. E. Bernardelli (1959)[3] showed that hypoxia created from barometric pressure depression caused an erythrocyte stimulation in intact experimental animals.

According to Root (1954)[120] exposure to lower oxygen tension is the better method for experimentally inducing red bone marrow hyperactivity. The author stated hypoxia does not directly affect the bone marrow but an oxygen deficiency of some yet unknown tissue or organ activates release of an erythroid stimulating agent. In 1959 Stohlman[128] suggested plasma erythropoietin titer is affected by the functional state of the erythroid marrow and severity of the hypoxic stimulus employed.

2. Anemic Anoxia

It is known that repeated bleeding or hemorrhage is an effective stimulus for erythropoiesis.[51] Anemic anoxia is that condition characterized by a reduction in tissue oxygen tension. It may be caused by decreased hemoglobin level or red cell count.[123] Many workers have persued this line of experimentation to study erythropoietin. Grant and Root (1947)[50] reported that oxygen capacity and hematocrit are decreased after hemorrhage, but a gradual recovery occurs during the following three weeks, indicating red blood cell regeneration with an increase in oxygen saturation.

Using anemic anoxia Erslev (1959)[28] observed that this stimulus operated in accelarating the maturity of pronormoblasts from stem cells. Similarly, Rambach, Alt, and Cooper in 1957[112] suggested that the factor found in anemic rabbit plasma increased

the rate of red blood cell mitosis and maturation in the bone marrow of experimental animals. In 1955 Erslev[24] demonstrated that dilution anemia induced by administration of dextran stimulates erythropoeisis and suggested that this stimulus must depend on oxygen saturation. After dextran treatment he noted an absence of erythropoietic activity even though a reduced oxygen content of the arterial blood was induced. Erslev et al. [31] observed a significant reticulocytosis in normal monkeys when anemic animal plasma was injected, and concluded these phenomena strongly implied the presence of a humoral factor in bled primates which stimulates an increase in red cell production.

In 1959 Naets[100] published results stating that bled dogs showed an erythropoietic factor in their plasma and urine. He observed the animals demonstrated a marked erythropoiesis when severe anemia was produced.

Phenylhydrazine has been reported to be a potent agent producing anemia. White et al.(1959)[150] reported that repeated injections of phenylhydrazine in sheep created a strong erythropoietic titer with an accompanying increase in the plasma pool. Finch et al. (1959)[35] produced anemia by red blood cell removal with replacement by nonviable erythrocytes, hemolysis, and phenylhydrazine. A greater percentage of reticulocytes cells and other nucleated red blood cell forms were observed in animals made anemic by hemolysis. Phenylhydrazine stimulation was second with red cell removal and

replacement not augmenting a noticeable erythropoiesis. Lowy et al. (1959)[85] noted that plasma from phenylhydrazine treated animals induced a striking erythropoietic response.

In 1954 Hodgson and Toha[60] observed a greater reticulocytosis in bled rabbits after injection of anemic rabbit plasma. The effect of anemic plasma disappeared after exposure to an atmosphere of 100% oxygen. They noted that injections of urine of anemic animals accelerated the rate of hemoglobin synthesis after a standard hemorrhage in normal rabbits.

Stohlman and Brecher (1956)[129] exposed rats to sublethal doses of x-rays immediately after the animals had been injected with plasma from anemic donor rats. They reported a significant increase in erythropoiesis as measured by Fe^{59} incorporation. Linman and Bethell (1957)[77] used a similar approach to study anemia as a stimulating agent. Protein free plasma from rabbits rendered anemic by phenylhydrazine followed by total body x-irradiation induced a marked erythrocytosis, reticulocytosis, and increased bone marrow erythropoietic activity in normal rats. Tkadlécek (1959)[142] induced anemia in normal mice through x-ray treatment. Urine from the anemic mice initiated an increased hematopoiesis, particularly of the red cell components, in normal non-irradiated mice.

3. Cobalt Administration

The use of cobalt as an erythropoietic stimulant is not a new concept. Cobalt in comparatively small amounts produces a decided polycythemia.[87, 95, 104] Stare & Elvehjem in 1933[127] found that the presence of .04 to .05 mg of cobalt in the rats body was sufficient to create this condition. Originally, cobalt's erythropoietic action was believed to be associated with bone marrow anoxia. However, the failure of various concentrations of cobalt to stimulate hemesynthesis or oxygen consumption strongly suggests that cobalt does not directly affect the bone marrow.[75] Brown and Meineke (1958)[13] and Goldwasser and co-workers (1958)[44] reported increased erythropoietin titer in the plasma of experimental animals after cobalt therapy. In an earlier investigation Goldwasser et al.[43] compared anemic plasma and cobalt plasma and suggested that they have grossly similar properties but are not identical. They postulated that the erythropoietic effect of the cobaltous ion was probably due to an increased formation of erythropoietin, enhancing red cell production.

4. Reduction of Erythropoietic Response

It has been demonstrated that rats subjected to hypophysectomy,[145] atmospheres of high oxygen,[45] starvation,[59, 63] and transfusion induced polycythemia[56, 67] show a decreased rate of erythropoiesis.

Berthard et al.[4] observed a decrease in blood volume, hemoconcentration, and a drastic depression of erythropoiesis in rats

after removal of protein from their diet. The author suggested that the hemoconcentration within the vascular system is an important factor in regulating the erythropoietic rate than red cell volume.

In 1951 Birkhill and co-workers[6] demonstrated that transfusion induced polycythemia caused a complete cessation of erythropoiesis. Tinsley et al.[137] noted a decrease in reticulocytosis when hemolytic anemic patients were subjected to an atmosphere high in oxygen. Linkenheimer et al. (1960)[73] reported that repeated administration of erythropoietin fails to elevate erythropoietic responses to anemic anoxic or hypoxic stimulation.

