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Erythropoietin: Pharmacology, Biogenesis and Control of Production*

JAMES W. FISHER

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana

I. Introduction

CARNOT and Deflandre (52) postulated in 1906 that a humoral factor controls erythropoiesis and that this factor which they designated "Hemopoietine" is secreted into the blood stream to stimulate red blood cell formation directly in

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the bone marrow. These workers demonstrated an increase in the peripheral red cell count in normal rabbits given injections of plasma from donor rabbits made anemic by bleeding. This hormone has since been more appropriately named "erythropoietin" (erythropoiesis stimulating factor, ESF) (27). For many years after the report of Carnot and Deflandre (52) many people questioned the existence of this hormone because several investigators failed to confirm the presence of an erythropoietic factor in blood after phlebotomy. This may have been due to the lack of sensitivity of the assay systems being used during this period.

It was for many years a well known fact that animals which were exposed to hypoxia developed an increased erythroid cellularity in their bone marrows (18, 22, 75, 186, 212, 378). However, Grant and Root (187, 188) were unable to demonstrate a direct relationship between oxygen concentration in the marrow and the regulation of erythropoiesis. Reissmann's intriguing experiments (356) awakened a renewed interest in a possible humoral control of erythropoiesis when he reported that the exposure of one member of a pair of parabiotic rats to reduced oxygen tension while the remaining partner breathed air at normal oxygen tension resulted in erythropoietic stimulation in both animals. He suggested that a humoral agent had passed across the parabiotic junction from the hypoxic to the non-hypoxic animal to stimulate erythropoiesis in the animal breathing normal air. For many years the Carnot experiments (52) could not be confirmed by some workers. However, Erslev (81) injected large quantities of plasma from phlebotomized rabbits into normal recipient animals and found erythropoietic stimulation (Stohlman *et al.* (397)). The finding of polycythemia in a patient with a patent ductus arteriosus and regional hypoxia below the diaphragm was instrumental in stimulating a clinical interest in erythropoietin.

The recent work reported on the physicochemical characterization of erythropoietin (95–97, 159, 160) and the progress being made in the development of a radioimmunoassay (106–108, 135) of ESF are important advances in this field of research and should stimulate a significant increase in interest in this agent. Most of the work on erythropoietin and the control of erythropoiesis has been reported during the past 15 years. ESF has been found to be a glycoprotein hormone which is produced primarily in the kidney and induces differentiation of an erythropoietin-responsive stem cell in the bone marrow, produces a rapid release of reticulocytes from the bone marrow and a subsequent elevation in reticulocytes in peripheral blood, increases the red cell mass after prolonged injections, and increases the number of erythroid precursor cells in bone marrow which results in an increase in iron incorporation into heme in newly formed red blood cells.

Several books (109, 111, 112, 172, 215, 239, 272, 359, 369, 398, 401, 445) and reviews (26, 37, 82, 110, 166, 186, 191, 201, 245, 246, 255, 287, 399, 400, 412, 441) have been written on erythropoietin and erythropoiesis. The most recent extensive works on erythropoietin and erythropoiesis have been the books published by Krantz and Jacobson (239), Gordon (172) and Fisher (109) as well as the proceedings of recent conferences in Tel Aviv, Israel, edited by Matoth (290), papers presented at a conference on erythropoietin and erythropoiesis in Prague,

Czechoslovakia, edited by Travnicek and Neuwirt (417) and an International Conference on Erythropoiesis in Capri, Italy, edited by Condorelli and Gordon (53). The purpose of this review is to summarize the present state of our knowledge on erythropoietin, especially pharmacological agents which modify ESF production, and to attempt to give direction to future investigations in this field.

II. Assay and Standardization of Erythropoietin

Since erythropoietin is like other protein and polypeptide hormones, in that it cannot be assayed by chemical techniques, bioassay methods have been used to determine the levels of erythropoietin in urine, plasma, tissue extracts, and other body fluids. Assay methods for erythropoietin have been discussed completely by Cotes *et al.* (60, 61), Camiscoli *et al.* (50) and Shore *et al.* (392).

