

EFFICACY OF SHEEP ERYTHROPOIETIN IN HISTIDINE
DEFICIENT RATS

by

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INTRODUCTION

That protein depletion causes a reduction in the rate of Erythropoiesis has been shown by many investigators. Bethard et al. (4) demonstrated that essential amino acid depletion drastically reduced Erythropoiesis as judged by the incorporation of Fe^{59} into red cells. Orten and Orten (52) showed that anemia of protein depletion responds favorably to cobaltous ion. Hallegren (33) showed that the protein depleted rats retain the capacity to respond to hemorrhage by a reticulocytosis and erythroblastic hyperplasia.

On the basis of the above observations, Jacobson et al. (38) hypothesized that Erythropoietin[®] may be the common denominator that causes the reduction in Erythropoiesis and the response to such stimuli as cobalt and bleeding in the protein depleted animal.

It is believed that the reduced rate of Erythropoiesis in protein depleted animals, is in part secondary to the reduced production of Erythropoietin. However the mechanism of reduced titre of Erythropoietin in protein depleted animals is not clearly understood.

Jacobson et al. (38) found that normal plasma elevated both the Fe^{59} incorporation and reticulocyte count to about one-half the extent shown by anemic plasma in amino acid depleted rats. They concluded that the partial response to normal plasma may reflect the effect of adding the essential amino acids that are needed for protein synthesis, thus supplying the building blocks for the production of the hormone as well as the other body proteins necessary for the growth and development of red cells.

[®]The term "Erythropoietin" is used to designate the factor(s) found in plasma of animals made anemic by bleeding or other methods, which accelerates Erythropoiesis when such plasma is injected into assay animals.

The present investigation was concerned with the production of Histidine depletion and testing the efficacy of Erythropoietin and iron singly and in combination in such deficient animals and in animals on complete control diets.

PURPOSE OF STUDY

The experiment was designed to:

1. Demonstrate the effect of phenylhydrazinized sheep Erythropoietin in histidine deficient and normal rats.
2. Study the utilization of iron under the influence of Erythropoietin in histidine deficient and normal rats.
3. Elucidate the possible correlation of the particular amino acid to the suggested polypeptidic nature of Erythropoietin.

REVIEW OF LITERATURE

Significance of Erythropoietin in Erythropoiesis

The Humoral Theory. The concept of humoral control of Erythropoiesis has been given serious consideration since the original observations of Garnot and Deflandre (10) in 1906. In a typical experiment these investigators withdrew 30 cc. of blood from a rabbit, bled the animal again on the next day, and injected 9 cc. of the serum of that blood into a normal rabbit whose red blood cell count rose from 5.5 millions to 8 millions/cmm. They named this hypothetical substance in the anemic serum that induces the polycythemia "hemopoietin."

The contemporary state of affairs began with the experiments of Krundieck (41) in 1943, who reported that the serum of the rabbits rendered

severely anemic by repeated bleedings caused a reticulocytosis when injected into normal rabbits, the peak occurring on the third day of injection. Neither the hemoglobin nor the red cell count rose noticeably. Reissmann (57) in 1950 published convincing evidence of a circulating erythropoietic factor based on a classical experiment using parabiotic rats. His report that hyperplasia of bone-marrow developed in both partners of a parabiotic pair of rats - only one of which was subjected to hypoxia, renewed the interest in the existence of a circulating erythropoietic factor which serves to mediate the anoxic stimulus. In the same vein Grant (30) reported that nursing mice kept at low oxygen tension except when suckling their young secrete in milk a substance that causes polycythemia in nurslings. Grant and Root (31) also wrote an excellent review concerning the fundamental stimulus for Erythropoiesis in 1952, and strongly suggested a blood borne substance capable of stimulating Erythropoiesis.

Erslev (14) in 1953 pointed out the need for administering large quantities of plasma or serum in order to elicit an erythropoietic response in recipient animals.

An important advancement was made when Borsook and his coworkers (6) demonstrated an erythropoietic stimulating factor in the filtrate obtained from heat-denatured anemic rabbit plasma, after deproteinization by boiling for 10 minutes at pH 5.5.

Many investigators (24, 29, 28, 27, 40, 42, 50, 53, 56) reported similar observations subsequently. Stohlman and Breacher (62, 63) and Erslev (15) found plasma so treated to be ineffective.

In the last few years, Jacobson and his colleagues (38) have used increased incorporation of Fe^{59} into the blood to demonstrate an increase

in Erythropoietin in the blood of normal or hypophysectomized rats--and in rabbits with anemia due to hemorrhage. In order to demonstrate this effect it was necessary to increase greatly the sensitivity of the assay in rats or mice by such means as hypophysectomy, exposure for a number of days to 85 to 95 percent oxygen, making them polycythemic by the injection of red cells, or starvation. These animals have a decreased metabolic rate, and a marked decrease in tissue demand of oxygen--exists, without appreciable change in the number of circulating Erythrocytes. Thus a state of relative polycythemia developed in such animals which led to a depressed erythropoietic base line and increased responsiveness to anemic plasma.

Toha and coworkers (66) found that plasma from rabbits anemiated by bleeding produced a significant increase of the reticulocytes, red cells, and hemoglobin in the normal animal.

Underbjerg and his associates (68) demonstrated similar phenomena with plasma filtrate of anemiated bovines, which produced a reticulocytosis at an average of 283 percent, as compared to 69 percent in normal bovine plasma. Lowy and coworkers (44) produced a true polycythemia in normal adult rats at ordinary altitudes by injection of the filtrate of boiled plasma of rabbits made anemic with phenylhydrazine. They observed increases in red cell count, hematocrit and hemoglobin concentration, as well as a reticulocytosis and evidence in the bone marrow of stimulation of erythropoiesis. Gurney and his associates (32) demonstrated the presence of Erythropoietic stimulating factor in normal plasma in 1957.