C. Assay of Erythropoietin

Increases in hematocrit value, hemoglobin concentration, red cell count, nucleated red cell components, reticulocytosis, and hyperplastic bone marrow are indicative of erythropoietin activity. Other methods used to demonstrate erythropoiesis include nitrogen-mustard treated rats,[5] red cell circulating values in normal, hypohysectonized on new born rats,[18] irradiation after anemic plasma infusion,[129] and Fe^{59} incorporation in hemoglobin of normal, hypophysectomized[54] or starved rats.[61]

No totally accepted method of assay has yet appeared. Finch[34] stated that the existing concepts of red cell production

related to erythropoiesis are primative and superficial. It has been suggested that insufficient amountsof anemic plasma have been used for bioassay.[30] Hodgson et al.[60] proposed that the plasma of anemic animals not only contains erythropoietin but also other factors which elicit erythropoiesis. Linman et al.[79] postulated that the conflicting experimental findings of other investigators are attributed to the presence of two plasma erythropoietic factors of different natures and modes of action. Another apparent discrepancy is the lack of critical evaluation, objectivity, and consistency in red cell and reticulocyte counts, which are two of the more commonly employed methods to measure the degree of erythropoiesis.[30]

In 1955 Plzak et al.[111] used Fe^{59} incorporation to measure effects of erythropoietin. They reported that three doses (two ml. each) of anemic rat plasma, which was about equal to one half the rat's blood volume, gave the best stimulation. The authors suggested the blood samples should be obtained twelve hours after injection, and the Fe^{59} must be injected a few hours after the final injection of anemic plasma. Fried and co-workers[37] also used Fe^{59} incorporation to assay erythropoietin. They reported that administration of anemic plasma in hypophysectomized rats increased Fe^{59} uptake three to seven fold.

An in vitro erythropoietin study was conducted by Thomas in 1955.[138] Using radioactive glycine uptake and oxygen consumption,

Thomas simultaneously measured hemin synthesis in rabbit bone marrow. Thomas reported a slight decrease in the first twenty hours with optimal pH of approximately 8.0. An interesting dissociation between hemin synthesis and oxygen consumption was suggested.

Schroeder et al. (1958)[125] suggested that the practical objections to ESF assay methods, i.e. expense in time, animals, test materials, can be somewhat eliminated by using Fe^{59} incorporation into bone marrow cell suspensions of rats. These investigators observed a seven fold increase of Fe^{59} uptake into suspension cells in the presence of anemic serum in contrast to normal donor serum. However, Thomas and his associates (1960)[141] have been unable to confirm these experimental findings. Thomas et al.[141] have attempted to develop an in vitro bone marrow assay for erythropoietin using phenylhydrazine induced anemic rat plasma and hypoxic rat plasma. It was reported that no erythropoietic activity could be demonstrated in vitro by measuring oxygen consumption, hemesynthesis, DNA synthesis, or Fe^{59} incorporation. These investigators did not advocate the prescribed assay techniques of Matoth et al.[86] and Rosse and Gurney.[122] The latter group proposed an assay of erythropoietin in which the number of erythropoietic precursors in tissue culture and normal serum cultures are compared with serum believed to contain erythropoietin. Korst and colleagues[71]

reported a correlation between the marrow cultures, Fe^{59} incorporation, and the erythropoietic activity in incubated plasma cultures. Thomas et al.[141] were unable to confirm these findings.

In 1958 Lowy, Keighley, and Borsook[83] presented a method to concentrate erythropoietically active plasma. They reported the major active part of anemic plasma represented less than 0.5% of plasma proteins. Yet, they suggested only a fraction of this concentrated plasma represents erythropoietin.

Garcia and Van Dyke[40] attempted to quantitate the erythropoietic response to graded dosage of human urinary erythropoietin and to compare different methods of assaying erythropoietin. This investigation employed two assay methods, red cell radioactive iron assay and red cell volume assay. They suggested the former method to be used as a "screening" method for erythropoietic activity while the latter be used as a confirming assay method. This investigation compared erythropoietic response to injections of plasma containing erythropoietin with the response to administered erythropoietin via hypoxia, and Fe^{59} red cell turnover assay to normal, hypophysectomized, and starved rats after prolonged and graded injections of anemic plasma.

A method for purification of erythropoietin was proposed by Ramback et al. in 1959.[114] This procedure utilized diethylaminoethyl (DEAE) cellulose in exchange columns. Erythropoietin from the

filtrate of acidified, boiled plasma prepared from rabbits rendered anemic by phenylhydrazine was shown to be active erythropoietically.

Winkert and associates[151] reported a method of partial purification of urinary erythropoietin by kaolin absorption preceeding ammonium acetate buffer serial elution. Using this procedure the investigators produced a 39% yield with a two-hundred and thirty fold purification of the active factor.

In 1960 Weissman et al.[148] proposed a method for quantitation of erythropoiesis in dogs. They determined the intravascular reticulocyte maturation time, measured erythropoiesis by total circulating red cell volume, and obtained red cell life span, and iron turnover.

Gurney and Pan (1960)[57] compared methods of bioassay of erythropoietin in human plasma. Plasma and heat-denatured extracts of anemic patients' plasma were assayed for their ability to stimulate erythropoiesis in hypophysectomized and starved rats. They found that the most sensitive method was the whole plasma assay.

D. Proposed Biochemical Nature and Properties of Erythropoietin

The exact biochemical nature of this humoral agent is unknown. It is believed to be non-protein in nature.[12] However,

this is not totally accepted by all workers.[85] It has been proposed that erythropoietin is a mucoprotein,[10, 92, 112, 113, 151] glucoprotein,[115] lipid or serum protein,[30] polypeptide,[143] trioxo-molalcoholic sterol,[42] similar to a-n-octa decylglyceryl ether 78, or other chemical substances.

Erythropoietin has been shown to be non-species specific and nonantigenic.[77] An appreciable titer of erythropoietin is shown in protein-free filtrate of anemic plasma.[76] Rambach and associates[114] have partially characterized an active erythropoietic factor from anemic rabbits. They suggested that erythropoietin might be a low molecular weight acidic glycoprotein with a low glucose content and with a portion of the hexose as galactose.