Blood reticulocyte levels in normal animals given injections of erythropoietically active plasma or partially purified urinary erythropoietin was one of the earliest methods used to assay erythropoietin (81, 209). Measurable increases in hemoglobin, hematocrit and marrow erythroblasts in bone marrow of normal animals injected with extracts of plasma were also used in early assays for erythropoietin (167). A significant advance in the search for assay methods for erythropoietin was the technique developed by Plzak *et al.* (349), in which the percent of radioactive iron incorporation into new red cells was measured after injections of erythropoietically active plasma into normal rats.

One of the most difficult problems in erythropoietin research at the present time is the variation in assay methods used in different laboratories throughout the world. For example, reticulocytes may appear in the circulation due to an effect of ESF on a release phenomenon occurring in the reticulocyte compartment of the bone marrow. On the other hand, an increase in radioactive iron in red cells reflects the appearance of the label in newly synthesized hemoglobin in the circulating erythrocytes which have been recently released into the peripheral circulation from the erythroid compartment in the marrow or spleen. These two actions of erythropoietin may differ in that one may require much higher dosages of ESF to initiate. Therefore, discrepancies in the activities of various erythropoietin preparations assayed in different laboratories might be expected.

The most sensitive assay methods for erythropoietin have been those in which the test animal has been prepared in such a way that endogenous erythropoietin production has been suppressed by decreasing tissue oxygen demand by fasting (141) or increasing tissue oxygen supply by the induction of polycythemia with transfusion (216) or hypoxia (62). As pointed out by Cotes (61), a dose of 0.5 to 1.0 International Reference Standard (IRS) units of ESF in the starved rat (377) or a dose of 0.05 to 0.10 (17, 50, 168, 377) IRS units in the polycythemic mouse is necessary to induce a detectable elevation in the percent of ^{59}Fe incorporation in newly formed red cells. In comparison, a dose of 20 to 25 (150) IRS units is required in the normal rat to induce a measurable increase in total red cell mass or hemoglobin. The starved rat assay (141) for erythropoietin is still used in some laboratories but the inability to detect small amounts of erythropoietin with this assay is its major disadvantage. Cotes (61) has dis-

cussed the advantages of the exhypoxic polycythemic mouse (62) and has pointed out that it is more sensitive and possibly less expensive than the starved rat or the normal rat for the assay of ESF. The normal animal is not a satisfactory animal for ESF assay because of the effects of the high background of erythropoiesis which is reflected in the very high iron incorporation in red blood cells (RBC) of the normal animal and may mask the response to ESF.

Some modification of the polycythemic mouse assay for erythropoietin is the most commonly used and probably the most sensitive assay method for erythropoietin. From an economic standpoint, the high cost of transfusions was reduced with the development of the method of Cotes and Bangham (62) in which polycythemia was induced in mice by exposure to an atmosphere of reduced oxygen tension. The exhypoxic rather than the hypertransfused polycythemic mouse is now probably the most commonly used assay system for ESF. The mice are made polycythemic by placement in an atmosphere of either 0.40, such as that recommended by Weintraub *et al.* (435), or 0.50 atmosphere according to the method of Cotes and Bangham (62). The marrow in the exhypoxic polycythemic mouse clears of erythroid cells during the first 4 to 5 days after removal from the hypobaric chamber. At this time the mice are very sensitive to a test dose of erythropoietin and usually ESF is injected in divided doses on days 4 to 5 after removal from the hypoxic atmosphere. A more pronounced erythropoietic response is usually seen when the total dose of ESF is injected in divided doses on separate days (10, 137, 151). Radioactive iron is injected 24 hr later and blood is removed after 2 days to determine the percent of radioactive iron incorporation in newly formed red blood cells. The period that the animals remain in an hypoxic atmosphere has varied in several studies between 2 to 3 weeks. The animals have usually remained at ambient pressure for 1 to 7 days to allow sufficient time for endogenous erythropoietin secretion to cease and to permit a very low basal level of erythropoiesis to be reached.

A very interesting method has been used to expose animals to a low oxygen environment with the use of silicone rubber membrane enclosures (256) which are more permeable to carbon dioxide than to oxygen. Exposure of mice in a tank containing carbon monoxide gas (137) has also been used as the hypoxic stimulus. Linman and Pierre (273) have utilized hyperbaric hyperoxia where the animals are exposed to four atmospheres for 192 hr to reduce endogenous production of erythropoietin and to suppress basal ^{59}Fe incorporation in RBC.