As studies with experimental animals have progressed, increasing interest has been centered on the significance of these findings in human patients with various types of anemia and polycythemia. This phase, the study of human

patients has been aided tremendously by the demonstration (32) that boiled extracts of human plasma retain their erythropoietic-stimulating properties. These boiled extracts of human plasma and urine could be assayed using small amounts of plasma and urine in other animal species without detectable reactions due to foreign proteins.

Incontrovertible evidence from recent studies in different laboratories has thus been accumulated in the past few years, supporting the existence of a factor in the plasma of anemic donors that is capable of increasing erythropoiesis in normal animals. However the role of these factors commonly referred to as Erythropoietin (s) in the dynamic equilibrium of the erythron has not yet been defined clearly.

Fundamental Stimulus for Erythropoiesis. Oxygen transfer from the atmospheric air to the cytochrome system in the cells is accomplished by the integrated action of a number of organ systems. Impairment in the supply of oxygen to the cells is associated with compensatory changes in the functions of these systems; in the pulmonary vital capacity, in the cardiac output, in the production of red blood cells, and in the distribution of blood among the tissues. Undoubtedly the degree of tissue oxygenation must be influential in the mobilization of these compensatory mechanisms. They have shown that although the nutritional and hormonal environment, conditions the rate of the red cell production and hemoglobin synthesis, but does not constitute the primary Erythropoietic stimulus.

The role of arterial oxygen tension as the basic determinant of Erythropoietic activity had been widely confirmed by observations on experimental animals and human subjects. However Erslev (15) has demonstrated that a reduction in the concentration of the arterial blood, by itself, as

in dilution anemia induced by dextran, does not result in increased Erythropoiesis. The more important factor appears to be the tissue O_2 tension. The original theory that this was due to hypoxia of the bone marrow has also been disproved.

It has been conjectured that the Erythropoietic centre is located in some extra-medullary organ or cellular system and that it controls the red-blood cell production by releasing a certain amount of Erythropoietic factor into the general circulation. Since the principle function of Erythropoietic tissue is to synthesise, carry and protect oxygen carrying-hemoglobin, it appears most probable that the degree of tissue oxygenation not only influence but controls the red cell production.

Jacobson and his associates (39) developed a concept that it is the relation between the oxygen tension of the blood and the oxygen demand by the tissues that determines the rate of Erythropoiesis and provides a mechanism for the production of the factor. Thus it is explained that immediately after hypophysectomy an overall metabolic reduction occurs. As a result of this reduced metabolic requirement the hypophysectomised animal is more or less comparable to an animal that has been made polycythemic by red cell injections. The increased O_2 supply in such animals without any effect in the O_2 demand results in decreased erythropoiesis stemming from diminished elaboration of the erythropoietic stimulating factor(s). If this explanation is valid the same would be true of transfusion induced polycythemia or starvation or any other condition that produces a relative plethora of red cells. To support their hypothesis the Jacobson group induced these conditions experimentally in lab. animals and found that in all states there was a reduction in the rate of Erythropoiesis and the animals

gave an exaggerated response to the injection of anemic plasma. The hypothesis is also strengthened by the fact that in response to anemic anoxia produced by bleeding or by hemolysis induced by Phenylhydrazine, plasma erythropoietin levels are increased. In these circumstances the available oxygen supply is reduced without any appreciable alteration in the demand. Metabolic stimulants like dinitrophenol and triiodothyronine, act by increasing the O_2 demand by the tissues, and elevate the levels of Erythropoietin and RBC production. Such animals showed a decrease in the sensitivity to exogenous erythropoietin.

Increased sensitivity of the Erythropoietic tissue results when the levels of Erythropoietin and the rate of Erythropoiesis are reduced. Lowered response occurs when the levels are raised and the rate is augmented.

Stohlmann recently (64) called attention to situations in which enhanced Erythropoiesis does not appear to be associated with measurable hypoxia. It is conceivable that more than one mechanism operates in the production of RBC.

It is interesting to note in this connection that some members of the VIII group of the periodic table, like cobalt, increase the rate of Erythropoiesis. Mechanism of Erythropoietic stimulating action of cobalt is largely unknown. While it seems logical that cobalt may exert its effect by producing an anoxia directly in the sensitive organ that elaborates the hormone.

The following chart (65) in short delineates the nature of the fundamental stimulus and control of dynamic erythrocytic equilibrium.

Conditions	Effects
1 < Demand for oxygen while supply is normal (starvation, hypophysectomy).	< Erythropoiesis ²
> Supply of oxygen while demand is normal (Transfusion-induced Polycythemia, hyperoxia).	< Erythropoiesis ²
> Demand for oxygen while supply is normal (Dinitrophenol, Triiodothyronine)	> Erythropoiesis
< Supply of oxygen while demand is normal (Bleeding, phenylhydrazine-induced hemolysis). ³	> Erythropoiesis
1 >, < is increase or decrease respectively.	
2 Exaggerated response to Erythropoietin (anemic plasma).	
3. Standard procedure to obtain plasma with high erythropoietic activity in rats.	

Chemistry of Erythropoietin. The exact nature of Erythropoietin is still unsettled. There have been many discrepancies in the observations which resulted in diverse views on the possible chemical nature of the factor. However bulk of the evidence indicates that the humoral factor is polypeptidic in nature.

Slavn White et al. (61) employed boiled filtrate of plasma from phenylhydrazinized rabbit found the ESF to be nondializable, stable to high temperatures in weakly-acid (pH 5.5) or alkaline (pH 9) solution. The factor is however inactivated by heat in strong acid (pH 1) or basic (pH 13) solution. It is digested by pepsin, trypsin and chymotrypsin. It is not soluble in chloroform. From these observations the authors concluded that the factor may be polypeptidic in nature. They used Fe^{59} incorporation into red cells as a criterion for the assay of these materials.

Rambach et al. (56) employed the continuous flow electrophoresis as

a means of separating the active fraction, using plasma filtrates from rabbits rendered anemic by phenylhydrazine. The active fractions separated as O₂ globulins as determined by paper strip electrophoresis. It was also reported that the factor was dializable, is precipitated by 75 percent saturated ammonium sulphate, and is non-extractable from the lyophilized state by repeated washing with ether. It was concluded that the factor belongs to the class of mucoproteins.