In 1954 Erslev[30] found this factor to be nondialisable, to remain in solution during fibrinogen-fibrin transformation, and to persist during alpha globulin salt precipitation. Other workers have found the ESF to be dializable.[43] Erslev[25] reported eryhtro-poietin stable at temperatures between 4°C and -21°C. He suggested these findings imply that erythropoietin is linked or has similar action to an alpha globulin, beta globulin, or serum albumin. The author postulated the nondialysable factor might be a portion of the serum protein.

Observations by Hodgson and Toha[60] showed that anemic plasma lost its erythropoietic activity, when boiled at pH 5.5,

but remained active at pH 9.0. The reverse condition was reported when urine of anemic rabbits was boiled. The authors cited early work done by Tei[133, 134, 135, 136] who supported the existence of two factors capable of stimulating erythropoiesis. According to Tei, one factor present in bled rabbits blood and phenylhydrazine anemic rabbits resists boiling and is soluble in acetone, ether, and alcohol. The second erythropoietin is present in the blood of rabbits with anemia induced from Allium scordorprasum extracts and in polycythemic animals secondary to exposure of depressed barometric pressure. This factor is thermolabile, insoluble in ether and was found in the globulin fraction of blood serum.

Linman and co-workers[78, 79, 80, 81] reported results which support the existence of two humoral factors capable of stimulating erythropoiesis. They maintained one factor is thermolabile and insoluble in ether. This factor initiates hemoglobin synthesis. The second erythropoietic substance is thermostable and ether soluble and seems to increase the activity of the precursors of erythropoiesis and may be closely associated with batyl alcohol. Sandler (1949)[124] found that isolated batyl alcohol from yellow bone marrow of cattle initiated erythropoiesis increase in normal animals. This has been confirmed by more recent investigation.[132]

Rambach and colleagues[115] reported that their findings did not correspond to Linman's either soluble factor and to the supposition of one erythropoietin being chemically allied to batyl alcohol.

These workers isolated an acidic glycoprotein which was capable of stimulating red cell production. They postulated that only one erythroid stimulating factor exists. This single factor acts like a glucoprotein.

Van Dyke et al.[146] supported the theory of a second erythropoietic hormone of pituitary origin which is similar to ACTH chemically but neither chemically nor biologically identical.

Preliminary biochemical observations by Toká and associates[143] showed their purified acetone extracts of anemic plasma gave negative reactions with ammonical silver nitrate, mercuric nitrate reagent, and ninhydrin and a positive reaction with Sudan IV (fatty acid esters).

In 1959 Gordon et al.[48] used a urinary source for studying the properties of ESF. They found that it may be absorbed on kaolin in acid pH ranges and eluted in the alkaline range. Ultraviolet light studies revealed the active fractions absorb a great amount of light at 280 millimicrons. Their report stated proteolytic enzymes destroyed its biological activity. A mucoprotein nature was their proposed biochemical composition for erythropoietin.

Loo, Lee, and Flemming (1960)[82] found that crystalline papain and crude bromelin were capable of stimulating erythropoiesis, while other proteolytic enzymes showed no such activity. This work was conducted to determine the relationship between certain

enzymes and the activity of erythropoietin. They did not postulate possible mechanisms or implications.

Borsook et al.[11] have described erythropoietin as a plasma factor which is not precipitated or its activity impaired at boiling temperatures. According to this group, ESF plasma factor increases the rate of incorporation of amino acids in reticulocyte proteins in vitro observations, particularly hemoglobin. However, in 1960 Nakao and co-workers[102] proposed a factor found in human plasma, which accelerated hemesynthesis, was not identical to the factor suggested by Borsook et al.[11]

Lowy, Keighley, and Borsook (1960)[84] suggested the active erythropoietic factor found in animals with phenylhydrazine induced anemia is related to a mucoprotein and proposed that sialic acid is an essential member of the molecule.

E. Site of Production and Release of Erythropoietin

The location of synthesis and release of erythropoietin remains undefined. In 1956 Gordon et al.[47] tested acidified boiled filtrates of plasma, liver, spleen, thymus, lung, brain, skeletal muscle, bone marrow, and packed red cells from anemic rabbits for erythropoietic activity in an attempt to find this location. Their results indicated only plasma was capable of

initiating erythropoiesis in normal rats. They suggested that the erythropoietic factor might appear in the plasma resulting from enzymatic action upon substrates of blood forming organs.

The reticuloendothelial system,[72] red blood cell,[7] and stomach[33] have been suggested as possible centers for the release of erythropoietin. It has been proposed that the spleen might release the factor causing erythropoiesis[152] or function in erythropoiesis to regulate the numerical erythropoietic units in the bone marrow, to determine the release age of young red blood cells from marrow tissue, and to exert an influence on hemoglobin or stromal protein synthesis from enzymatic processes.[21] Linkenheimer et al.[74] observed an increased P^{32} uptake of DNA of the spleen following hemorrhage and anoxic anoxia. The workers did not postulate possible mechanisms involved. Waldmann and associates[147] observed a decrease of plasma and red cell volumes, hematocrits, and Fe^{59} uptakes after splenectomy of dogs. The authors reported that the resultant anemia was due to either removal of the splenic red cell reservoir or removal of the organ producing erythropoietin.

In 1954 Van Dyke et al.[145] presented evidence suggesting that the anterior pituitary exerts a zenith control on circulating red cell volume. They reported a mild anemia was induced after combined or individual removal of thyroid, adrenals, and gonads. Animals under these conditions were capable of responding erythropoietically

to hypoxic stimulus. Severe anemia followed hypophysectomy with an inability to respond to hypoxia. Oral administration of fresh anterior lobe stimulated erythropoiesis in hypophysectomized, adrenalectomized, and normal rats. These results confirmed the observations of Flaks et al. (1937),[36] Meyer et al. (1940),[89] and Contopoulos et al. (1953).[19] Bernardelli[3] noted depressed vitro bone marrow reactions through hypoxic stimulation and reported hypophysectomized animal bone marrow showed similar depressions to hypoxia. In 1927 Schulhof and Matthies[126] suggested that the vegetative centers of the brain play a role in the regulation of circulating red cells. Moehlig and Bates (1933)[93] suggested the importance of the pituitary on red blood cell production.

