

THE EFFICACY OF BOVINE ERYTHROPOIETIN IN THE RAT
FED A VALINE DEFICIENT RATION

by

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INTRODUCTION

Recognition of the importance of dietary protein in red cell production is not recent. Whipple (98) began his classical experiments in dynamics of protein metabolism and hemoglobin formation in 1918, when he described continued but reduced hemoglobin formation during starvation in anemic dogs. Orten and Smith (66), in 1937, reported the appearance of hypochromic anemia in rats, subjected to chronic protein deficiency. Similar observations have been reported by Orten and Orten (67) and Metcalf *et al.* (61). However, with the isolation, purification and manufacture of synthetic amino acids, it has been made possible to evaluate the physiological effects of individual amino acids, on various processes in a living organism. Studies on the effects of various amino acids on the process of erythropoiesis in rats are meager. Sebrell and McDaniel (84) have reported the effects of diets deficient in a single essential amino acid in rats, depleted of blood protein and formed elements of the blood, by multiple bleeding, while receiving a protein-free diet. They found that each amino acid deficiency, except that of arginine, had some adverse effect on blood regeneration but these effects were quite variable. They reported that valine deficiency had a significant effect on the recovery of red cells. Benditt *et al.* (3), while using synthetic amino acid and using a diet lacking valine, reported that there is no significant gain in serum protein and in production of erythrocytes.

The experiments reported hereunder were designed to investigate the role of valine in the production of erythrocyte stimulating factor and to study the action of bovine erythrocyte stimulating factor in the process of

erythropoiesis, when administered alone and in combination with iron.

REVIEW OF LITERATURE

Role of Proteins in Erythropoiesis

General considerations. Whipple (98) began his classical experiments in the dynamics of protein metabolism and hemoglobin formation, in 1918, when he described continued but reduced hemoglobin formation during starvation in dogs. Orten and Smith (66), in 1937, reported the appearance of hypochromic anemia in rats, subjected to chronic protein deficiency. Orten and Orten (67) have shown that the anemia secondary to protein deficiency is reversible. Metcalf et al. (61) have reported anemia, in rats, fed a low protein diet. This condition is associated with hemoconcentration and a significant decrease in total circulating hemoglobin. McGay (60) reported that the rate of blood regeneration in rats, made anemic by repeated hemorrhages, is accelerated by an adequate dietary intake of proteins.

Role of Amino Acids in Erythropoiesis. Benditt et al. (3), while studying the influence of various amino acids on the fabrication of serum proteins and of erythrocytes reported that protein depleted rats can synthesize new plasma proteins and erythrocytes on a diet in which the sole source of amino nitrogen is a mixture of crystalline amino acids which includes tryptophan, lysine, methionine, histidine, threonine, valine, leucine, isoleucine, phenyl alanine and arginine in relative proportion equivalent to that found by chemical analysis of casein, the extent of such synthesis is being comparable to that produced by a diet containing casein at an equivalent nitrogen level. With a synthetic diet identical to the above

but lacking one of the above mentioned amino acids other than arginine, there is poor diet consumption and no significant gain in serum protein, just as with the complete absence of protein from the diet. The same is true concerning the production of erythrocytes. Frazier et al. (30) while studying the amino acid utilization in adult albino rats found that omission singly of histidine, lysine, tryptophan, phenyl alanine, methionine, threonine, leucine, isoleucine or valine from the ration led to a marked loss of weight in a 10 day period, coincident with a prompt loss of appetite, as considered by the consumption of only one-third to one-half of the daily ration offered. When, however, the missing amino acid was added to the ration, the rats quickly recovered lost appetite and rapidly regained lost weight. The rapid decline in appetite suggests the development of a state of acute amino acid deficiency.

As is now generally accepted, ten amino acids are found to be essential dietary components for the growing rats (82). These are leucine, isoleucine, threonine, methionine, phenylalanine, tryptophan, lysine, histidine, arginine and valine. The exclusion of any one of these, other than arginine, leads to nutritive failure, loss in weight, diminished appetite and eventual death. In contrast to these spectacular effects, arginine deprivation merely decreases the rate of gain. This is accounted for by the fact that arginine can be synthesized by the rat but not at a rate commensurate with the needs of the organism for maximum growth. Mixtures of the ten indispensable amino acids are effective sources of nitrogen not only for growth but for reproduction of the rat as well and for the maintenance of nitrogen equilibrium of the dog.

Sebrell and McDaniel (84) reported the effects of diets deficient in a

single essential amino acid, in rats depleted of blood proteins and formed elements of the blood, by multiple bleedings, while receiving a protein free diet. They found that each amino acid deficiency, except that of arginine, had some adverse effects on blood regeneration but these effects were quite variable. Histidine, valine and leucine deficiency had significant effect on the recovery of red blood cells. Isoleucine and lysine deficiency also affected the regeneration of hemoglobin while arginine, methionine and tryptophan deficiency had the least effect. The animals failed to gain weight on diet deficient in each single amino acid except arginine. The growth of the animals on the arginine deficient diet was less than on a diet containing all ten essential amino acids. Tremors develop on rations deficient in valine, leucine, tryptophan, isoleucine, and phenyl alanine. Maddy and Swift (59) reported that a period of adaptation to the amino-acid diets was required by the rats since, during the first seven days on experiment, the rats receiving the optimum amino acids diet grew at a significantly lower rate than the control rats receiving the casein ration. After this adaptation period, the two groups grew at the same rate.

Role of Amino Acids in Hemoglobin Formation. The formation of erythrocytes and hemoglobin should be considered as one process, namely erythropoiesis, although both are independent of each other. Hemoglobin is formed by the developing erythrocytes in the bone marrow. The level of hemoglobin in the blood represents the balance between the production and destruction of the hemoglobin molecule. Hemoglobin is a conjugated protein, consisting of an iron-containing pigment called heme combined with a protein of the histone class called globin. The heme constitutes about four percent and

the globin about 96 percent of the hemoglobin molecule.

Protein intake, when limited, can restrict the production of hemoglobin in anemia (43). The problem of feeding a diet consisting of a particular protein or amino acids is beset with numerous difficulties. Few proteins are analytically completely understood and the body apparently can break down amino acids and from these fragments can synthesize other amino acids (83), therefore no protein diet can be called a simple diet. Even when the protein intake is reduced to zero, the fasting anemic dog can synthesize new hemoglobin (40 to 50 gm), which must come from protein stores and protein wastage (20). Exchanges between the plasma proteins, body proteins and hemoglobin goes on readily in the dog (96) and it is to be assumed that it goes on in the rat but no experimental data are available.