In more recent experiments (54) these investigators described the fractionation of the factor in boiled plasma filtrates in a diethylaminoethyl cellulose ion-exchange column. Its behavior indicated that it has a low isoelectric point probably below (pH 3.9). Ultra-centrifuge data suggest a molecular weight in the vicinity of 10,000. Paper electrophoretic studies show that the more purified material migrates as a single component with a mobility between alpha and alpha₂-globulin. The factor has the following composition:

Protein	69.3 percent
Sialic Acid	15.6 "
Hexose	7.7 "
Glucosamine	10.0 "

The authors concluded that the factor is a low molecular weight acid glycoprotein, erythropoietically active in daily doses of 10 μ gm per day.

Gley (21) claims to have extracted two fractions using acetone treated anemic horse serum. One called hematostimulin is water soluble. The second factor, called 'hematopoietin' is soluble in organic solvents (alcohol, chloroform, ether, and acetone) and it displays an infra red absorption pattern

similar to that of 11-dehydrocorticosterone or 11-desoxycortisone. Consequently hemopoietin was said to be a mono-alcoholic Triketonic steroid. Increase in the number of reticulocytes was used as a criterion for assay of the fractions.

Hogdson and his collaborators (36) support the view that Erythropoietic stimulating activity is retained in boiled filtrates of plasma from phenylhydrazinized rabbits as judged by their ability to augment plasma iron turnover and Fe^{59} incorporation into the RBC of normal rabbits. The factor in boiled plasma is admitted to be non-dialyzable, and flocculates on treatment with saturated ammonium sulphate at pH 4.5 (35). Active fractions could be obtained from both urine and boiled plasma of anemic rabbits by alcoholic precipitation techniques. Chemical analysis of the active fractions indicated that they contained more than 10 mg neutral sugar (orcinol) per mg protein (Biuret). The latter findings support the mucoprotein nature of Erythropoietic stimulating factor.

Erslev and Lavistes (17) showed that the factor is non-dialyzable, is precipitable from whole serum or plasma with 50 percent ammonium sulphate, and is associated with the alpha-and beta-globulin fractions.

Brecher (9) believed that the Erythropoietic factor was a mucoprotein, that is, a substance with an electrophoretic mobility between alpha-1 and alpha-2 globulin.

Linman and Bethell (42) supported the view that this factor may be similar to or identical with batyl alcohol, claimed to stimulate Erythropoiesis in rats. In fact they reported that daily injections for 20 days of 12.5 - 25.0 mg of batyl alcohol to normal rats resulted in an Erythropoietic response similar to that evoked by their 'anemic' plasma filtrates.

Brecher (9) stated that the dose of batyl alcohol used by Linman and Bethel was far more than could possibly be present in any plasma.

More recently Evenstein et al. are reported to have administered synthetic batyl alcohol in doses ranging from 1 to 5 mg daily to rats. No significant changes were observed in the RBC, reticulocyte, hemoglobin, and hematocrit values over a period of 1-4 weeks (25).

Gorden et al. (26, 70, 23) found that strong erythropoietic activity is present in boiled filtrates of plasma from subjects with Cooley's and hypoplastic anemias. The unconcentrated urine of these patients contains considerable quantities of the factor and showed that the urinary erythropoietic factor may be absorbed in kaolin in acid pH and eluted in several pHs in the alkaline range. The greatest activity is obtained in the 1M NH_4OH eluate, in which a purification of 230 X has been achieved. The addition of Toluidine blue to this eluate results in strong metachromasia. UV absorption spectra studies indicate that the biologically active fractions absorb considerable light at 280 m μ . These properties support the contention that the Erythropoietic stimulating factor is a mucoprotein or an associated substance. Biological activity was destroyed with proteolytic enzymes, trypsin and chymotrypsin.

Borsook et al. (6) are sometimes erroneously credited with stating that Erythropoietin is non-proteinaceous, although they refrained from making any dogmatic statement concerning the nature of Erythropoietin, and mention was made in their discussion that some protein, precipitable with 7 percent TCA, remained in the boiled plasma filtrates.

The plasma was precipitated with 80 percent saturated ammonium sulphate. Active principle was found to be dissolved in the filtrate. Ammonium sulphate

was removed by dialysis. The solution was then concentrated by freeze drying to 2 percent protein, after which ammonium sulphate was added to give a 70 percent saturation. The filtrate containing the activity was dialyzed salt free, and lyophilized to dryness. Three hundred milligrams of active lyophilizate was collected from approximately 3 litres of original whole plasma.

Electrophoretic techniques revealed the active material as moving faster than alpha globulins and slightly slower than alpha₂ globulins. The Erythropoietic activity of the potent plasma filtrates and fractions was always associated with protein. Activity was lost after treatment with trypsin or chymotrypsin and after exposure to pH 1.6 at 36° C for 16 hrs.

Grant et al. (31) have partially purified the active fractions in boiled filtrates of plasma from rabbits treated with phenylhydrazine, by alcohol precipitation techniques. Activity was precipitated between 60-80 percent ethanol. The precipitate was dissolved in water and reprecipitated at pH 5.5 with the same percentages of ethanol. Concentration obtained was approximately 3500 times that of original plasma solids. The active fractions were non species specific, heat stable, nondialyzable, non-ultrafiltrable, non-antigenic, and had 4 percent nitrogen and were digestible by proteolytic enzymes and ineffective orally. A molecular weight of approximately 40,000 was estimated. Rate of incorporation of radio iron into the RBC, reticulocyte and hematocrit values were the parameters employed for activity.

Thus it may be construed that Erythropoietic stimulating factor moiety derived from both plasma and urinary sources is protein in nature. Findings are not inconsistent with the contention that the ESF is a small molecular

weight glycoprotein with the possibility of an associated active polypeptide grouping. The possibility that Erythropoietin is a complex system comprising of multiple factors is not ruled out. Different methods of demonstrating the erythropoietic stimulation, source and species differences, various techniques utilized in the preparation of extracts and the possibility of the existence of more than one factor, were some of the explanations forwarded for the discrepancies.