Cotes (61) has emphasized the importance of the selection of a strain of animals which is reproducibly responsive and sufficiently sensitive to exogenous erythropoietin and has called attention to the necessity of insuring homogeneity by the use of an inbred strain of animal. She has also stressed the importance of age in the use of the starved rat assay in that young and old rats are less tolerant to starvation than rats of intermediate age. Older female rats do not appear to tolerate a period of starvation which is sufficiently long to suppress erythropoiesis. The development of gastric ulceration in the fasted rat also presents a problem. The hypertransfused or exhypoxic polycythemic mouse is the most widely used and the most sensitive biological assay being used at the present time.

Assay methods *in vitro* for erythropoietin have also been reported during the past few years. Because of the expense and the amount of material required for the assay system *in vivo*, a simple and inexpensive system *in vitro* would have great appeal. Krantz *et al.* (237) reported a dose-response relationship with erythropoietin on heme synthesis in bone marrow cultures and significant increases in ^{59}Fe incorporation in heme with as little as 0.05 IRS units of human or sheep erythropoietin in human bone marrow cultures (238). The linear part of his dose-response curve was in a range of 0.05 to 1.0 IRS units of erythropoietin. A disadvantage of this system lies in the large amounts of plasma with small concentrations of erythropoietin that must be added to the culture medium *in vitro*. In addition, non-specific inhibition of the system *in vitro* may occur due to impurities present in certain erythropoietin preparations (161). Neuraminidase (275, 385) or acid hydrolysis have been reported (352) to destroy the biological activity of erythropoietin in the system *in vivo* apparently because of the removal of sialic acid. On the other hand, the desialated form of erythropoietin retains its activity when assayed in the marrow system *in vitro* (159). Dukes *et al.* (79) found that different erythropoietin preparations exhibited the same activity in the polycythemic mouse assay but differ from each other in their ability to stimulate heme synthesis and glucosamine incorporation in bone marrow cells in culture. They suggested that the action of erythropoietin may result from the separate stimulation by different factors of specific processes in erythroid cell differentiation. This very interesting difference in the activities *in vivo* and *in vitro* of apparently different molecular species of erythropoietin further emphasizes the problems which arise in interpreting assay data from bone marrow cultures *in vitro* and the polycythemic mouse systems.

Immunochemical assays for erythropoietin have also been reported for erythropoietin and include a double diffusion technique by Krugers Dagneaux (241) and Goudsmit *et al.* (184) for the assay of human plasma erythropoietin, the hemagglutination of tanned red blood cells which have been sensitized with a partially purified erythropoietin (257), and preliminary results with a radioimmunoassay for erythropoietin (106–108, 135). With the use of the double diffusion technique (184), ESF-antiserum was found to cross-react with rat, rabbit and human erythropoietin by neutralizing the biological activity. This assay system appeared to be 1000 times more sensitive than other methods for detecting erythropoietin. Good agreement was seen between the bioassay and the above immunoassay systems in estimating erythropoietin. Goudsmit *et al.* (184) and Krugers Dagneaux (241) by using the double diffusion technique estimated the normal human plasma levels of erythropoietin to be between 0.5 and 2.0 mU/ml.

The reports by some investigators that there may be non-precipitating antibodies to erythropoietin (257, 258, 379) raises certain questions as to whether precipitation parallels neutralization of biological activity in the immunoassay system. Furthermore, Lange *et al.* (257) have reported one antibody which neutralizes the biological activity of erythropoietin and a second antibody which causes hemmagglutination of tanned red blood cells previously sensitized to erythropoietin. Lange *et al.* (257, 259) found that erythropoietin inhibited the

hemagglutination reaction of tanned red blood cells which had been previously sensitized by partially purified erythropoietin. The inhibition correlated well with the amount of erythropoietin found when the same preparation was assayed in the polycythemic mouse. These workers reported a range of 6 to 50 mU of ESF per ml of serum from normal human donors (259). Even though the hemagglutination assay appears to be very promising, until a specific antibody directed solely against erythropoietin is available, it will be difficult to determine whether the hemagglutination inhibition seen is correlated directly with biological activity. Perhaps this method of assay for erythropoietin could be improved if a highly purified erythropoietin were used for the sensitization of red cells.

Fisher *et al.* (106–108, 134, 135) have reported the use of a radioimmunoassay for erythropoietin with a sample of purified human urinary erythropoietin with a specific activity of 8300 units/mg protein. This radioimmunoassay was sensitive enough to detect as little as 0.01 mU of the International Reference Standard erythropoietin (135). This very sensitive system for the detection of erythropoietin should be useful in measuring normal human plasma and urine levels of erythropoietin.