The amino acid composition of the globin molecules has been shown to contain all of the essential amino acids as well as many of the non-essential ones. Hemoglobin contains the following 15 amino acids: leucine, isoleucine, lysine, histidine, aspartic acid, phenyl alanine, tyrosine, arginine, alanine, tryptophan, proline, glutamic acid, serine and cystine. Glycine, although not represented in the hemoglobin analysis, was found to be as effective as any one noted above. It is assumed that it plays its part by means of combination with other amino acids or other split products of amino acids to form those amino acids required for the hemoglobin formation.

Glycine, glutamic acid, aspartic acid, cystine, histidine, phenylalanine and proline when given in one gram doses daily for two weeks increase hemoglobin output on the average 23 to 25 gm above the control level.

Alanine, valine, isoleucine and arginine in the same dosage increases the hemoglobin output on the average 13 to 17 gm per two weeks over the control level. Leucine, methionine, lysine, tryptophane and tyrosine increase the hemoglobin output on the average to about 20 gm (99).

These workers tested the individual amino acids in well standardized anemic dogs to see how much new hemoglobin would be produced over and above the control base line output. Various doses were tried but one gm per day seemed to be the optimum intake for most of the amino acids for a two week period. Larger doses rarely increased the output of hemoglobin but smaller doses, in some experiments, do decrease the output. Of the 17 amino acids found in the hemoglobin, seven are essential amino acids but these are no more potent than the others, when measured by their capacity to promote hemoglobin production in this type of anemia due to blood loss. On the contrary, Orten and Orten (68) reported that the effects of the administration of various amino acids on the hemoglobin content, in rats, maintained on a low protein diet, consisting of 3.5 percent lactalbumin, no consistent sustained increase in hemoglobin values occurred following supplementation with any of the ten essential amino acids or with glycine, cystine, glutamic acid, proline or tyrosine. They interpreted these results as evidence that no single amino acid can be regarded as a 'key' amino acid, in hemoglobin synthesis. It is logical to assume that if more than one amino acid is lacking, then hemoglobin synthesis will not proceed until all of the missing amino acids are supplied. They concluded that a combination of amino acids in, as yet undetermined proportion, is essential for the in vivo fabrication of the hemoglobin molecule. Pearson et al. (71) state that hemoglobin formation in rats was more vital than growth. Whipple

(97) states that standard anemic dogs, on basal low protein diets, could make 40 to 50 gm. of hemoglobin per week, for several weeks, in the absence of infection. Orten and Orten (69) emphasize the importance of both the quality and quantity of dietary proteins for hemoglobin formation in the rat. Their results indicate that those proteins which are qualitatively best constituted for supporting somatic growth are also most effective for hemoglobin formation. If rats are fed on synthetic diet containing human or beef globin as the protein, poor growth occurs and an anemia develops. If the chief qualitative deficiency in the amino acid composition of globin is remedied by the addition of isoleucine, an increased rate of growth occurs and anemia is corrected. The same is true if the dietary intake of tryptophan or lysine is inadequate. Whipple *et al.* (100) reported that dogs with sustained anemia and hypoproteinemia due to bleeding and a continuing low protein or protein-free diet with abundant iron will continue to produce new hemoglobin and plasma protein for many weeks. The stimulus for double depletion anemia and hypoproteinemia leads to raiding of body and tissue protein to fill the demands for new hemoglobin and plasma protein. The blood protein takes priority over the organ and tissue proteins.

Bethard *et al.* (5) observed that removal of protein from the diet was followed promptly by hemoconcentration, diminution of blood volume and drastic reduction in erythropoiesis. These changes were reversible after 35 days, upon addition of protein to the diet. They reported that protein intake is more essential for maintenance of normal erythropoiesis than is total caloric intake. They point out that their data suggests that hemoglobin concentration within the vascular system is more important than red

cell volume in regulating erythropoietic rate.

The Role of Valine in Erythropoiesis. Valine ($C_5H_{11}O_2N$) or α -amino isovaleric acid is an indispensable amino acid for rats. Despite its relative simplicity in structure, it cannot be synthesized by the organism of the rat. Rose and Eppstein (81), during their study of indispensibility of valine in rats, reported that rats fed a diet deficient in valine, the animals manifested a profound nutritive failure with rapid decrease in weight and marked loss of appetite. The animals were kept on a valine deficient diet for 28 days. The growth rate improved immediately on the administration of valine. The most striking features of valine deprivation is the development of peculiar symptoms. The rats became extremely sensitive to touch and displayed a severe lack of coordination in movement. They walk with a staggering gait. As the animals attempt to walk, the left foreleg is raised inordinately and the head is retracted. Frequently the subject show a rotary motion resembling that of a dog chasing its tail. This may be either clockwise or counterclockwise and may continue until the animal falls to the floor of the cage from sheer exhaustion. The symptoms readily disappear on the administration of valine without any other therapeutic measure. This indicates that valine is an indispensable amino acid.

Madden *et al.* (58) have reported that when the blood plasma protein of dogs is depleted by bleeding, with return of the washed red blood cells (plasmapheresis), it is possible to bring dogs to a steady state of hypoproteinemia and a constant level of plasma protein production, if the diet protein intake is controlled and limited. When the protein intake of such

dogs is completely replaced by the growth mixture of crystalline amino acids, plasma protein production is excellent, weight and the nitrogen balance is maintained. The omission of valine from the growth mixture is quickly followed by a sharp decline in plasma protein formation and by a negative nitrogen balance. Robscheit-Robbins et al. (80) reported that dogs fed abundant iron and protein-free or low protein diets with sustained anemia and hypoproteinemia, valine has a moderate influence upon urinary nitrogen balance towards nitrogen conservation.

ROLE OF ERYTHROCYTE STIMULATING FACTOR (ESF)

General considerations

The primary function of the red blood cells is the transport of respiratory gases. In order to fulfill this function, there must be sufficient circulating erythrocytes to meet the metabolic needs of the body. When there is a decrease in the amount of oxygen supply to the tissues, there is a compensatory increase in the number of peripheral red blood cells. The number of red blood cells circulating in the blood is controlled by the regulation of erythropoiesis. At present it appears that tissue oxygen tension controls erythropoiesis, this control being mediated by a humoral factor(s), which has been variously termed as, erythropoietin (51, 78), plasma erythropoietic stimulating factor (55), plasma erythropoietic factor (EPF) (75, 77), erythropoietic stimulant (52), etc. In view of the present unsettled status of its chemical nature and the possibility that it might represent more than a single substance, the material will be referred to, in this discussion, as the erythropoietic stimulating factor(s) (ESF).