In opposition to the role of the pituitary as the source for erythropoietin, Jacobson and colleagues[69] showed that plasma of hypophysectomized rats, subjected to bleeding, initiated erythropoiesis in other hypophysectomized rats. A similar reaction occurred upon injection of anemic plasma in hypophysectomized animals. Administration of anemic plasma to hypophysectomized rats showed a reticulocyte increase much greater than in normal or saline treated controls.[66] Fried et al.[37] observed a metabolic depression after pituitary removal and suggested red cell percentage, before reduction in metabolic requirements occurs, is similar or identical to transfusion induced polycythemia. They reported that as a new erythrocyte equilibrium is established an increase of reticulocytes and

Fe^{59} uptakes is noted after administration of anemic plasma to hypophysectomized animals. Linman[79] observed plasma from hypophysectomized rats after hemorrhage was erythropoietically active. Crafts and Meineke[20] concluded that the anemia resulting after hypophysectomy is a secondary effect acquired through general metabolic decrease. These workers showed by varying the doses of thyroxin erythropoiesis could be stimulated in hypophysectomized animals.[88] They reported thyroxin returns bone marrow and erythroid elements to near normality in hypophysectomized rats by increasing the oxygen need and general metabolism. Piliero[109] suggested the pituitary, adrenal, ovarian glands effect hemopoietic organs secondary to their metabolic actions.

Contopoulos and associates[16] proposed the existence of two separate and distinct erythropoietic hormones. One hormone being closely related to ACTH and also of pituitary origin, the second factor from an unknown site. Garcia and associates[41] observed an increased red cell volume in normal and hypophysectomized rats after ACTH therapy. In 1959 Van Dyke[144] stated the biological and chemical differences of erythropoietin and corticotropin found in plasma and urine. He proposed that the erythropoietic stimulatory ability of plasma from anemic hypophysectomized animals eliminate a possible erythropoietin from a pituitary source. Van Dyke suggested that the terms corticotropin and "pituitary erythropoietic hormone" are

identical. He concluded the erythropoietic activity of pituitary extracts are merely secondary erythropoietic effects of ACTH and other adrenal steroids.

Jacobson and co-workers[63, 64] have strongly supported the kidney as the site for erythropoietin release and production. They suggested that the rate of erythropoiesis is determined by the amount of circulating erythropoietin produced in the kidney and released by oxygen supply-demand levels of the body. Administration of cobaltous ion, severely induced anemia, or hypoxic anoxia failed to stimulate erythropoiesis in bilaterally nephrectomized rats, whereas rats with bilateral ureter ligations responded to appropriate stimuli. Jacobson et al.[65] reported removal of adrenal, gonad, stomach, intestine, spleen, pancreas, thymus, and 90% of the liver followed by induced anemic or cobalt stimulation did not impair erythropoietin release. Only bilateral nephrectomy caused a cessation of erythropoiesis in these animals. However, Mirand and Prentice[91] found no significant change in erythropoietin titer through hypoxic anoxia stimulation with or without the kidney and/or spleen. Jacobson et al.[68] demonstrated that plasma from nephrectomized animals, exposed to hypoxic anoxia, showed a small erythropoietic response in transfusion induced polycythemic mice. This plasma showed no erythropoietic activity when injected into starved rats and measured by Fe^{59} incorporation.

J.P. Naets[97, 98] reported that bilateral nephrectomy abolishes erythropoiesis in dogs. He observed a disappearance of bone marrow erythroblasts in bilaterally nephrectomized dogs kept alive by peritoneal dialysis.[96] Naets suggested that erythropoietin might appear in the urine as the result of kidney filtration.[98] The findings obtained indicated the resultant depression of erythropoiesis in nephrectomized dogs is the effect of removal of the organ which is the source of erythropoietin.[99] In a more recent investigation Naets[101] starved mongrel dogs, which had been bilaterally nephrectomized and kept alive by peritoneal lavage, and noted a rapid depletion of erythroblasts from the erythroid marrow tissue. Dogs with bilateral ureter ligations, starved to the same degree, showed essentially normal erythropoiesis. Both nephrectomized and ligated dogs demonstrated elevated blood urea nitrogen to the same degree. Marrow erythroblasts were reported to have remained after injection of the erythropoietic factor in one bilaterally nephrectomized dog. Erslev (1960)[29] noted a supression of red cell production in rabbits after bilateral nephrectomy. Erslev reported some erythropoietic activity remained until the animal died and that bilateral ureter ligations induced erythropoietic supression of the same degree as nephrectomy.

Osnes (1958)[105] found the ultra filtrate of active serum from nephritic mice gave no reticulocytosis in normal mice and proposed the substance active in nephritic mice is different from

erythropoietin. Osnes[106] produced anemia in mice by irradiating both kidneys. Partial nephrectomy impaired reticulocyte response to hemorrhage which was restored by serum of nephritic mice. Osnes reported the presence of another factor in the serum of normal mice which was only effective in animals with intact kidneys.

Anemia often accompanies chronic renal insufficiencies, with morphologically normal bone marrow. However, in acute renal insufficiencies the condition is different.[96] Kaye[70] correlated the severity of anemia with the degree of renal failure. He noted a marked change in iron metabolism and decreased erythrocyte survival time in subjects with renal failure. Naets (1957)[96] and Ricket et al. (1954)[119] have observed erythroblastopenia in the bone marrow, although nitrogen retention was similar to the chronic condition. Erslev[26] found that uremic rabbits failed to respond to anoxic anoxia with accompanying reticulocyte and marrow normoblast increases. He concluded that this and other findings of his experiment indicated the uremia and the anemia of uremic rabbits are associated through changes in metabolism and not through the presence or absence of the kidney. Muirhead et al. (1952)[94] noted bilateral nephrectomy was accompanied by anemia with lower hematocrit, erythrocyte count, hemoglobin concentration, and red blood cell volume decrease in rabbits.