The earliest standard unit for erythropoietin was the "cobalt unit" which was adopted by White *et al.* (441). The erythropoietic effect of cobalt is mediated through enhanced kidney production of erythropoietin (56). One cobalt unit was proposed to be the amount of erythropoietin producing a response in fasted rats which was equivalent to the response seen with 5 μM of cobalt. In the fasted rat assay cobalt showed a variable response depending upon the particular strain used. In addition, some strains have been reported not to respond to cobalt (232). The cobalt unit provided a useful interim standard but has now been abandoned in favor of an International Reference Standard preparation of erythropoietin maintained at the Bureau of Standards, National Institutes of Medical Research, Mill Hill, London, England.

The first International Reference Standard of erythropoietin was a freeze-dried step 4 anemic sheep plasma erythropoietin that contained 23 units/mg of protein (233). The slopes of the regression lines with rabbit, sheep, human and monkey erythropoietin were found not to differ significantly (17, 63) from the International Reference Standard A. One unit of Standard A erythropoietin was roughly 0.05 mg and produced a response which was very close to that of 1 cobalt unit (63, 169). The Standard A material was soon depleted and an International Reference Standard B which was a crude freeze-dried preparation of human urinary erythropoietin (63) was made available. One International Unit of erythropoietin is the activity contained in 1.48 mg of the International Reference Preparation B. Standard B (freeze-dried) has been demonstrated to be stable at 20°C for 2 years (61, 63). More recently a Second International Reference Standard which is human urinary erythropoietin has been made available for use (64). Studies on the mechanism of action and the role of erythropoietin in the pathophysiology of anemias associated with several disease entities have been hampered because of the lack of a sufficiently sensitive assay method to detect levels of erythropoietin in normal human plasma and urine. A more sensi-

tive assay with increased precision and specificity is needed for ESF. A new assay method is also needed for erythropoietin because of the high cost of the polycythemic mouse and fasted rat bioassays.

III. Purification and Physicochemical Characterization of Erythropoietin

Purification of erythropoietin has been hampered because of the limited supply of erythropoietin available for study. Most of the studies on purification of erythropoietin have been with a plasma erythropoietin preparation obtained from phenylhydrazine anemic sheep and human urinary erythropoietin from patients with various types of anemia. Gordon *et al.* (167) and Borsook *et al.* (28) were among the earliest workers to study the chemical composition of erythropoietin and found that erythropoietically active plasma was resistant to boiling. Several workers reported that the active principle in plasma was confined to the globulin region (247, 276, 441). Slaunwhite *et al.* (394) found that erythropoietin was destroyed by trypsin and suggested that lysine or arginine was contained in the polypeptide chain of erythropoietin. In studies on an erythropoietically active fraction of boiled filtrate from anemic rabbit plasma, Rambach *et al.* (353) demonstrated that erythropoietin is associated with an α_2-globulin. Erythropoietin is considered to be a glycoprotein and sialic acid is a part of the active molecule. Lowy *et al.* (275) and Schooley and Mahlmann (384) reported that erythropoietin is inactivated by neuraminidase when assayed *in vivo*. Goldwasser (161) found in further studies that erythropoietin is still capable of stimulating heme and stromal synthesis in a bone marrow culture preparation *in vitro* after removal of sialic acid by either acid hydrolysis or the enzyme neuraminidase. It was postulated from the above studies that sialic acid may protect erythropoietin *in vivo* from inactivation and therefore is necessary for transport in plasma (161) and that the sialic acid moiety is not an important determinant in the combination of ESF with its antibodies (384).

Allen *et al.* (11) have also reported studies on partially purified erythropoietin from phenylhydrazine-anemic sheep plasma and mouse plasma erythropoietin with a flat bed discontinuous acrylamide gel electrophoresis system. In separating the active material from the acrylamide gel, the protein band migrating between the α_1-globulin and albumin was found to be erythropoietically active. These workers postulated that two molecular species of erythropoietin were present in their sample, one having a molecular weight of 40,000 and a second of approximately 100,000. These workers (11) also applied their acrylamide gel technique to human urinary erythropoietin and reported erythropoietic activity in the postalbumin region (11). Rosse (370) reported that erythropoietin was elongated in shape and had a molecular weight of about 27,000 when he used the technique of inactivation by ionizing radiation for his molecular weight studies. However, Hodgson (210) found a target size of 68,000 when he used *gamma* ray inactivation for his studies.