The hypoxic stimulus for erythropoiesis would lead one to think that it is the primary stimulus for erythropoiesis on the assumption that the loss of blood and hemolytic anemias lower the oxygen delivery to the tissues, thus directly stimulating the red blood cell formation by hypoxia of the marrow. Alternately it is also likely that hypoxia, though acting as the primary stimulus, might act indirectly by stimulating the release of a humoral factor, which in turn may stimulate erythropoiesis. Reissman (73) in 1950 furnished a convincing proof that stimulation of erythropoiesis by hypoxia is indirect. He exposed one partner of a pair of parabiotic rats to an atmosphere containing 7.6 percent oxygen while the other partner was allowed to breathe room air. Both parabionts developed marked erythroid hyperplasia. Since the oxygen saturation of the blood of the parabionts breathing room air was 97 percent, hypoxic stimulation of its marrow could be excluded. Transfer of a plasma factor released in response to hypoxia was implicated and interest in the concept of humoral regulation of erythropoiesis revived. A further impetus to the study of the postulated plasma factor came from the clinical observations by Stohlman *et al.* (88) of instances of polycythemia secondary to the reverse flow in patients with patent ductus arteriosus. Borsook (7) demonstrated that acidified boiled extracts of plasma from rabbit with phenylhydrazine anemia were capable of stimulating erythropoiesis in normal rats. These observations excluded a direct hypoxic stimulation of erythropoiesis and supported the concept of humoral regulation by erythropoietic stimulating factor(s).

Experimental confirmation of the existence of ESF has come from a large number of laboratories (10, 38, 45, 54, 55, 73, 74, 86, 90, 91).

Donor and Recipient Animals

Donor Animals. Rabbits, rats, dogs, guinea pigs, monkeys, cattle, human subjects, etc., have been used as donors of ESF. The minimal effective period of subjection of the donor animal to the hypoxic stimulus for evoking the appearance of detectable amounts of the ESF varies with the type of hypoxia employed. Thus, exposure to lowered barometric pressure for two to 48 hours (75) is sufficient. One injection of a large dose of cobalt (94), or a single bleeding (87) also results, within one to 24 hours, in detectable amounts of ESF in the circulating blood. With hemolytic agents like phenylhydrazine, however, an intensive anemia, brought about through daily administration of the drug for approximately one week, appears essential for the consistent production of the ESF. Repeated withdrawal of blood is not necessarily more effective than single bleedings in evoking the factor. A humoral concept of the control of erythropoiesis also demands the demonstration of the regulatory factor in the blood of normal as well as hypoxic animals, although its concentration might be expected to be considerably less in the former than the latter. Opinion is divided as to whether ESF activity exists in the blood of normal animals. Several of the reports are negative in this regard (7, 13), whereas others claim that the ESF is present in normal plasma or serum (42, 52). Borsook (6) interprets the generally slight stimulatory effects of normal plasma concentrates as a response to a hemolytic action of the antibodies evoked by nonerythropoietic proteins of plasma. It remains to be determined whether these effects are due to known erythropoietic stimulating hormones (e.g., hypophyseal growth, adrenal, steroidal and thyroidal) or to the specific ESF.

Recipient Animals. Rats, rabbits and mice have been most frequently used for the assessment of the ESF and to some extent guinea pigs, monkeys and man have also been employed. It has been reported that female rats exhibit a greater sensitivity than males (7), but others (55) could find no such difference.

Criteria for determining ESF activity in the materials tested include changes in the peripheral erythrocytic indices (red cell counts, hemoglobin, hematocrit and reticulocyte levels); Fe^{59} incorporation into the red blood cell of normal (73), hypophysectomized, starved or polycythemic (31, 47) rats and a variety of other methods. The wide diversity of methods employed may account, in part, for some of the conflicting results obtained by the various investigators.

Although the intact animal is relatively insensitive, it has the advantage of reacting in a more specific manner to the ESF. The only factors truly capable of augmenting the circulating red blood corpuscles volume above the normal levels in intact animals are lowered barometric pressures, metals like cobalt, the ESF and possibly some adrenal cortical steroids (100). Factors such as folic acid, liver and vitamin B_{12} and other vitamins, iron and copper, although specifically effective in animal lacking these principles, exert little or no action upon erythropoietic processes in intact animals subsisting on a normal diet and possessing a normal responsive marrow.

However, for purposes of screening and to have available assay procedures which are more sensitive and less time consuming, hypophysectomized or starved rats are employed as recipients. In these animals the erythropoiesis is at a low ebb and the quantity of ESF present is presumably reduced. Such animals respond to smaller amounts of the ESF and in a shorter period of

time than do intact animals. Care, however, must be applied in the interpretation of information gained from the use of hypophysectomized or starved animals when they are treated with whole plasma or crude extract derived therefrom. Thus, the hypophysectomized rat responds with increased erythropoiesis to a variety of hormonal factors, particularly those of thyroidal, adrenal cortical and adenohipophyseal origin (41, 98). Some of these may be present in the plasma of hypoxic (stressed) donors, in amounts sufficient to stimulate red blood cell formation in the hypophysectomized recipients.

The antigenicity of the plasma of one species of animals, when used in case of heterologues species of animals, is a factor of considerable importance. However, the use of boiled filtrates of plasma (55, 66), containing little or no antigenicity has permitted the assessment of their contained activity in other species. This has the advantage that when limited amounts of materials are available, larger concentrations of the factor can be achieved in the body of smaller sized species, used as recipients.

There is a need for an ESF standard preparation to which activity of material studied in various laboratories may be compared and expressed in terms the standard. Recently Hodgson *et al.* (46) have indicated the feasibility of a dose response analysis in starved rats treated with boiled filtrates of plasma from phenylhydrazinized rabbits and with alcoholic extracts of urine from the animals. A significant linear correlation exists between the ratio RBC:liver radioiron, and the dose of extract administered. This ratio is claimed to permit detection of smaller amounts of ESF than is possible with Fe⁵⁹ RBC incorporation method alone.

Role of Endocrines in Erythropoiesis. Gordon (36) showed that anemia of mild to moderate severity develop in experimental animals following the the removal of the pituitary, adrenals, thyroid and testes and can be prevented by suitable replacement therapy. In a concise and comprehensive review of the extensive literature and his own work on this subject, Gordon (36) concluded that the hormones act by mobilizing factors needed for the manufacture of blood cells and that their action is best interpreted as an indirect one, secondary to their influence on metabolic processes.

Much of the early work relating the endocrine system to the blood elements centered about the possible role of the pituitary. Its removal results in the development of a peripheral anemia, in a few species, including the rat (14, 85, 95). The anemia has been shown to develop as a result of the absence of the anterior rather than the posterior and intermediate lobes of the gland (93). Peripheral erythrocyte augmenting qualities of ACTH have been reported by several workers (33, 101). If daily injections of ACTH are initiated on the first day of hypophysectomy, the decrease in total circulating red cell volume is prevented (33). Contopoulos *et al.* (11) have reported that extracts of beef, sheep, pig, horse pituitary, when given parenterally, increased the red cell mass of hypophysectomized animals. It is claimed that the active factor was associated with ACTH but could be separated by autolytic digestion and is effective in adrenalectomized animals (95).