Erythropoietin in Iron Metabolism. The experimental results presented by Hodgson et al. (34) show that plasma iron turnover in animals with normal Erythropoiesis is independent of plasma iron concentration over wide ranges. This suggests that the bone marrow works with iron concentration in the region of excess substrate in relation to enzymes involved in the incorporation of iron into hemoglobin. This relationship between plasma iron concentration and turnover in rabbits confirms the findings of Bothwell et al. (7) and demonstrates that the rate of disappearance of Fe^{59} from plasma, used alone, is not a valid criterion for hemoglobin synthesis.

Hodgson et al. (35) in their experiments found that extracts of boiled plasma of phenylhydrazine treated donors produce a definite increase in plasma iron turnover and a drop in plasma iron. The increase of percentage Fe^{59} appearing in Erythrocytes is striking, an effect that is not apparent in normal animals. Extracts of boiled plasma from normal rabbits show no effect. They also showed the effects of varying doses of Erythropoietin on the percentage Fe^{59} in Erythrocytes and in liver, 24 hours after tracer injection. In starved rats an increase of Erythrocyte Fe^{59} and a drop of liver Fe^{59} is produced.

The percentage of Fe^{59} in Erythrocytes is found to be proportional to

log dose while the Erythrocyte Fe^{59} liver Fe^{59} ratio is proportional to dose of Erythropoietin. This finding is significant in the sense it permits quantitative bioassay. Thus the effects of extracts of plasma obtained from anemic rabbits on iron metabolism of normal and starved animals show that plasma of anemic animals not only contains factors that influence Erythropoiesis, but also affects iron metabolism per se.

Clinical Significance of Erythropoietin. One of the ultimate aims of studies on Erythropoietin is to develop a preparation, ideally the crystalline material, which could be used in the treatment of blood dyscrasias. The factor might find application in certain forms of 'secondary anemias' such as those associated with non-detectable amounts of Erythropoietin in plasma. Erythropoietin might also be expected to stimulate Erythropoiesis in anemic states associated with the first few weeks of life in normal and, especially, premature infants. Here Erythropoiesis, and probably the quantity of circulating Erythropoietin have been reduced as a result of the relative Polycythemia present at birth. In fact relatively few attempts have been made to test its effectiveness. This was probably due to an unavailability of an abundant source of Erythropoietin. More recently Swanson (65) has demonstrated an abundant source of Erythropoietin in the bovine plasma.

Ullrey et al. (67) have used intramuscular injection of Erythropoietin in pigs at 2 and 5 days of age upon the hemopoietic response to intramuscular iron-dextran injections. They found that in one litter which was anemic at 2 days, Erythropoietin produced a slight but statistically insignificant hemopoietic response. All other litters were more nearly normal at two days and Erythropoietin injections did not produce any observable change in the criteria measured. Sporadic reports regarding the use of

clinical states in man give results of a positive and negative nature. Five-3 daily infusions of approximately 300 cc. of plasma from bled human subjects resulted in Erythropoietic stimulation in two animals with congenital hypoplastic anemia. In both recipients the reticulocyte and marrow-response was greater than that obtained with any previous treatment (25). On the other hand Luhby et al. (25) have found that a total of 735 cc. of Cooley's plasma, given intravenously over a period of nine days, failed to stimulate RBC formation in a child with chronic hypoplastic anemia. Obviously more work is required.

Significance of Proteins and Amino Acids in Erythropoiesis

General Considerations. Most of our knowledge concerning the requirements of red cell production have been derived from evidence gained by observing the effects of dietary restrictions. Details concerning the building stones and the manner in which they are put together are meagre. The development of anemia when the diet is abnormally low in protein is fully recognized. Protein deficiency has been shown to lead to decreased formation of hemoglobin, red blood cells, and plasma proteins (69, 45, 46). This is quite understandable when one considers the size, complexity and amino acid content of the globin fraction of the hemoglobin molecule. Rats fed a diet low in protein but adequate in all other aspects develop anemia, which responds to protein therapy (11). The anemia is characterized by a distinctly sub-normal hemoglobin content of the blood and a normal erythrocyte count.

Bethell (5) suggested that the anemia which occurs in pregnant women and is characterized by a normal color index and by red cells of normal or increased volume is due to protein deficiency.

Amino Acids. Studies on the effects of individual amino acids have been made possible only after the isolation, purification and manufacture of synthetic amino acids.

It is now generally accepted that at least ten are indispensable for rats. These are Leucine, Isoleucine, Threonine, Methionine, Phenylalanine, Tryptophane, Lysine, Histidine, Arginine and Valine.

Studies on the role of amino acids in blood formation have been sporadic but significant. The relationship of each individual amino acid to Erythropoiesis is however not yet established. The effect of administration of various amino acids on the hemoglobin content of rats maintained on a low protein diet consisting of 3.5 percent lactalbumin have been studied by Orten and Orten (51). No consistent, sustained, increase in the values occurred following supplementation with any of the ten essential amino acids. They interpreted these results as evidence that no single amino acid can be regarded as a "key" amino acid in hemoglobin synthesis.

Whipple and Robschiet-Robbins (69) have shown that certain amino acids given to dogs made anemic by repeated hemorrhage cause an increase in new hemoglobin production over the basal level. Threonine, glycine, glutamic acid, aspartic acid, cystine histidine, phenylalanine and proline caused an increase in hemoglobin output of 23 to 26 gms above the control levels for a two week period. Leucine methionine, lysine and tryphophane caused an average increase of 20 gms and tyrosine, valine, isoleucine, arginine and hydroxyproline increased hemoglobin to output 10 to 17 gms. over the control level for the two week period. There was no correlation between the quantity of an amino acid found in globin and its hemoglobin regenerating capacity.