Goldwasser *et al.* (159, 160, 162, 163) have reported a high degree of purification of erythropoietin from phenylhydrazine-anemic sheep plasma. The initial

step in this procedure involved column fractionation on DEAE-cellulose and IRC-50 which gave a specific activity in the first fractions in a range of 0.8 to 1.6 units/mg of protein (159). The passage through an XE-97 column yielded an eluate with a potency of 2 to 4 units/mg (step 3) (159). Ammonium sulphate precipitation of this step 3 material was used to yield a step 4 material with a specific activity of 30 units/mg of protein (159). Goldwasser and Kung (159, 160) have reported more recently the apparent complete purification of erythropoietin from anemic sheep plasma with a purification factor of more than 1 million. Their technique involved further fractionation of the step 4 anemic sheep plasma erythropoietin by dissolving in lithium chloride, precipitation with alcohol, desalting and chromatographing on sulfoethyl-Sephadex, elution with acetate buffers, adsorption on and elution from calcium phosphate gel, chromatographing on a methylated albumin-Kieselguhr (MAK) column and elution with a phosphate buffer (159). When the final fraction which was eluted from the MAK column was examined by micropolyacrylamide gel electrophoresis, the specific activity obtained was reported to be 8250 units/mg of protein with a 1,180,000 purification factor. The only significant contaminant contained in this purified preparation was desialated erythropoietin. In that the small amount of desialated erythropoietin in this purified preparation was not active *in vivo*, these workers inferred that the active material in their final preparation had a potency of 9200 units/mg of protein. This is considered to be both sialic acid containing erythropoietin and desialated erythropoietin which was found to be active in a rat bone marrow culture system. In a further report (160) Goldwasser and Kung reported a molecular weight of 45,800 for sheep plasma erythropoietin with an ^{125}I labeled erythropoietin which was subsequently studied with electrophoresis in sodium dodecyl sulphate-containing polyacrylamide gel. They also suggested that the molecule was a single peptide chain containing approximately 30% carbohydrate (galactose, mannose, glucosamine, glucose and sialic acid) with the remainder being protein. Micro determination of amino acid composition showed that there was no methionine or cysteine present and only a trace of proline. The carbohydrate content was a total of 30% of which 10.8% was sialic acid.

An impressive contribution to the chemistry of human urinary erythropoietin has recently been the work reported by Espada and Gutnisky (95, 96) with erythropoietically active urine from patients with anemia associated with hookworm disease (*Necator americanus*). These workers (95, 96) precipitated the erythropoietically active protein in urine with benzoic acid and ethanol, heat-reacted the protein precipitate, fractionated on DEAE-cellulose, hydroxyapatite, Sephadex-G-25, a second fractionation on DEAE-cellulose, Sephadex G-100 column fractionation and recycling of the erythropoietically active fractions from Sephadex G-100 at least three times to arrive at a specific activity in the final Sephadex G-100 fraction in the range of 8300 units/mg of protein. Proof of purity was based on the symmetry and superimposition of the biologically active and protein curves (95); continuity of the identity lines of the purified erythropoietin with less pure preparations (71); and the localization of all of the biologi-

cal activity in a single protein band when the purified erythropoietin was subjected to polyacrylamide gel electrophoresis (97). The electrophoretic mobility was reported to be slightly less than that of human serum albumin. This purified human urinary erythropoietin was reported to contain 65.5% protein, 13.0% total hexoses, 8.9% hexosamine and 7.5% sialic acid (97). The amino acid composition was aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine (97).

This high specific activity erythropoietin was labeled with ^{125}I for radioimmunoassay studies and separated on Sephadex G-100 (107, 108, 135). The front edge of the protein peak in the fraction which immediately followed the void volume from the Sephadex G-100 column (108) contained most of the erythropoietic activity. The isolation and purification of erythropoietin reported by Goldwasser and Kung (159, 160) and Espada and Gutnisky (95) are important advances in this field of research. It is of utmost importance that sufficient amounts of purified erythropoietin be made available for further physicochemical studies and for use in the treatment of erythropoietin deficiency anemias. It is also important that sufficient amounts of purified erythropoietin be made available before the widespread use of radioimmunoassay for erythropoietin can be achieved.