Proof of a separate existence of the pituitary erythropoietic hormone has been suggested by Van Dyke *et al.* (95). He suggests that although it is recognized that a number of factors are involved in the mechanism controlling red blood cell formation, the anterior pituitary furnishes one factor of

importance. This pituitary factor is apparently a hormone distinct from other trophic hormones. It is allied chemically only to ACTH and yet both biological and chemical evidence indicates that the pituitary erythropoietic hormone and ACTH are not the same. The major problem resolves itself into developing a satisfactory method of preparation of erythropoietic hormone which will distinguish it from ACTH. It is true that no ACTH has been made which does not possess some erythropoietic activity and no pituitary erythropoietic hormone has been prepared which is entirely devoid of ACTH. Nevertheless, the preparation of these two activities in different preparations has varied so greatly as to assume their separate existence. Autolytic digestion consistently reduces the ACTH activity while erythropoietic activity is retained. It has been found that oxycellulose is a better absorbant of ACTH than of erythropoietic hormone. These observations add clinical evidence to the accumulated histological evidence establishing the separate existence of these two pituitary principles.

If indeed there is a specific pituitary erythropoietic hormone, it is not clear what physiologic role it plays. The anemia that develops following hypophysectomy can be corrected by the administration of anabolic agents, a high protein diet, testosterone and thyroxine (15). It seems likely, as emphasized by Houssay (48) that the main function of the pituitary is to maintain the metabolism necessary for normal erythropoiesis in the absence of a specific protein anabolic agent and that neither can an anabolic agent be effective in the absence of a specific stimulus. Its exact role is yet to be determined.

Crafts and Meinke (19) reported that thyroid does have an influence on erythropoietin and that hypothyroidism alone cannot account for hypophysectomy

anemia.

Adrenalectomized rats also exhibit an anemia but it is of a temporary nature (72) which may be due to the adrenal remnants. Injections of cortisone acetate daily do have an effect on the blood picture of hypophysectomized rats but do not return it to absolutely normal figures. Cortisone seems to stimulate delivery of erythrocytes to the peripheral blood.

It has been shown that injection of growth hormone in hypophysectomized rats stimulates the bone marrow (32, 62). But the peripheral anemia was not completely eliminated (92). Meineke and Craft (63) reported that combination of growth hormone with thyroxine-cortisone therapy completely eliminated post hypophysectomy anemia. In summary it can be said that the anemia following hypophysectomy would seem to be due to the lack of thyroid and adreno-cortical activity and to loss of growth hormone in such animals.

Site of Production of ESF. A number of organs have been suggested as the possible source of ESF and the consensus of opinion now seems to be in favor of kidneys as the source. Jacobson (50) assayed the plasma of nephrectomized rats and rabbits stimulated by bleeding or cobalt ESF could not be demonstrated in such plasma when treated in starved or hypophysectomized rats or mice. Reismann *et al.* (79) reported that ureter ligated rats responded nearly like normal controls, to bleeding or phenylhydrazine anemia and ESF was demonstrated in their plasma, thus eliminating the urea retention as a depressing factor of ESF in uremia. The presence of functioning renal (tubular) tissue appears to be necessary for a normal ESF response. Very recently Naets (64, 65) reported that removal of both kidneys was followed by a rapid disappearance of erythroblasts from the marrow and by a decrease

in iron utilization and turnover while in the ureter ligated animals, the ESF remained essentially normal. Fisher and Birdwell (29) similarly reported that dog kidney when perfused *in situ*, was stimulated with cobalt to produce ESF.

Chemistry of ESE. In a comprehensive review, Gordon (37) suggests the view that the ESF moiety derived from both plasma and urinary sources is protein in nature. The factor appears to be appreciably heat resistant, nondialyzable, nonultrafiltrable, nonprecipitable by perchloric acid or boiling. But precipitable with 70 to 80 percent saturated ammonium sulphate. It has an electrophoretic mobility characteristic of alphaglobulins. Its activity is destroyed by proteolytic enzymes. It is possible that ESF is a complex system comprising multiple factors. The process of erythropoiesis encompasses proliferative activities of stem cells, maturation of these elements, synthesis of hemoglobin and the release of the RBC from the blood forming organs. It seems unlikely that all of these processes are regulated by one principle. Discrepant views in the field of the chemistry of ESF may perhaps be due to the different investigators extracting with different procedures, the component parts of a complex system, all necessary for complete erythropoietic activity. In addition, the use of different animal species, both as donors and recipients, may contribute to the divergent results.

Effect of Temperature on ESE.

Heat. Since most investigators who have reported on the use of the acidification-boiling procedure for the preparation of plasma extracts have found activity retained in such preparations, it seems valid to conclude that

the active principle or an attached protective moiety, or both, displays resistance to elevated temperatures. However, the persistence of activity in boiled plasma filtrates does not preclude the possibility that some inactivation might occur. The assays employed in most of these studies have utilized arbitrary dosages and it is conceivable that the quantities of the material administered might have exceeded those necessary to evoke a maximal biological response. However, Linman and Bethell (56, 57) reported that although boiling does not alter the ability of the anemic plasma to stimulate RBC proliferation, it destroys its capacity to increase the hemoglobin and hemotocrit values. Partly on this basis (57), they have postulated the existence of two principles: one, heat stable and ether soluble, which stimulates erythroblastic cell division, and the second, relatively thermostable and ether insoluble which stimulates hemoglobin synthesis. The effect on hemoglobin is reported demonstrable in unmodified anemic plasma and after boiling for 10 minutes but extracts boiled for 30 minutes or longer lose this activity.

Freezing. There is consistent agreement that the ESF present in whole untreated plasma and serum or acidified boiled filtrates prepared from the fluids can withstand freezing temperatures ranging from -5°C . to -20°C . for indefinite periods of time. This is true of whole plasma or serum from animals subjected to bleedings (23, 55).

Mode of Action of ESF. In spite of the considerable interest in the subject of ESF, little is known concerning its mode of action. One possibility is that it acts by mobilizing, in certain organs, specific nutrient factors, minerals and perhaps enzymes, which are transferred to the blood

forming tissues for fabrication of red blood cells. On the other hand, a growing literature tends to favor a direct action on RBC formation. Preliminary observations have indicated that a boiled plasma filtrate from phenylhydrazine-treated rabbits stimulated the respiration of bone marrow cells more strongly than did similarly prepared filtrates from normal rabbits (39).

Metabolism of ESF. The ESF undergoes a rapid clearance from the blood. Urinary excretion of ESF has been demonstrated by a number of investigators (92, 102). It is not yet known whether a renal threshold exists for ESF. Rapid disappearance of ESF from the blood is not only due to its excretion in the urine but it also is due to its rapid utilization and prompt inhibition.