In dogs, both anemic and hypoproteinemic, Robschiet-Robbins and his associates (59) have shown that globin can be readily formed from pure amino

acid mixture, plasma, serum digests, casein, hemoglobin given l/P, and hemoglobin digests given by mouth.

A slight anemia has been reported in growing rats maintained on a diet where protein was furnished by acid hydrolysed casein. The blood could return to normal when Tryptophan was administered (1). The bone marrow is not particularly hyperplastic. If dogs are rendered anemic by bleeding, accelerated hemoglobin formation takes place when Tryptophan is administered (69). Tryptophane leads to prompt regeneration of plasma regeneration of plasma proteins in hypoproteinemic plasmapheresed dogs (47).

Slight through definite decreases in red blood cells and hemoglobin content are seen as a result of lysine deficiency (8).

There is evidence that Arginine is necessary for plasma protein formation in the protein depleted dog, (47) and for hemoglobin formation in dogs rendered anemic by bleeding (69).

When rats were placed on diets deficient in phenylalanine, an average reduction in hemoglobin from 14.7 gms. to 9.9 gms. and a slight fall in plasma protein concentration from 5.58 gm. percent to 4.71 gm. percent are described (48). In dogs phenylalanine appears to be necessary for plasma protein production (47).

Leucine has been shown to be of importance in the corrections of red blood cell and hemoglobin regeneration after severe hemorrhage (60). This amino acid is also necessary for hemoglobin synthesis (69) in the dog.

The significance of Isoleucine for hemoglobin and plasma protein production in rats, depleted by bleeding was shown by Sebrell and McDanniel (60). Isoleucine has been shown to be necessary for hemoglobin formation in the dog (69).

Whipple and Robschiet-Robbins (69) also showed the necessity of threonine for hemoglobin formation. When methionine deficiency was produced in the growing rat by utilizing a mixture of purified amino acids, a retardation of growth, and a reduction in the hemoglobin and plasma protein production was seen. Methionine has been shown to be necessary for hemoglobin formation in the dog (69). Further studies in rats (22) and dogs (58) reported reduction in hemoglobin content, red blood cell count, and plasma protein concentrations. When groups of rats were placed on diets whose protein content was supplied by mixtures of crystalline amino acids containing varying amounts of methionine and cystine, the following differences were noted. When inadequate amounts of methionine but no cystine were added to the diet, the animals lived a little longer, but succumbed due to liver necrosis even though no anemia or hypoproteinemia developed. When methionine intake was restricted but dietary cystine was adequate no liver necrosis was observed although during life, disturbances in hemoglobin and plasma protein formation developed. Thus methionine deficiency appeared to interfere with hemoglobin and plasma protein formation, while lack of cystine resulted in liver necrosis (22).

Sebrell and McDanniel (60) demonstrated the need of valine for hemoglobin and red cell regeneration in the rat made anemic by hemorrhage. Valine has also been shown to be necessary for plasma protein and hemoglobin formation in the dog.

Animals acutely deficient in histidine develop a moderate anemia; hemoglobin values falling from the normal of 14 gms. to about 10 gms. percent (49). Histidine promotes hemoglobin formation in dogs made anemic by bleeding (47). Histidine is important in the sense that it furnishes the

seat of attachment for one of the coordination valencies of the iron of hemoglobin. The iron atom in Heme has a coordination valence of six, four of which are attached to the 4 pyrrol nitrogens, another bond is thought to be attached to the nitrogen of the imidazole group of histidine in the globin and the sixth coordination link is held reversibly by an oxygen molecule.

The amino acid composition of hemoglobin is now fully known. Thus it has been shown that with the possible exception of Arginine all of the amino acids are required for hemopoiesis in the rat. In the order of decreasing importance they are Histidine, Valine, Leucine, Isoleucine, Lysine, Arginine, Methionine, Tryptophane (71).

MATERIALS AND METHODS

Source Material

Source of Erythropoietin: Sheep Erythropoietin^{*} obtained from phenylhydrazine treated animals was employed in this experiment. It was a desiccated sample of plasma and each vial made up to 20 ml. contained 200 units of the active fraction.

Source of Iron: Iron dextran complex, Armidexan^{*} constituted the source of iron. Each ml. of "Armidexan" contained 50 mg. of elemental iron.

^{*}Armour Veterinary Laboratories, Division of Armour and Company, Kankakee, Illinois.

Preparation of Assay Animals

Young virgin female rats of the Sprague Dawley strain were used in the experiment. The body weights ranged from 115 to 178 gms. The rats were then randomly assigned to eight groups with seven rats in each group, as per the design of the experiment in Table 1.

All rats were then maintained on a basal nitrogen free diet for the first seven days. A provision of eight gms. per rat per day was made for the computation of the rations. The basal "nitrogen free" diet had a percentage composition shown in Table 2. At the end of the seven day period Groups I, II, III, and IV were placed on the experimental diet and the other four groups on complete control diet until the end of the experiment. The composition of the control diet is shown in Table 3. This purified synthetic diet was mixed with histidine deficiency to obtain the experimental diet.

Seven days after changing to the above diets, respective groups of rats were given intra-peritoneal injections of Erythropoietin and iron dextran for two days consecutively. The exact treatment of the groups and the total dose of Erythropoietin and iron received by the rats is delineated in Table 4. On the third day after starting the injections, post-experimental weights were recorded. Approximately 2-4 cc of blood was collected into heparinized syringes by cardiac puncture from each rat and placed in tubes containing dried heller's and Paul's anticoagulant.

The criteria for effectiveness employed were increases in the hemoglobin and hematocrit levels. Hemoglobin determinations were made by acid hematin method employing spectrophotometer.* Hematocrit values were determined by

*B. Cohen and A. H. Smith, Jour. Biochem. 39:489, (1919).

Table 1. Experimental design.