IV. Sites of Production of Erythropoietin

The search for the site of erythropoietin production has been the subject of numerous investigations involving organ excision experiments and studies of the erythropoietic activities of various organ and tissue extracts. Gordon *et al.* (170, 171) studied extracts from liver, spleen, lung, bone marrow, muscle, brain, thymus, and red blood cells of anemic rabbits and were unable to demonstrate erythropoietic activity. Jacobson *et al.* (217) removed the gonads, adrenals, stomach, intestines, pancreas, and thymus and found that the response to cobalt was unaltered from that of sham-operated controls. These workers (217) also removed 90% of the liver in rats and found that the erythropoietin titers in plasma after administration of cobalt were the same in the partially hepatectomized as in the sham-operated animals. However, Katz *et al.* (230) reported that the erythropoietin levels in plasma were lower in partially hepatectomized than in sham-operated controls exposed to hypoxia. Perfusion of livers with hypoxemic blood (357) or perfusion of livers from animals after administration of cobalt (41) have been reported with increased erythropoietin titers in the perfusates. However, Kuratowska *et al.* (248) by use of a hypoxemic perfusion system with a rabbit liver and Fisher *et al.* (113) with the dog liver perfused with blood containing cobalt, were unable to demonstrate an increase in erythropoietin titers in the perfusates. Splenectomy did not modify the erythropoietic response to cobalt in rats (217) nor was erythropoietic activity demonstrated in splenic extracts (171). Neither did perfusion of the spleen with hypoxemic blood increase erythropoietin titers in the perfusate (248). In that irradiation of the bone marrow of rats did not modify their erythropoietic response (274, 297) to hypoxia, the

bone marrow would not appear to be a site of ESF production. It is of further interest that red cell hemolysates have been reported to stimulate erythropoiesis (91, 376). Erslev (91) reported that red cell hemolysates increased radioactive iron utilization in normal rats and that nephrectomized recipients failed completely to respond to hemolysates with an increase in the rate of iron utilization. Erslev (91) also reported that the erythropoietic response of hypertransfused rats to red cell hemolysates was completely blocked by anti-ESF serum. Bilirubin has recently been reported to stimulate radioiron incorporation in red cells of fasted rats (366) and the erythropoietic effect was partially blocked by the antibody to ESF. Hemoglobin and hemin have also been found to stimulate erythropoiesis in rats and gerbils and the erythropoietic effects of hemoglobin were blocked in gerbils by anti-ESF (208).

The pituitary gland was once postulated to be a site of production of an erythropoietic factor (54–56). However, extensive investigations, with hypophysectomized and adrenalectomized rats, have clearly demonstrated that the pituitary gland is not a site of erythropoietin production (65, 100, 142, 197, 346). ACTH from the anterior pituitary has been demonstrated to exert its actions secondarily through an action on the adrenal cortex (418).

The kidney is now known to be the major site of production of erythropoietin. The classical experiments of Jacobson *et al.* in 1957 (218) first clearly demonstrated in both rats and rabbits that bilateral nephrectomy abolished the erythropoietic response of these animals to bleeding or cobalt administration (219). Rats with their ureters ligated showed an elevation in blood urea nitrogen but still demonstrated an erythropoietic response to cobalt or bleeding. The kidneys would appear to be the sole site of erythropoietin production in the dog. Erythroblasts almost completely disappear (314) from the bone marrows of nephrectomized dogs and a marked suppression in radioactive iron incorporation into newly formed red cells is seen in peripheral blood (312). In addition, no change in plasma erythropoietin levels was seen in nephrectomized dogs subjected to a bleeding stimulus (315, 316). On the other hand, other species such as rabbits (83), tamarins (298, 299), and rats (143, 380), exhibited a slight to moderate elevation in plasma erythropoietin titers after nephrectomy when exposed to an hypoxic stimulus. Fried *et al.* (143) demonstrated that the extrarenal erythropoietin produced in rats is very similar to kidney erythropoietin in that it is capable of stimulating heme synthesis in marrow cultures and can be neutralized by erythropoietin antiserum. Halvorsen *et al.* (197) found that nephrectomy almost completely abolished the erythropoietic response of rats and rabbits to hypoxia. A slight increase in erythropoietic activity was seen in plasma of nephrectomized rabbits exposed to hypoxia which was not abolished by hypophysectomy. Thus, the pituitary does not appear to be essential for the slight erythropoietic activity found in nephrectomized rabbits exposed to hypoxia (197). Fried (140) has recently reported that hepatectomy abolished the extrarenal erythropoietin produced in nephrectomized rats exposed to hypoxia and suggested that the liver plays a major role in extrarenal ESF production.