F. Clinical Observations Concerning Erythropoietin

Some anemias of varied etiology have been shown to possess a relatively high titer of erythropoietin in their plasma.[79] Low blood values, observed in most hemolytic disorders, have been proposed to result from low levels of circulating erythropoietin.[128] With isolation, purification, identification, and synthesis of erythropoietin, clinical application of this factor would be invaluable in treatment of various anemias.

Gurney, Jacobson, and Goldwasser in 1958[55] suggested that a deficiency in circulating erythropoietin is related to the anemia noted in malnutrition, chronic renal disease, chronic infection, and rheumatoid arthritus. They observed erythropoietic activity in human plasma after concentration.[54] Gallagher et al.[39] reported fifteen of sixteen uremic subjects demonstrated a humoral erythropoietic stimulating factor in their plasma. Piliero et al.[110] showed erythropoietic stimulation of normal rats after administration of acidified boiled filtrates of plasma from seven Cooley's anemia and two sickle cell anemia patients. Urine of the subjects with Cooley's anemia, similarly prepared as their plasma, was active erythropoietically. They found plasma filtrates from patients

with hypoplastic anemia were incapable of stimulating erythropoiesis. In 1957 Gurney and associates[58] administered plasma from bled patients to two patients with congenital hypoplastic anemia. After treatment an increase in reticulocyte response was reported. One patient showed an increased hemoglobin concentration. They concluded this chronic anemia results from a congenital deficiency of some plasma factor. Gurney et al.[53] reported a high titer of erythropoietin in one patient with hypoplastic anemia. The titer decreased after blood transfusion.

Erythropoietin has been demonstrated in the plasma of patients with polycythemia vera and secondary polycythemia.[17] Linman et al.[80] administered plasma, prepared from thermostable or ether soluble fractions of sixteen polycythemia vera patients, to normal recipient rats. They observed increased red cell production, reticulocyte count, and erythroid hyperplasia. All erythropoietic parameters measured returned to normal after cessation of injections. These workers proposed that polycythemia vera may be the result of an over production of a humoral factor.

Mirand and colleagues[92] reported boiled filtrates of plasma from Cooley's and hypoplastic anemia patients showed erythropoietic stimulatory effects. Unconcentrated urine from these subjects showed similar activity in some instances. They proposed that the lack of erythropoietin in the urine of some cases may be due to

the change of threshold levels of the kidney for this factor in normal or abnormal conditions. The presence of erythropoietin in urine has been confirmed by other investigators.[60] In 1959 Osnes[106] reported that anemic serum from subjects with chronic glomerulone nephritis and chronic infection initiated a reticulocytosis in normal mice. This response was not seen in nephritic mice.

In 1960 Payne et al.[108] reported that a unilateral hydronephrosic male subject and another male subject with unilateral polycystic kidney were polycythemic. However, four months after unilateral nephrectomy was performed, neither case showed this condition. Testing their plasma for erythropoietic activity in black hooded rats, this group observed a mean 5.9% reticulocyte count and 18.3 gms% hemoglobin resulting from pre-nephrectomy plasma injections of the hydronephrosis case. After nephrectomy of the hydronephrosic patient injection response of the experimental animals induced a 3.4% reticulocyte count and 14.6 gms% hemoglobin. The unilateral polycystic kidney case showed similar results. Plasma from normal donors showed a 3.3% reticulocyte count in the tested rats.

PART III

MATERIALS AND METHODS

White rats of the Br_{16} strain were used throughout this work. For reticulocyte slides blood was removed from the end of the tail by clipping the distal part. The blood smears for determining reticulocyte percentage changes were made on 0.3% Brilliant Cresyl Blue pre-stained slides. The blood smear was then counterstained with Wright's stain. This staining procedure, prescribed by Whitby and Britton,[149] was employed for all reticulocyte determinations. Unless otherwise designated all injections into the experimental animals were made through the tail vein.

A. Erythropoietic response to bleeding and cobalt therapy in unilaterally nephrectomized and "sham" operated white rats.

Male white rats from different litter but of approximately the same age and weight were used.

On day I 35% of the blood volume was removed by cardiac puncture, using heparin as an anti-coagulant. Immediately following bleeding, unilateral nephrectomy was performed on one half of the series. A similar "sham" operation was conducted on the remaining rats, the kidney merely being exposed, manipulated and replaced in the peritoneal cavity. In both unilateral nephrectomy and "sham" nephrectomy, precaution was taken to minimize blood loss from the animals.

Blood smears were made each day of the experimental period to determine reticulocyte percentage changes.

Hematocrit values were determined on the day of bleeding and on the final day of observation in three groups of young female rats.

A comparison of erythropoietic response of unilaterally nephrectomized and "sham" operated adult male white rats to cobalt therapy was attempted. The levels 1.5 mg of cobalt chloride/250 gm body weight, 3.8 mg of cobalt chloride/250 gm body weight, and 5.0 mg of cobalt chloride/250 gm body weight were used for this comparison. Unilateral nephrectomy and the "sham" operation were done just preceeding the first injection. In each case cobaltous ions were introduced into the rat by peritoneal injections. Each day for two days injections were given.

On one series Fe^{59} injections were made the third day of observation, and twenty-four hour uptake were counted in a well-type scintillation counter in an effort to establish a proper stimulatory level of cobalt.

B. Erythropoietic response of white rats injected with rabbit kidney incubates and other substances.

Slices of kidney from domestic rabbits were incubated in Mammalian Tyrode's. Thin slices of kidney were placed in 50 ml erylenmyer flasks which contained 7 ml of pre-warmed Tyrode's and

attached to the manometer of the Warburg apparatus. Approximately 2 gm of kidney slices were placed in each flask. R.Q. values were determined using one manometer flask containing kidney, Tyrode's and 70% KOH (.2ml) as a CO_2 absorbant. Barometric pressure fluctuations were corrected for by using a thermobarometer. When O_2 consumption showed marked reduction the tissues were removed from the flasks to prevent possible release of toxins from dying tissue. Three such kidney incubations were conducted with resulting R.Q. values of 0.89, 0.95, and 0.82. After incubation the supernatant was centrifuged to remove cellular debris then frozen until its experimental use. For the last incubation part of the kidney slices were incubated in 3.5 ml Tyrode's plus 3.5 ml rabbit plasma (removed just preceeding nephrectomy). Domestic rabbit skeletal muscle and liver slices were incubated using the same procedure as incubation of kidney slices. The R.Q. values for the incubated skeletal muscle and liver slices were 0.76 & 0.88, respectively.