Group	Number of rats	Description
1	7	Histidine deficient
2	7	Histidine deficient + Erythropoietin
3	7	Histidine deficient + iron
4	7	Histidine deficient + Erythropoietin + iron
5	7	Complete control ration
6	7	Complete ration + Erythropoietin
7	7	Complete ration + iron
8	7	Complete ration + Erythropoietin + iron

Table 2. Basal nitrogen free diet.*

Composition

Ingredient	:	Percent
Cerelose		15.0
Dextrin		60.8
Crisco		14.0
Mazola		2.0
Cellulose		2.0
Salt Mix**		4.0
Vitamins (2.2 gm/kilo vit)		2.2
A		4.5
D		.2
E		5.0
C		45.0
Inositol		5.0
Choline Chloride		75.0
k		2.25
Paba		5.0
Niacin		4.5
Pyridoxin		1.0
Riboflavin		1.0
Thiamin		1.0
Ca. Pant.		3.0
Biotin		0.02
Folic Acid		0.09
B ₁₂		0.002

*M. Richcigl, Jr., et al., Jour. Biol. Chem. 231:829, (1958).

**D. M. Hagsted, et al., Jour. Biol. Chem. 138:459, (1941).

Table 3. Composition of control diets.¹

Ingredient	Percent
DL-Alanine	0.346
L-Arginine-HCl	0.816
DL-Aspartic Acid	0.558
L-Cystine	0.334
L-Glutamic Acid	5.664
Glycine	1.588
L-Histidine-HCl-H ₂ O	0.676
DL-Isoleucine	1.420
L-Lysine-HCl	1.368
DL-Methionine	0.468
DL-Phenylalanine	0.840
L-Proline	0.392
DL-Serine	0.732
L-Tyrosine	0.502
Threonine	*
Tryptophan	**
Valine	***
Salts ¹ ****	4.00
Corn oil*****	5.00
Vitamins*****	+
Starch***** to total 100	-

* Contained 1.020% of DL-threonine.

** Contained 0.216% of L-tryptophan.

*** Contained 1.464% of DL-Valine.

**** Hegsted, et al.: Jour. Biol. Chem. 138:459, 1941.

***** Masola, Corn Products Refining Company, New York.

***** Vitamins: in protein to starch in mg/100 gm of diet; Thiamine - 1 mg; Riboflavin - 1 mg; Pyridoxine - HCl 1 mg; Nicotinic acid - 10 mg; i-inositol - 20 mg; PABA - 20 mg; Folic acid - 0.1 mg; Biotin - 0.1 mg; Menadione - 2.0 mg; Ca pantothenate - 4 mg; choline Cl - 150 mg; B₁₂ - 0.004 mg. Each rat received weekly 2 mg α-tocopherol acetate dissolved in 2 drops of corn oil. Vit. A and D (25 ml corn oil with 2,000 I.U. - Vit. A, 200 I.U. - Vit. D).

***** Corn Products Refining Company, New York.

¹F. N. Hepburn, W. K. Calhoun, and W. B. Bradley: A Growth Response of rats to Glutamic Acid when Fed an Amino Acid Diet. Jour. Nutr. 72 (2): 163-168, 1960. (American Institute of Baking, Chicago.)

Table 4. Treatment of the groups with iron and Erythropoietin.

Group	: Erythropoietin : cc./day	: Iron : mg/day	: Total dose of Erythropoietin : of iron
Histidine deficient	-	-	-
Histidine deficient + Erythropoietin	2	-	4
Histidine deficient + Iron	-	50	-
Histidine deficient + Iron + Erythropoietin	2	50	4
Complete control ration	-	-	-
Complete ration + Erythropoietin	2	-	4
Complete ration + iron	-	50	-
Complete ration + iron + Erythropoietin	2	50	4

microhematocrit technique.

RESULTS

The results of the experiment on the effect of sheep erythropoietin with and without iron on histidine deficient and normal rats and the data on each individual rat of the eight groups as outlined in Table 1 is presented in Tables 5 to 12.

The average of the hematocrit and hemoglobin values was then delineated in Table 13.

Figs. 1 and 2 summarize the results of the effect of phenylhydrazinized sheep erythropoietin and iron dextran injections on hematocrit and hemoglobin values.

The results summarized in Figs. 1 and 2 show that histidine deficiency lowers both the hematocrit and hemoglobin values in rats. Intraperitoneal erythropoietin injections increase both the hemoglobin and hematocrit values considerably above the control levels in histidine deficient rats. Injections of intraperitoneal iron dextran increased the hemoglobin levels but have little effect on hematocrit values. The results with the injections of erythropoietin with iron dextran show a notable increase in hemoglobin levels. The combination also had a considerable effect on hematocrit values.

Erythropoietin in normal animals produces a slight increase in hemoglobin levels and hematocrit levels. Iron dextran injections in normal rats increase notably both the hemoglobin and hematocrit values.

Results with the injection of erythropoietin in combination with iron, show a negative effect on the hemoglobin and hematocrit levels in normal animals.

Table 5. Effect of histidine depletion on Erythropoiesis in female Sprague Dawley rats.

Animal number	Weight in grams	Amount of Erythropoietin injected	Amount of iron injected	Hemoglobin gm/100 cc	Hematocrit %
1	144	106		14.7	42.3
2	146	113		13.5	38.8
3	153	108		10.2	32.0
4	149	112		13.2	42.0
5	140	118		—	33.0
6	178	132		12.2	33.2
7	162	118		13.5	45.6

Table 6. Effect of sheep erythropoietin on histidine deficient female Sprague Dawley rats.

Animal number	Weight in grams	Amount of Erythropoietin injected	Amount of elemental iron injected	Hemoglobin	Hematocrit
	Pre-expt. Post-expt.	cc.	mg.	gm/100 cc	%
1	150 116	4		12.0	45.5
2	150 110	4		15.1	46.0
3	170 124	4		14.3	43.0
4	150 112	4		14.7	43.0
5	134 114	4		12.6	38.8
6	162 122	4		15.1	40.6
7	156 103	4		12.0	28.0

Table 7. Effect of iron on Erythropoiesis in histidine deficient female Sprague Dawley rats.