Erythropoiesis in bilaterally nephrectomized human subjects awaiting kidney

transplantation was reported to be decreased but still measurable by Nathan *et al.* in 1964 (323). They concluded that the human subject was able to maintain a low basal level of erythropoiesis even in the absence of the kidney (323). In further studies Nathan *et al.* (324) reported a decrease in the rate of appearance of ^{59}Fe in red cells of uremic patients before nephrectomy as well as in anephric patients. More recently (325) these workers concluded that deficiency of renal erythropoietin may contribute to the anemia of renal failure but postulated that this defect alone could not explain the anemia or the multiple defects of cell proliferation seen in this condition. They felt that the presence of myelosuppressive toxins retained, perhaps generated in the gastrointestinal tract by bacterial metabolism, would seem to be just as important an offender as deficiency of kidney erythropoietin. It is of interest that detectable erythropoietin levels could not be demonstrated in bilaterally nephrectomized patients before kidney transplantation (1, 70, 281). However, after kidney transplantation, measurable levels of erythropoietin were seen in plasma of most of the patients studied. In fact, one patient was reported (437) who developed an erythrocytosis after kidney transplantation and the hematocrit eventually increased to 60% with a concurrent elevation in erythropoietin levels in urine. Several investigators have reported elevations in plasma levels of erythropoietin in anephric patients when the hematocrit was reduced to extremely low levels (84, 114, 300, 317, 318). Therefore, it would appear that if the erythropoietic stimulus is sufficiently potent or the hematocrit is reduced to very low levels, extrarenal erythropoietin production or activation in the anephric human subject can be evoked.

More direct evidence for the role of the kidney in erythropoietin production is the finding by Kuratowska (248, 249) of increased titers of erythropoietin in perfusates of the isolated rabbit kidney perfused with hypoxemic blood and the findings of Fisher *et al.* (115, 116) of increased erythropoietin levels in the perfusates of the isolated dog kidney perfused with cobalt or hypoxemic blood. Other investigators have confirmed that the isolated perfused rabbit (357) or dog kidneys (116, 449) perfused with hypoxemic blood results in increased titers of ESF in the perfusates. In addition, Pavlovic-Kentera *et al.* (339) completed an impressive experiment in which blood was shunted from the right atrium of dogs to perfuse the kidney and found significant elevations in erythropoietin titers in the perfusates after 7 to 8 hr in dogs breathing an atmosphere of 10% oxygen. Histological examination of kidneys were performed in the perfusion studies reported by Fisher *et al.* (116) and no correlation could be found between erythropoietin production and damage to the kidney. These investigators (116) suggested that erythropoietin released from the kidney during perfusion was not likely due to a degenerative change in renal cells. However, Erslev *et al.* (85) were unable to demonstrate an increase in plasma erythropoietin titers in the isolated perfused dog-lung-kidney preparation with hypoxemic blood.

Studies on the cytological localization of erythropoietin within the kidney has provided evidence for both a renal cortical and a renomedullary source for erythropoietin. Sokabe and Grollman (395) have found that removal of the kidney cortex resulted in a decrease in peripheral red cell counts while removal