White female rats of approximately the same weight and age were starved for four days. On day IV tail vein injections (1 ml/ 100 gm body weight) were performed on three groups: (1) the supernatant of kidney incubated in Tyrode's, (2) The supernatant of kidney incubated in Tyrode's and rabbit plasma, (3) Tyrode's solution. Reticulocyte percentages were determined on days IV, V, and VI. Using starved adult male rats an identical experiment was conducted, five rats, being injected with kidney incubate and five with liver incubate.

Twnety-four hour Fe^{59} uptakes were determined in young immature male and female rats (weighing approximately 60 gm). The rats had previously received two injections (1 ml/100 gms body weight) incubate. On the third day an injection of the incubate with the Fe^{59} was given. A control group was similarly injected for the same period with Tyrode's using the same injection volume.

Kidney slice incubates were injected in two non-starved groups of young immature and older adult male white rats. Reticulocytes were counted on the day of injection (day I) through day III, the final day of observation.

Other substances were tested for their erythropoietic activity, using reticulocyte counts as the parameter for response. These substances include (1) Anemic rat plasma (from four adult male rats bled 33% of blood volume for 3 days with final average Hct of 24% and Reticulocyte counts of 10.7% on day IV, (2) plasma and centrifuged urine from a polycythemic vera patient, and (3) Tyrode's. Comparisons of reticulocyte changes before and after treatment were noted.

PART IV

RESULTS

A. Erythropoietic response to bleeding and cobalt therapy in unilaterally nephrectomized and "sham" operated white rats.

It was demonstrated that unilaterally nephrectomized and "sham" operated control white rats differ in magnitude of reticulocyte response to bleeding of 35% of their blood volume. This variation in response is in Figure 1. Nine rats represented each group. The mean reticulocyte percentage is summarized in Table 1. The % Hct regenerated on days III, V, and VI after bleeding of different groups was calculated in Table 2.

Table 1. Reticulocyte response comparison of male white rats bled 35% of their blood volume on Day I.

Day	Number of animals	Unilateral Nephrectomized % Reticulocyte	"Sham" Operated Controls % Reticulocytes	
I	9	3.5 ± 0.5[1]	3.4 ± 0.2	t cal.[2] = 1.743 t.20 = 1.337
II	9	6.4 ± 0.3	10.3 ± 1.9	t cal. = 2.131 t.05 = 2.120
III	9	12.0 ± 1.7	8.4 ± 1.6	t cal. = 1.608 t.20 = 1.337
IV	9	13.2 ± 4.8	8.9 ± 1.5	t cal. = 0.860 t.50 = 0.690
V	9	11.7 ± 2.9	8.4 ± 0.9	t cal. = 1.311 t.40 = .865

1. Method from Dodson, E.O., Genetics, Philadelphia, 1956, p.53.
2. "t" calculated by "Students" method; Snedecor, G.W., Statistical Methods. The Iowa State College Press, Amer. Iowa, 1956, p.45.

Table 2. % Hct regenerated in young female white rats bled 35% their blood volume on day I.

Group		% Regenerated	Day
1.	4 unilateral nephrectomized	1.4 ± 0.1[1]	III
	4 sham operated control	2.0 ± 0.2	
2.	3 unilateral nephrectomized	9.7 ± 0.3	V
	3 sham operated control	12.6 ± 1.4	
3.	6 unilateral nephrectomized	13.2 ± 2.2	VI
	6 sham operated control	13.6 ± 1.6	

1. Method from Dodson, E.O., Genetics, Philadelphia, 1956, p.53.

The reticulocyte response of unilaterally nephrectomized and "sham" operated controls to cobalt therapy did not show any significant differences. The mean response of four rats of each group to injections of 3.8 mg/250 gm body weight of $CoCl_2$ is shown in Table 3. with their final hematocrit on day III. The mean Fe^{59} uptake for five rats treated with Co (1.5 mg/250 gm body weight) was 41.2 ± 3.0% while five untreated controls showed an average uptake of 45.0 ± 2.1%. The values were determined on day III after two preceding injections. The level of $CoCl_2$ at 5.0 mg/250 gm body weight was lethal in four of the ten rats injected on day I. The experiment was discontinued.