Animal number	Weight in grams	Pre-expt. Post-expt.	Erythropoietin injected	Amount injected	cc.	Erythropoietin injected	Amount injected	Hemoglobin gm/100 cc	Hematocrit %
1	168	128					100	15.7	44.0
2	156	102					100	—	—
3	146	108					100	15.8	40.1
4	154	128					100	14.7	39.0
5	138	112					100	12.6	33.0
6	150	—					100	—	—
7	156	118					100	—	—

Table 8. Effect of iron and sheep Erythropoietin on Erythropoiesis in histidine deficient female Sprague Dawley rats.

Animal number	Weight in grams : Pre-expt. :	Weight in grams : Post-expt. :	Amount : Erythropoietin : injected : cc.	Amount : elemental : iron : injected : mg.	Hemoglobin : gm/100 cc.	Hematocrit : %
1	152	124	4	100	13.25	35.8
2	126	—	4	100	—	—
3	138	120	4	100	15.0	39.5
4	148	116	4	100	14.9	40.1
5	126	104	4	100	15.25	40.9
6	162	130	4	100	—	—
7	115	100	4	100	19.7	56.5

Table 9. Control group on complete rations.

Animal number	Weight in grams	Amount of Erythropoietin injected	Amount of iron injected	Hemoglobin gm/100 cc	Hematocrit %
	Pre-exp't.	Post-exp't.	cc.	mg.	
1	146	130		14.5	41.0
2	162	124		15.7	45.5
3	138	110		15.0	41.3
4	142	110		14.3	40.9
5	146	112		12.5	—
6	150	120		13.5	35.0
7	162	128		12.5	33.0

Table 10. Effect of sheep Erythropoietin on Erythropoiesis in normal female Sprague Dawley rats.

Animal number	Weight in grams	Pre-expt.:	Post-expt.:	Amount Erythropoietin injected	Amount elemental iron injected	Hemoglobin gm/100 cc	Hematocrit %
1	144	108	4	4	---	34.5	
2	162	120	4	4	13.8	42.0	
3	136	108	4	4	15.1	43.6	
4	138	96	4	4	16.2	43.0	
5	140	102	4	4	---	26.8	
6	174	126	4	4	15.4	42.0	
7	136	100	4	4	14.2	41.0	

Table 11. Effect of iron on Erythropoiesis in normal female Sprague Dewley rats.

Animal number	Weight in grams	Pre-expt.	Post-expt.	Amount : Erythropoietin : injected	Amount : elemental : iron	Hemoglobin	Hematocrit
:	:	:	:	: cc.	: mg.	: gm/100 cc	: %
1	150	105		100	100	17.1	45.5
2	160	104		100	100	18.1	45.2
3	154	--		100	100	--	--
4	160	120		100	100	14.0	37.0
5	166	114		100	100	14.9	38.5
6	152	105		100	100	15.7	40.1
7	150	110		100	100	16.0	42.6

Table 12. Effect of iron and sheep Erythropoietin on Erythropoiesis in normal female Sprague Dawley rats.

Animal number	Weight in grams	Pre-expt.:	Post-expt.:	Amount Erythropoietin injected	Amount elemental iron injected	Hemoglobin gm/100 cc.	Hematocrit %
1	116		114	4	100	—	—
2	134		104	4	100	13.1	35.5
3	124		102	4	100	12.5	34.0
4	160		127	4	100	11.4	31.5
5	138		120	4	100	13.7	42.0
6	152		102	4	100	16.2	46.0
7	126		104	4	100	12.7	39.8

Table 13. Effect of Erythropoietin and iron dextran on erythropoiesis in normal and histidine deficient rats.

No. of rats	Erythropoietin : cc.	Iron : mg.	MT.	Hb.	Remarks
1. Histidine Deficient					
6	-	-	38.4	12.9	-
2. Histidine Deficient + Erythropoietin					
7	4	-	42.6	14.05	-
3. Histidine Deficient + Iron					
4	-	100	39.3	14.7	-
4. Histidine Deficient + Erythropoietin + Iron					
5	4	100	42.3	15.62	-
5. Complete Control Ration					
6	-	-	39.4	14.0	-
6. Complete Ration + Erythropoietin					
5	4	-	38.9	14.96	-
7. Complete Ration + Iron					
6	-	100	41.4	15.99	-
8. Complete Ration + Erythropoietin + Iron					
6	4	100	37.3	13.29	-

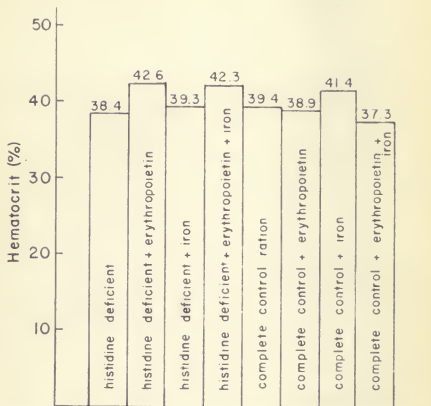


Fig.1. Effect of sheep erythropoietin and iron dextran on the hematocrit values of histidine deficient and normal rats.

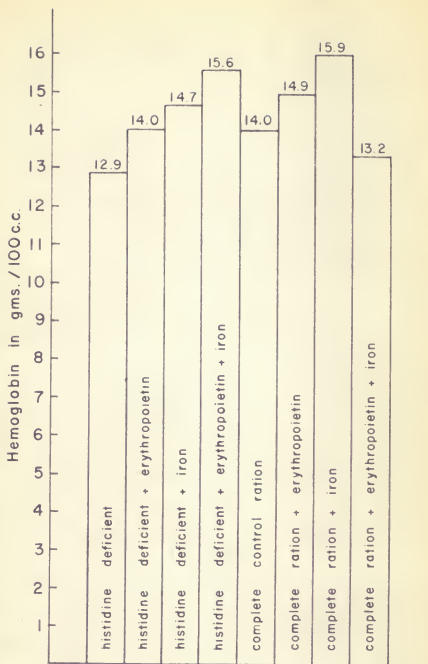


Fig. 2. Effect of sheep erythropoietin and iron dextran on the hemoglobin values of histidine deficient and normal rats.