Iron Metabolism in Erythropoiesis. Since the radioactive isotopes have been available, a great deal of effort has gone into studying the pathways of iron metabolism and utilization of iron. Several techniques have been proposed for the evaluation of erythropoietic function, using, for example, the plasma iron turnover time (49), rate of appearance of labeled red cells (21), etc. Technique of radio autography for evaluating iron incorporation at the cytological level has been described by Austoni (2), and recently by Alpen and Cranmore (1). Hodgson *et al.* (47) studied the effects of plasma and extracts of plasma and urine from anemic animals on iron metabolism, to evaluate indirectly the intensity of erythropoiesis in assays of hemato-poietin, in which Fe^{59} has been employed as a tracer. They reported that plasma iron turnover in animals with normal erythropoiesis is independent of plasma iron concentration over wide ranges. 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 contain factors that influence erythropoietin, but also affects iron metabolism per se, not just as a consequence of the modification of hemoglobin synthesis. Plasma from phenylhydrazine treated donors contains a factor (hematopoietin) stimulating hemoglobin synthesis. It is probable also that the plasma of anemic animals contain a factor(s) other than hematopoietine, that influences plasma iron level.

Hematopoietin injected into starved animals produces a notable increase in iron turnover and marked changes in the distribution of iron between marrow and depots.

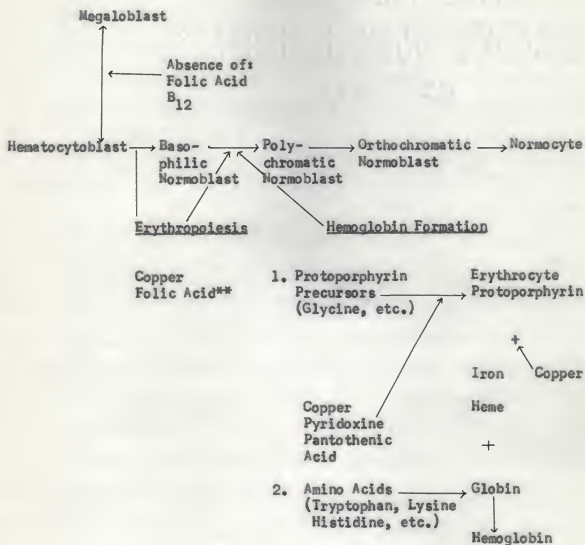
Other Additional Factors Needed for the Action of ESF. In addition to an adequate amount of protein, the following factors are needed for the action of ESF (9):

Pyridoxine: Harris *et al.* (44) have reported that a severe hypochromic microcytic anemia in man, due to deficiency of pyridoxine and which is corrected by the administration of pyridoxine.

Vitamin B₁₂ and Folic Acid: Both are necessary for the normal maturation of the red cells. Their function is not fully known. Both are concerned in the formation of purine and pyrimidine bases which are consistent parts of nucleic acid (Fig. 1).

Ascorbic acid: It appears to participate in metabolic activities. It has intense reducing properties and plays an important role in the oxidation-reduction reactions of the body (22).

Cobalt: Administration of cobalt is known to stimulate



* Follis, Jr., Richard H.: Deficiency Disease. Charles C. Thomas Publisher. Springfield, Illinois. p. 464, 1958.

** Nucleic Acid Synthesis

Fig. 1. Showing essential nutrients and blood cell formation.

erythropoiesis in man and animals. However, its mechanism of action is not known (50, 17). Its importance lies in the fact that it is a part of vitamin B₁₂ molecule.

Copper: It appears to function in the absorption, mobilization and utilization of iron in hemoglobin synthesis.

Clinical Applications of ESF. On theoretical grounds the ESF might have application in conditions in which the rate of RBC production is sub-normal provided the marrow activity is not limited by any toxic material. It may find application in various kinds of anemias, specially hypoblastic and secondary anemias. It might prove of some use in anemic states associated with the first few weeks of life in normal and specially premature young ones. Its use in radiation anemia and as a 'booster' in blood transfusions might also be feasible.

MATERIALS AND METHODS

Erythrocyte Stimulating Factor (ESF)

The source material of ESF (90) was the pooled plasma of 12 Hereford heifers, collected and preserved, in 1957, by the Department of Physiology, Kansas State University. Prior to preserving, the plasma had been acidified with 1 N hydrochloric acid to a pH of 5.5 and heated at 99.9°C. for 10 minutes to precipitate the proteins, in order to obtain a partial protein-free filtrate (PPFF). The clear PPFF was stored aseptically in glass containers at 5°C. until it was used for experimental purposes.

Source Material of Iron

The iron utilized in this experiment was Armidexan*. It contained the equivalence of 50 mg. of elemental iron as ferric hydroxide in complex with a low molecular weight dextran fraction, per ml.

Preparation of Assay Animals

Fifty-six young virgin female rats of Sprague-Dawley strain were selected as test animals and were divided at random, in eight groups, each group consisting of seven animals. The groups were designated as shown in Table 1. The average pre-experimental weight of the rat was 154.4 gm. These groups were maintained on a Basal nitrogen-free diet for a period of eight days. The composition of the diet is given in Table 2. After the eight days, the rats were kept on their respective experimental diets, the composition of which is given in Table 3, for 11 days. After eight days, the animals were placed on their experimental diets; they were injected for three consecutive days as per details shown in Table 1. On the eleventh day, the rats were post experimentally weighed and approximately 2 to 4 ml. of intra cardiac blood was collected under ether anesthesia. The blood was collected in heparinized syringes and tests were conducted immediately for hemoglobin values, hematocrit values and red blood cell counts. The hematocrit determination were conducted by the use of Adam's microhematocrit centrifuge.

The duplicate hemoglobin determination were made by acid hematin method employing spectrophotometer (10). The red blood cells were counted by the

*Manufactured by Armour Veterinary Laboratories, Division of Armour and Company, Kankakee, Illinois.

Table 1. Design of experiment.

Group	Description
I	Complete balanced ration
II	Complete balanced ration + ESF
III	Complete balanced ration + iron
IV	Complete balanced ration + ESF + iron
V	Valine deficient ration
VI	Valine deficient ration + ESF
VII	Valine deficient ration + iron
VIII	Valine deficient ration + ESF + iron

Note: Each group consists of six rats.