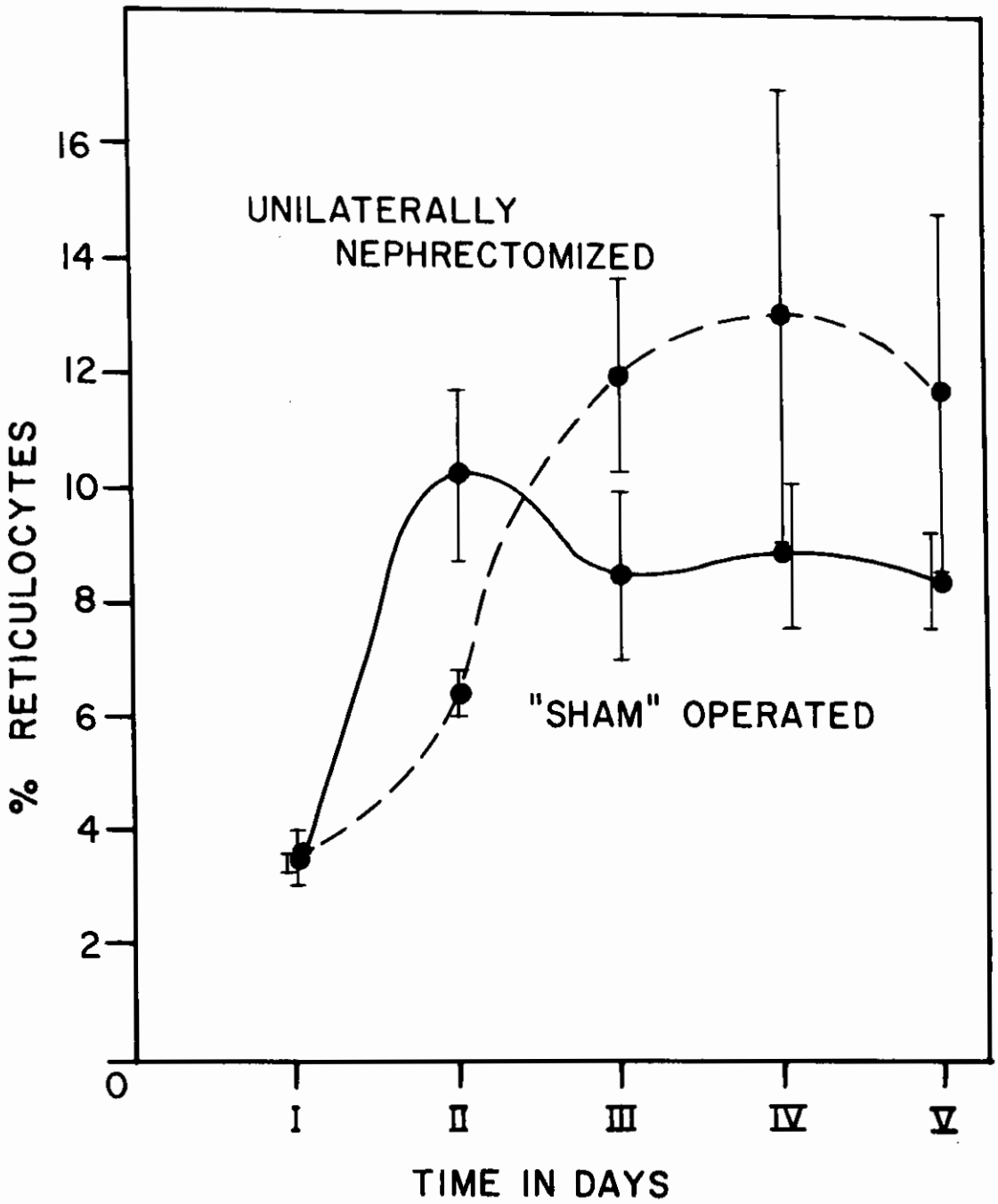

FIGURE I. RETICULOCYTE RESPONSE OF RATS BLED 35% OF THEIR BLOOD VOLUME ON DAY I.

Table 3. Reticulocyte response comparison in adult male white rats treated with $CoCl_2$ (3.8 mg/250 gm body weight).

	4 Unilateral Nephrectomized, adult	4 "Sham" Operated Control, adult
Day I % Reticulocyte	3.8 ± 0.6[1]	3.6 ± 0.1
Day II % Reticulocyte	3.8 ± 0.4	3.9 ± 0.7
Day III % Reticulocyte	4.0 ± 0.3	3.9 ± 0.6
Hct	48.0 ± 1.6	46.6 ± 2.2

1. Method from Dodson, E.O., Genetics, Philadelphia, 1956, p.53.

B. Erythropoietic response of white rats injected with rabbit kidney incubate and other substances.

The reticulocyte response of the starved experiment rats to injections of kidney incubate, kidney incubate and plasma, liver and skeletal muscle incubates, and Tyrode's is summarized in Table 4. Figure 2. shows the plotted variation of response of five starved males injected with kidney incubate and five starved males injected with liver incubate. The erythropoietic activity of substances injected in non-starved series is shown in Table 5.

The Fe^{59} uptake of five kidney incubate injected rats and five Tyrode injected controls showed considerable overlapping of percentages, the mean Fe^{59} uptake for the former group being 37.5 ± 2.4% and the latter 35.4 ± 6.3%.