DISCUSSION

The observations on histidine deficient rats clearly show that there is a notable decrease in the rate of Erythropoiesis as reflected by low hemoglobin and hematocrit levels. Further the histidine depleted animals responded to exogenous Erythropoietin in an exaggerated manner indicating that in case of histidine depletion the depression of Erythropoiesis probably reflects low Erythropoietin titres. The possibility that lack of precursors for the production of Erythropoietin may affect its levels is a factor that cannot be denied, and needs further exploration.

Simultaneous study of the effect of iron and Erythropoietin in histidine deficient rats shows that Erythropoietin injection increases iron uptake by marrow as reflected by increase in hemoglobin levels. It is interesting in this connection to note that Hodgson et al. (36) working with starved rats found a significant linear correlation between Erythrocyte Fe^{59} and Log dose, and Erythrocyte/liver Fe^{59} ratio and dose of hemopoietin.

Analyzing the data obtained in normal rats some interesting conclusions can be drawn with respect to the behavior of Erythropoietin in normal animals. Thus the hemoglobin levels in the normal rats seem to correspond to the hemoglobin levels of histidine deficient rats which received 40 units (4 cc) of Erythropoietin. The normal animals which received the same dose of Erythropoietin showed an average hematocrit value of 38.9 while the histidine deficient animals under the influence of Erythropoietin had an average hematocrit value of 42.6. The gain in sensitivity obtained in histidine deficient animals is manifest.

The results obtained with the simultaneous use of iron and Erythropoietin in normal animals showed a deleterious effect on Erythropoiesis which cannot

at present be fully explained. Hodgson et al. (34) pointed out that plasma from bled animals appears to contain a mixture of substances, some of which lower plasma iron and possibly depress Erythropoiesis and another that stimulates hemoglobin synthesis. The role of Erythropoietin in relation to incorporation of iron into cells of the Erythron and cells other than those of the Erythron presents itself for further vista of investigations.

The evidence so far presented by various laboratories points to the protein or polypeptidic nature of Erythropoietin (s) and suggests that the factor (s) may be classified as a mucoprotein. Whether they are the same remains to be established.

A possible correlation between histidine and the polypeptidic structure of Erythropoietin has been postulated on the basis of increased sensitivity of histidine deficient rats to exogenous Erythropoietin, and the assumption that the lowered titre of Erythropoietin in histidine depleted rats may be due to the lack of precursors for the production of Erythropoietin.

These studies may serve as preliminary observations in this direction and clear the course of future experimentation.

SUMMARY

Fifty-six virgin female Sprague Dawley rats were utilized in this experiment to determine the effect of intraperitoneal injection of phenylhydrazinised sheep erythropoietin and iron dextran upon the hemopoietic response of normal and histidine deficient rats. Depressed Erythropoiesis in histidine deficient animals was manifested by low hemoglobin and hematocrit levels. Injections of Erythropoietin reversed these effects and raised both the hemoglobin and hematocrit values above the control levels. The sensitivity of histidine deficient animals to exogenous Erythropoietin was thus

evident.

Results of the study with the simultaneous injections of Erythropoietin and iron dextran in histidine deficient rats have shown an increased utilization of iron by marrow cells as reflected by a notable increase in hemoglobin levels.

The normal animals gave a partial response to Erythropoietin. Erythropoietin and iron dextran combination had a deleterious effect on Erythropoiesis.

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EFFICACY OF SHEEP ERYTHROPOIETIN IN HISTIDINE
DEFICIENT RATS

by

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AN ABSTRACT OF A MASTER'S THESIS

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MASTER OF SCIENCE

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1961

It has been shown by many investigators that protein depletion results in a reduction in the rate of Erythropoiesis. The response of protein depleted rats to such stimuli as cobalt and bleeding reflects that the anemia of protein depletion is mediated by Erythropoietin (s), the factor (s) concerned with the dynamic equilibrium of the Erythron. It is believed that the reduction in the rate of Erythropoiesis in animals fed diets deficient in essential amino acids is in part secondary to the reduced production of Erythropoietin.

The experiment was designed to study the effects of phenylhydrazinized sheep Erythropoietin in histidine deficient and normal rats. The procedure was designed also to study the utilization of iron, under the influence of Erythropoietin in both normal and histidine deficient animals.

A desicated sample of phenylhydrazinized sheep Erythropoietin, and injectable iron dextran complex were utilized for testing the efficacy. Virgin female Sprague Dawley rats were employed for the assay. Eight groups of rats were placed on special diets. The experimental diet was devoid of histidine and the control animals received the same diet with added histidine. The rats (in groups of seven) were then given on two consecutive days, intraperitoneal injections of Erythropoietin and iron dextran. On the day following the second injection, samples of blood were taken by cardiac puncture. The criteria for the efficacy measured were hemoglobin and hematocrit determinations.

The data revealed that Erythropoiesis was depressed in histidine deficient rats as judged by hemoglobin and hematocrit values. When the histidine deficient animals were given Erythropoietin their Erythropoietic rate was elevated. The animals manifested an increased sensitivity to

exogenous Erythropoietin. Normal animals gave a partial response to Erythropoietin. Increased utilization of iron under the influence of Erythropoietin was shown in histidine deficient animals, with a lowered rate of Erythropoiesis. The combination in the normal animals had a deleterious effect on Erythropoiesis.

The experiment was delineated to further the knowledge concerning the Erythropoietic stimulating substance. Erythropoietin apparently seems to be mainly concerned with the process of multiplication in the phenomenon of Erythropoiesis. Observations strongly suggest the possibility that lack of precursors for the building up of Erythropoietin in the form of essential amino acids, may result in lowered Erythropoietic titres, and reduced rate of Erythropoiesis. A correlation between the amino acid histidine and the suggested polypeptidic nature of Erythropoietin was postulated on the basis of increased sensitivity of histidine deficient rats to exogenous Erythropoietin.

Further investigations in this direction are needed. The observations made here may serve to clear the future course of experimentation.