Table 2. The composition of basal nitrogen-free diet.*

	Percent
Ceralase	15.0
Dextrin	60.8
Crisco	14.0
Mazola	2.0
Cellulose	2.0
Salt-mix	4.0
Vitamin A	4.5 gm. (200,000 IU gm.)
Vitamin D	0.2 gm. (400,000 IU gm.)
Vitamin E	5.0
Vitamin C	45.0
Inositol	5.0
Choline	75.0
Vitamin K	2.25
PABA	5.0
Niacin	4.5
Pyridoxin	1.0
Riboflavin	1.0
Thiamine	1.0
Ca. Pantothenate	3.0
Biotin	0.02
Folic acid	0.09
Vitamin B ₁₂	0.002

*Richeegl, M. et al., Journ. Biol. Chem. 231:829. 1958

Table 3. Composition of control diets.*

	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-Leucine	1.158
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
DL-Threonine	1.020
L-Tryptophan	0.216
DL-Valine ¹	1.464
Salts ¹	4.00
Corn oil ²	<u>5.00</u>
Starch to total	100.0
<u>Vitamin³</u>	
Thiamine Hcl.	1.0
Riboflavin	1.0
Pyridoxine Hcl.	1.0
Nicotinic acid	10.0
i-inositol	20.0
p-amino benzoic acid	20.0
Folic acid	0.1
Biotin	0.1
Menadione	2.0
ca-pantothonate	4.0
Choline chloride	150.0
Vitamin B ₁₂	0.004
a-tocopherol acetate	2 mg.
Vitamin A ⁴	2000 IU
Vitamin D ⁴	200 IU

¹The valine has been completely eliminated for experimental purposes.

¹Salt mixture Hegsted, Nutritional Biochemicals Corp., Cleveland, Ohio.

²Mazola Corn Products Refining Company, New York.

³Vitamins were supplied in a portion of the starch in mg/100 gm. of diet.

⁴Vitamins A and D requirements computed on weekly basis.

*Hepburn, F.N., W.K. Calhoun and W. B. Bradley.

A growth response of rats to glutamic acid when fed an amino acid diet. Jour. Nutri. 72(2):163. 1960. (Amer. Inst. of Baking, Chicago.)

hemocytometer method, utilizing duplicate readings of each sample for accuracy.

RESULTS

Results of the experiment are given in Tables 4 to 11, inclusive. Table XII shows the average values for all the groups. Figures 2, 3 and 4 summarize the results of the effect of ESF and iron, singly and in combination, on the red blood cell count, hemoglobin and hematocrit values respectively, in the different groups.

No data for rats kept on control ration with ESF (Group II) could be collected as the samples of blood collected had coagulated.

In case of rats receiving control ration and iron (Group III), the values for red blood cells, hemoglobin and hematocrit were the highest.

In case of rats receiving control ration together with ESF and iron, there was a significant increase in the values of red blood cells and hemoglobin while the value for hematocrit remained almost the same when compared with controls of Group I. It was noted that rats receiving the experimental ration (Group V) the values for hemoglobin and hematocrit were the lowest.

When ESF was administered to rats receiving the experimental ration (Group VI), the values for hemoglobin and hematocrit and red blood cells rose slightly.

When iron was administered to rats receiving the experimental ration (Group VII), the values for hemoglobin and hematocrit rose slightly while the values for red blood cells decreased.

When iron and ESF were administered to rats kept on experimental diet (Group VIII), the values for red blood cells, hemoglobin and hematocrit

Table 4. Effect of complete ration on erythropoiesis in rats.

Animal number	Weight in grams	Amount PPF injected (cc)	Amount dextran injected (mg.)	Amount Iron	Rbc 10^6	Hemo-globin	Hematocrit	Remarks
1	146	-	-	-	508	14.5	41.0	
2	162	-	-	-	615	15.75	45.5	
3	138	-	-	-	497	15.0	41.3	
4	142	-	-	-	421	14.3	40.9	
5	150	-	-	-	339	13.5	35.0	
6	162	-	-	-	305	12.5	33.0	

Table 5. Effect of complete ration with PPF on erythropoiesis in rats.

Animal number	Weight in grams	Post-exptl.	Amount PPF injected (cc)	Amount PPF injected	Amount iron dextran injected (mg.)	Rbc10 ⁶ s	Hemo-globins	Hematocrit	Remarks
1	156	124	6	-	-	-	-	-	All the samples
2	160	122	6	-	-	-	-	-	of blood had
3	148	116	6	-	-	-	-	-	coagulated, hence
4	148	120	6	-	-	-	-	-	discarded
5	158	122	6	-	-	-	-	-	
6	152	122	6	-	-	-	-	-	
7	156	124	6	-	-	-	-	-	

Table 6. Effect of complete ration with iron on erythropoiesis in rats.

Animal number	Weight in grams Pre-exptl.:	Post-exptl.:	Amount PFF: injected	Amount iron: dextran in- jected (mg.):	Rbc10 ⁶ :	Hemo- crit:	Hemato- crit:	Remarks
1	148	116	-	150	864	15.25	37.3	Samples of blood
2	168	108	-	150	902	16.00	40.0	of animals nos.
3	144	106	-	150	-	-	-	3, 4 and 5
4	156	116	-	150	-	-	-	coagulated
5	152	116	-	150	-	-	-	
6	156	116	-	150	980	16.00	39.4	
7	150	116	-	150	1013	17.75	47.8	

Table 7. Effect of complete ration with PPF and iron on erythropoiesis in rats.

Animal number	Weight in grams	Amount PPF injected (cc)	Amount PPF injected (cc)	Amount Irons dextran injected (mg.)	Hemo- $\text{Rbc} \times 10^6$	Hemato-crit	Remarks	
1	152	102	6	150	834	15.65	40.5	
2	144	120	6	150	848	16.25	43.8	
3	154	116	6	150	673	15.65	40.0	
4	156	110	6	150	706	15.65	35.8	
5	144	104	6	150	863	15.50	37.8	
6	156	114	6	150	852	15.00	37.9	
7	156	-	6	150	-	-	-	Died

Table 8. Effect of valine deficient ration on erythropoiesis in rats.

Animal number	Weight in grams	Amount PPF: injected	Amount PPF: dextran injected	Amount iron: Rbc10 ⁶	Hemo: globin	Hemato-crit	Remarks
Pre-exptl.	Post-exptl.	(cc)	(cc)	(mg.)	:	:	
1	164	-	-	-	-	-	Died
2	152	-	-	438	12.75	31.8	
3	146	-	-	538	11.75	34.0	
4	152	-	-	-	10.85	21.7	
5	166	-	-	400	8.75	23.0	
6	152	-	-	632	13.25	36.0	
7	156	-	-	538	13.75	33.0	

Table 9. Effect of valine deficiency with PFF on erythropoiesis in rats.

Animal number	Weight in grams	Pre-exptl.	Post-exptl.	Amount injected (cc)	Amount PFF injected	Amount iron dextran injected (mg)	RbclO ⁶	Hemo-globin	Hemato-crit	Remarks
1	144	126		6	-	664	13.75	36.6		
2	160	110		6	-	297	11.25	25.0		
3	164	122		6	-	288	12.50	30.8		
4	154	-		6	-	-	-	-		Died
5	160	126		6	-	577	13.75	35.5		
6	158	116		6	-	433	13.25	30.5		
7	160	124		6	-	834	13.75	37.5		

Table 10. Effect of valine deficiency with iron on erythropoiesis in rats.