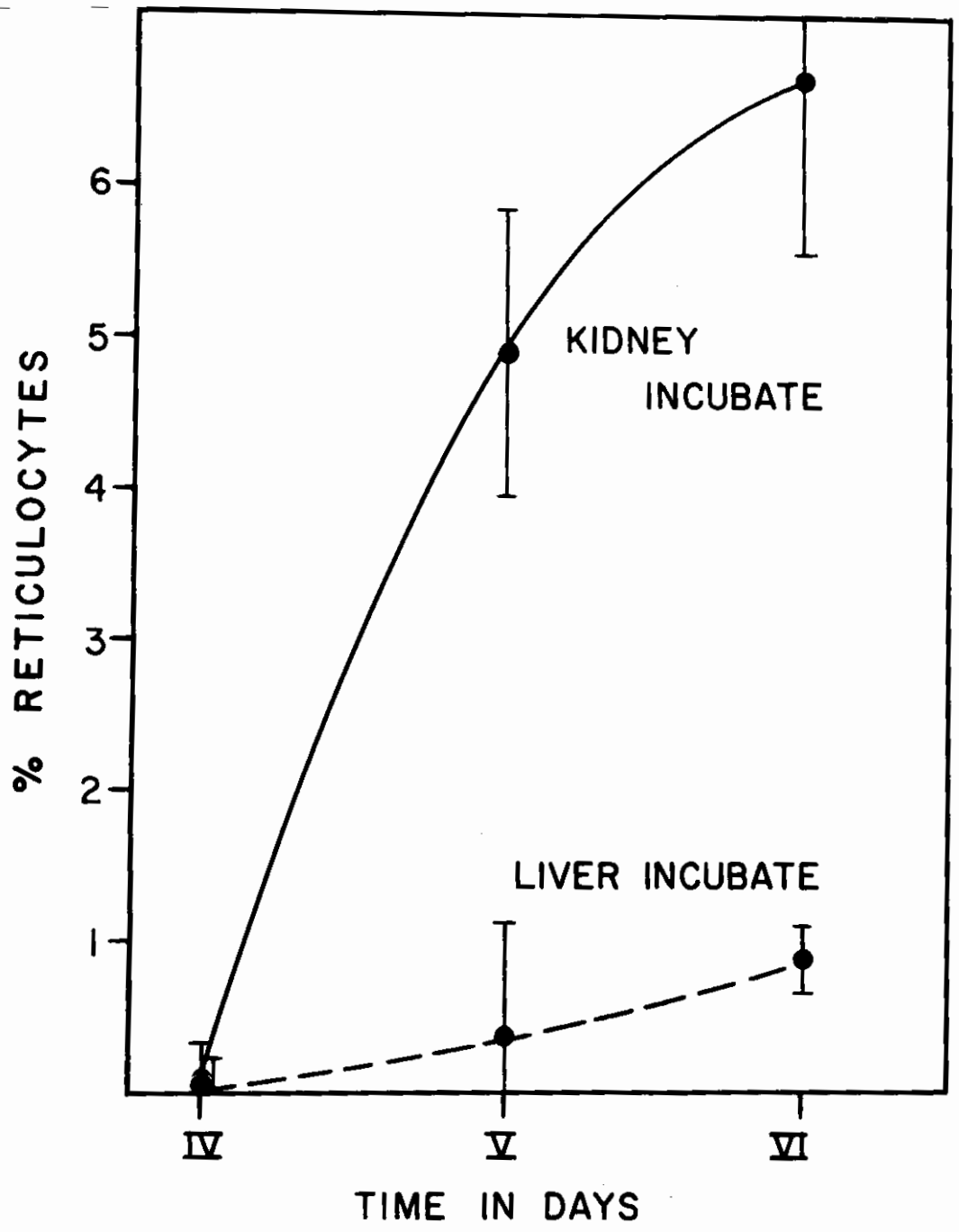

FIGURE 2. RETICULOCYTE RESPONSE OF RATS TO INJECTIONS OF KIDNEY & LIVER INCUBATES.

Table 4. Erythropoietic response of rats (starved 4 days) to injections of kidney incubate on day IV.

	No. of animals	Substances injected	Injection volume	Reticulocyte % Day IV	Day VI	
A.*	3 adult ♀	Kidney incubate (Tyrode's)	1 ml/100 gms	0.1±0.1[1]	5.3±1.2	t cal.[2]=6.666
	3 adult ♀	Kidney incubate (Tyrode's + plasma)	1 ml/100 gms	0.1±0.2	3.2±2.6	t.001 =5.041
	3 adult ♀	Tyrode's	1 ml/100 gms	0.3±0.4	0.9±0.1	
B.	4 adult ♂	Skeletal muscle incubate (Tyrode's)	1 ml/100 gms	0.3±0.2	0.6±0.1	t cal.=1.555
	4 adult ♂	Liver incubate (Tyrode's)	1 ml/100 gms	0.2±0.2	0.9±0.2	t.20 =1.440
C.	5 adult ♂	Kidney incubate (Tyrode's)	1 ml/100 gms	0.1±0.3	6.6±1.1	t cal.=6.823
	5 adult ♂	Liver incubate (Tyrode's)	1 ml/100 gms	0.0±0.1	0.8±0.4	t.001 =5.041

1. Method from Dodson, E.O., Genetics, Philadelphia, 1956, p.53.
2. "t" calculated for day VI of above groups by "Students" method; Snedecor, G.W., Statistical Methods, The Iowa State College Press, Amen, Iowa, 1956, p.45.

* for group A,"t" values calculated on kidney incubate (Tyrode's) and Tyrode's control.

Table 5. Erythropoietic response of non-starved rats to injection of various substances on Day I.

No. of animals	Substance injected	Injection volume	Reticulocyte % Day I	Day III
4 young ♂	Kidney incubate (Tyrode's)	1 ml/100 gms	8.1±1.2[1]	11.1±1.1
4 adult ♂	Kidney incubate (Tyrode's)	1 ml/100 gms	3.9±0.2	6.5±1.0
3 young ♂	Anemic Rat Plasma	½ ml/100 gms	7.1±0.7	11.6±0.3
4 young ♂	Polycythemia Vera Plasma	1 ml/100 gms	7.9±1.1	17.6±1.3
4 young ♂	Urine of Polycythemia Vera	1 ml/100 gms	8.2±1.5	11.0±1.2
4 young ♂	Tyrode's	1 cc/100 gms	5.8±0.5	5.5±1.2

1. Method from Dodson, E.O., Genetics, Philadelphia, 1956, p.53.

PART V

DISCUSSION

The results of this thesis indicate the kidney plays a role in controlling erythropoiesis in white rats. The first part of the experimental work, i.e. comparison of anemic rats, shows the animals with greater kidney mass have statistically a significantly greater reticulocyte response on the day following initial erythropoietic stimulation. This peak response is less than the nephrectomized group, which achieves peak reticulocytosis four days after induced anemia. A possible reason accounting for the greater percent of reticulocytes seen in the group having one kidney could be their inability to meet metabolic demands. As a result reticulocytes are being continually released from the red bone marrow. After the fourth day a decrease of reticulocytes is seen, possibly indicating a re-establishment of metabolic equilibrium.

It was noted also animals with a greater kidney mass have a greater ability to regenerate removed (induced anemia) red blood cells.

The comparison of unilaterally nephrectomized and "sham" operated control rats to Co therapy did not show any significant differences. No level of $CoCl_2$ was found which would produce polycythemic animals before suspected erythropoietic compensation of the remaining kidney.

The effect of kidney incubate injections in starved animals demonstrates the kidney possess an agent capable of inducing erythropoiesis. This agent was not found in liver or skeletal muscle incubates. Comparing the response resulting from kidney incubate injections in non-starved rats to known erythropoietic substances (Polycythemic Vera Plasma and Urine and Anemic Rat Plasma) a similar increase in circulating reticulocytes is observed, supporting the supposition that the kidney and the degree of erythropoiesis are directly related.

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