Animal number	Weight in grams	Amount PPF injected	Amount iron dextran injected	Rbc $\times 10^6$	Hemo-globin	Hemato-crit	Remarks
: Pre-exptl.	: Post-exptl.	: (cc)	: (mg)	:	:	:	:
1	152	116	-	150	142	12.75	29.8
2	156	120	-	150	418	14.50	31.8
3	162	118	-	150	332	14.00	31.0
4	164	130	-	150	448	14.75	38.8
5	168	124	-	150	616	13.50	33.0
6	150	108	-	150	623	14.00	34.0
7	160	124	-	150	408	13.50	34.0

Table 11. Effect of valine deficiency with PPF and iron on erythropoiesis in rats.

Animal number	Weight in grams : Pre-exptl.	Weight in grams : Post-exptl.	Amount PPF injected : (cc)	Amount PPF injected : dextran in-jected (mg.)	Rbc10 ⁶	Hemo- globin :	Hemato- crit :	Remarks
1	146	100	6	150	639	15.50	38.6	
2	152	-	6	150	-	-	38-	
3	156	118	6	150	696	13.25	37.0	
4	156	112	6	150	607	12.25	31.6	
5	148	124	6	150	359	14.25	36.0	
6	156	112	6	150	455	15.25	35.2	
7	158	126	6	150	391	12.50	32.8	

Table 12. Table showing average values.

Group	Weight in grams : Pre-exptl. : Post-exptl. :	Rbc10 ⁶ :	Hemoglobin :	Hematocrit :	Remarks
I Control ration	150	120.3	447.5	14.25	39.4
II Control ration with PFF					
III Control ration with iron	155.5	114	939.7	16.25	41.1
IV Control ration with PFF and iron	151	111	796	15.91	39.3
V Valine deficient ration	154.8	122	509.2	12.13	31.4
VI Valine deficient ration with PFF	157.6	120.6	515.5	13.04	32.6
VII Valine deficient ration with iron	158.8	119.4	426.7	13.8	33.1
VIII Valine deficient ration with PFF and iron	153.3	115.3	524.5	13.8	35.2

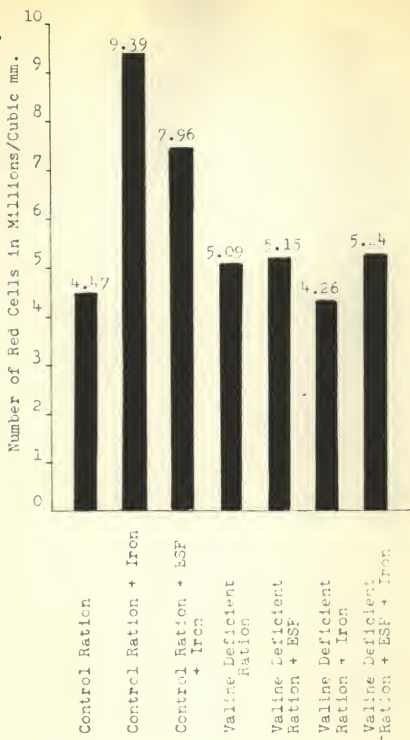


FIG. 2 SHOWING RED CELL COUNT

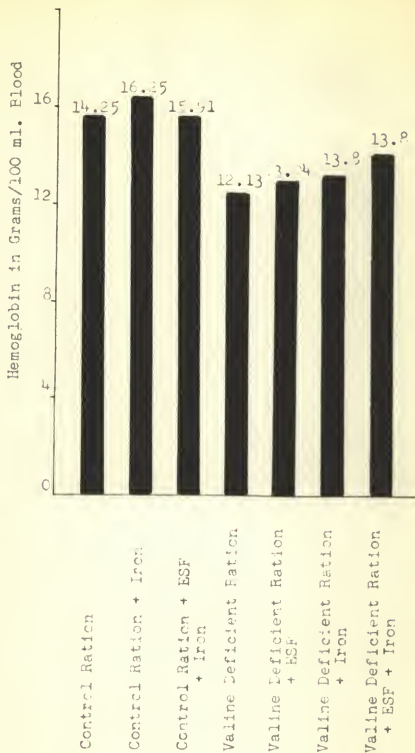


FIG. 3 SHOWING AMOUNT OF HEMOGLOBIN

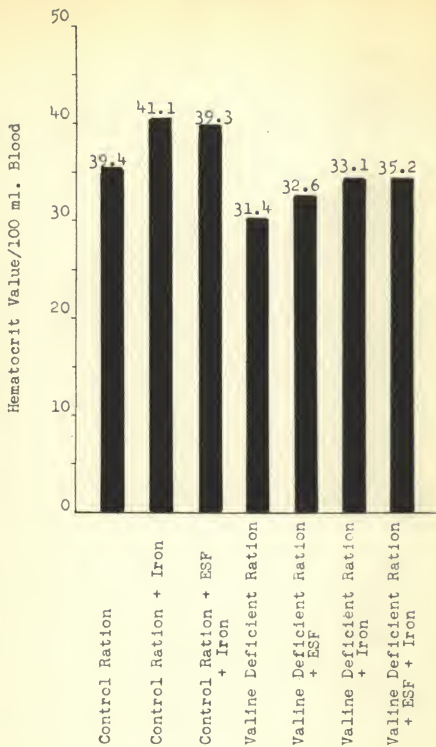


FIG. 4 SHOWING HEMATOCRIT VALUES

increased significantly.

DISCUSSION OF RESULTS

Results of the experiments with ration deficient in valine indicate the role of this amino acid in the process of erythropoiesis in rats.

Rats fed a valine deficient ration (Group V) had the lowest hemoglobin and hematocrit values while the values for red blood cells are next lowest, as compared with the rest of the group.

The red blood cell count in case of rats kept on valine deficient ration (Groups V, VI, VII and VIII) are considerably lower than that of rats kept on control ration (Groups I, III and IV) while the hemoglobin values for the former group do not show a corresponding decrease as compared to the latter group. This observation is in agreement with the view expressed by Bethard *et al.* (5) that hemoglobin concentration within the vascular system is more important than red cell volume, in regulating erythropoietic rate. Pearson *et al.* (71) stated that hemoglobin formation in rats was more vital than growth.

The administration of iron to rats kept on control ration (Group III) has resulted in a marked increase in the red blood cell, hemoglobin and hematocrit values while it has not produced any significant effect in case of rats kept on valine deficient ration (Group VII). The deficiency of valine might be responsible for the poor effect.

The data showing the effect of administration of ESF to rats kept on control ration (Group II), are not available. However, its effect on rats kept on valine deficient ration (Group VI) show a slight increase in red

blood cell, hemoglobin and hematocrit values when compared with those of Group V. The administration of ESF in combination with iron to rats kept on control ration (Group IV) has resulted in a marked increase in the red cell count, hemoglobin, and hematocrit values while a similar effect is seen in case of rats kept on valine deficient ration (Group VIII).

The administration of ESF either singly or in combination with iron has shown the expected results in case of rats kept on control ration (Group IV) and in those kept on valine deficient ration (Groups VI and VIII). The observation in case of rats kept on control ration are in agreement with those of other investigators (7, 38, 39, 55). The poor response in erythropoiesis in case of rats kept on valine deficient ration might be due to deficiency of valine, as this amino acid is required in the process of erythropoiesis (84). Valine deficiency seems to be concerned in the production of ESF and maturation of RBC, while the ESF seems to be concerned in the multiplication of red blood cells.

The writer did not observe the symptoms of valine deficiency as described by Rose and Eppstein (81), probably because the duration of valine deficiency regimen was short (11 days) as compared to theirs (28 days). However, the rats kept on valine deficient ration were sensitive to touch and some of them showed loss of hair under the neck, abdomen and thighs.

The PFFF plasma had been preserved for the last three years at 5°C. and had retained the erythropoietic activity to a moderate degree. The process of heating the plasma at 99.9°C. for ten minutes and its subsequent preservation at lower temperature does not seem to have affected its erythropoietic activity.

SUMMARY AND CONCLUSIONS

Fifty-six female rats of Sprague-Dawley strain, in eight groups, were utilized for determining the effect of a ration deficient in valine and to evaluate the efficacy of ESF, in the process of erythropoiesis, in the above experiment. Simultaneously, the utilization of iron in the process of erythropoiesis, either singly or in combination with ESF, was also determined.

The results of this study showed that red blood cell count, hemoglobin and hematocrit values in rats kept on the experimental diet were considerably low.

The administration of ESF to the above group resulted in slight increase in the red blood cell count, hemoglobin and hematocrit values. Similarly, the administration of iron produced slight increase in red blood cell, hemoglobin and hematocrit values.

The administration of ESF together with iron to rats kept on experimental ration has produced moderate increase in red blood cell, hemoglobin and hematocrit values.

The data showing the effect of the administration of ESF to rats kept on control ration are not available.

The administration of iron to the above group has shown significant increase in the red blood cell, hemoglobin and hematocrit values. In fact, the blood indices in this group were the highest of the groups studied.

The administration of ESF in combination with iron has produced moderate increase in red blood cell hemoglobin and hematocrit values.

The red blood cell count was considerably lower in case of rats kept on

the experimental diet as compared with controls while the hemoglobin values in case of the former do not show a corresponding decrease as when compared to the latter group.

The administration of iron alone to rats kept on control diet has produced a significant increase in the red blood cell, hemoglobin and hematocrit values while similarly treated rats on experimental diet show a slight increase.

The combined effect of the administration of ESF and iron to rats kept on control ration resulted in a significant increase in red blood cell count while hemoglobin and hematocrit values showed a moderate increase. The combined effect of the administration of ESF and iron in case of rats kept on experimental diet was slight.

The bovine PPF containing the ESF was found to be significantly potent since its storage at 5°C. for the last three years.

Iron seemed to be significantly utilized when given alone or when given in combination with ESF in the controls. However, its utilization in rats on experimental diet appears to be decreased, under similar conditions.

It is postulated that valine is concerned in the production of ESF and maturation of RBC while the ESF is concerned in the multiplication of red blood cells.

The ESF remains significantly potent during its storage at 5°C. since the last three years.

It is suggested that further investigations are needed to explore the role of valine in the process of erythropoiesis and also to elucidate information regarding the dosage and susceptibility of anemic animals of heterogeneous species to the action of ESF.

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THE EFFICACY OF BOVINE ERYTHROPOIETIN IN THE RAT
FED A VALINE DEFICIENT RATION

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Recognition of the importance of dietary protein in the process of erythropoiesis is not recent. Investigations in dynamics of protein metabolism and hemoglobin formation were started in 1918 and interest continues to remain unabated till the present time. With the advent of identification, purification and synthesis of amino acids on a commercial scale, the field of investigation has further widened. The discovery of an erythrocyte stimulating factor(s) in the blood of anemic animals has evoked a great deal of interest in the process of erythropoiesis, especially since 1950.

Valine is an essential amino acid for rats and is one of the many amino acids found in the hemoglobin molecule. These experiments were designed to evaluate its possible role in the process of erythropoiesis. The ESF present in the partial protein free filtrate (PPFF) of bovine plasma was used to trigger off the mechanism of erythropoiesis. Incident to this, the role of iron in erythropoiesis was also studied.

Fifty-six young female rats of Sprague-Dawley strain were utilized as test animals. These were divided at random, in eight groups, each group consisting of seven animals. After recording their pre-experimental weights, the groups were maintained on a basal nitrogen-free diet for a period of eight days. After this period, these groups were kept on their respective experimental diets for 11 days. Eight days later the animals were injected, for three consecutive days. Twenty-four hours after the last injection the rats were post experimentally weighed and approximately two to four ml. of intracardiac blood was withdrawn under ether anesthesia. The blood was collected in heparinized syringes and tests were conducted immediately for red cell count, hemoglobin and hematocrit values. Red cell count and hemoglobin values were determined in duplicate, for each sample.

The results of this study showed that red blood cell count, hemoglobin and hematocrit values in rats kept on experimental diet were considerably low. The administration of PPF plasma and iron, separately, to the above group, resulted in slight increase in red blood cell count, hemoglobin and hematocrit values. The administration of PPF plasma in combination with iron has produced moderate increase in red blood cell count, hemoglobin and hematocrit values.

The data showing the effect of the administration of PPF plasma to rats kept on control diet are not available. The administration of iron shows a significant increase in the red blood cell, hemoglobin and hematocrit values. In fact, the blood indices for this group were the highest.

The administration of PPF in combination with iron has produced a moderate increase in red blood cell, hemoglobin and hematocrit values.

The red blood cell count was considerably lower in case of rats kept on experimental diet as compared with the controls, while the hemoglobin values in case of the former do not show a corresponding decrease as when compared to the latter group.

The administration of iron alone, to rats kept on control ration had produced a significant increase in red blood cell count, hemoglobin and hematocrit values while in case of rats kept on experimental diet, the increase was slight. The effect of the administration of PPF in combination with iron, in case of control rats, resulted in a significant increase in the red blood cell count while the increase in hemoglobin and hematocrit values was moderate. Rats kept on experimental diet, when similarly treated, showed a slight increase in blood indices.

The bovine PPF containing ESF was found to be significantly potent

after being in storage at 5°C. for the last three years.

It is postulated that valine is concerned in the production of ESF and maturation of RBC while the ESF is concerned in the multiplication of red blood cells.

It is suggested that further investigations are needed to explore the role of valine in the process of erythropoiesis and also to elucidate information regarding the dosage and susceptibility of animals of heterogenous species to the action of ESF.