of the medulla failed to produce any change in peripheral red cell values. In addition, Muirhead *et al.* (312) demonstrated much higher levels of erythropoietic activity in cortical than in medullary kidney extracts from anemic dogs. Jensen and Thorling (224) found an isoenzyme pattern in the kidney cortex following an anemic or a cobalt stimulus which resembled the type of pattern seen with tissue hypoxia. Fisher *et al.* (117) localized erythropoietin in the glomerular tuft of the sheep kidney utilizing the indirect fluorescent antibody technique. The fluorescence was seen in the capillary wall of the glomerular tuft but not in the peritubular capillary beds, juxtaglomerular cells or capillary walls of spleen, liver or lung. This type of fluorescence in the peripheral portion of the anemic sheep kidney glomerulus was confirmed by Frenkel *et al.* (139) with the fluorescent antibody technique. More recently Busuttil *et al.* (44, 45) have demonstrated intense fluorescence in the glomerular tufts of anemic human and hypoxic dog kidneys. Busuttil localized the fluorescence in the peripheral portion of the glomerular tuft of the anemic human (44) and the hypoxic dog kidney (45) and suggested that the localization was in the epithelial cells of the tuft. Burlington *et al.* (42) produced erythropoietin in a renal glomerular culture and found an overgrowth of epithelial cells in the glomerular tuft. Further electron-microscopic studies are necessary to completely identify the cells in the glomerular tuft showing this fluorescence. Incubation of anti-ESF with highly purified erythropoietin blocked the fluorescence seen in the glomerular tufts of the human (44) and the dog (45) kidneys. In addition, elevating blood levels of erythropoietin in normal dogs (45) with an intravenous infusion of ESF failed to increase the fluorescence in the glomerular tuft indicating that a non-specific trapping of ESF by the glomerulus, when plasma ESF titers are elevated, is not likely involved. Photomicrographs of glomeruli from anemic human (44) and hypoxic dog (45) kidneys from Busuttil's experiments (44, 45) are shown in figures 1A and 1B.

The juxtaglomerular (JG) apparatus was first postulated by Osnes (330) to be a site of control of erythropoietin production. He found that daily bleeding produced a decrease in the number and the size of the granules in the juxtaglomerular cells (330). On the other hand, Hirashima and Takaku (207) noted an increase in the JG granules in the early stages of hypoxia and a decrease in the granularity of the JG cells in rats after hypertransfusion. The work of Hirashima and Takaku was confirmed by Imamura (213) who found in addition that the increase in granularity of the JG cells after removal of the adrenals was reversed when the animals were made anemic by administering phenylhydrazine. On the other hand, Goldfarb and Tobian (157, 158) found no change in JG granularity following an increase in erythropoietin titers in rats exposed to acute hypoxia. However, Demopoulos *et al.* (69) found if hypoxia was increased further that a decrease in granularity of the juxtaglomerular cells was seen concurrent with an elevation in erythropoietin titers in plasma. Donati *et al.* (72) reported that the secretion of renin and erythropoietin could be dissociated. Salt restriction increased and desoxycorticosterone decreased renin secretion without changing the production of erythropoietin. On the other hand, salt loading, acute

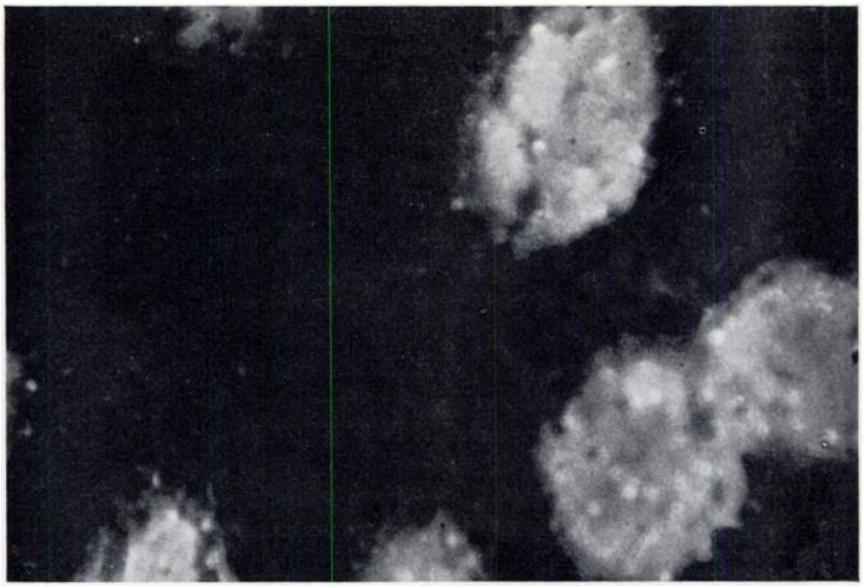

FIG. 1A. Low power (100 ×) fluorescence photomicrograph of an anemic human kidney section treated with anti-erythropoietin serum. (Busuttil *et al.*, Proc. Soc. Exp. Biol. Med. **137**: 327, 1971.)

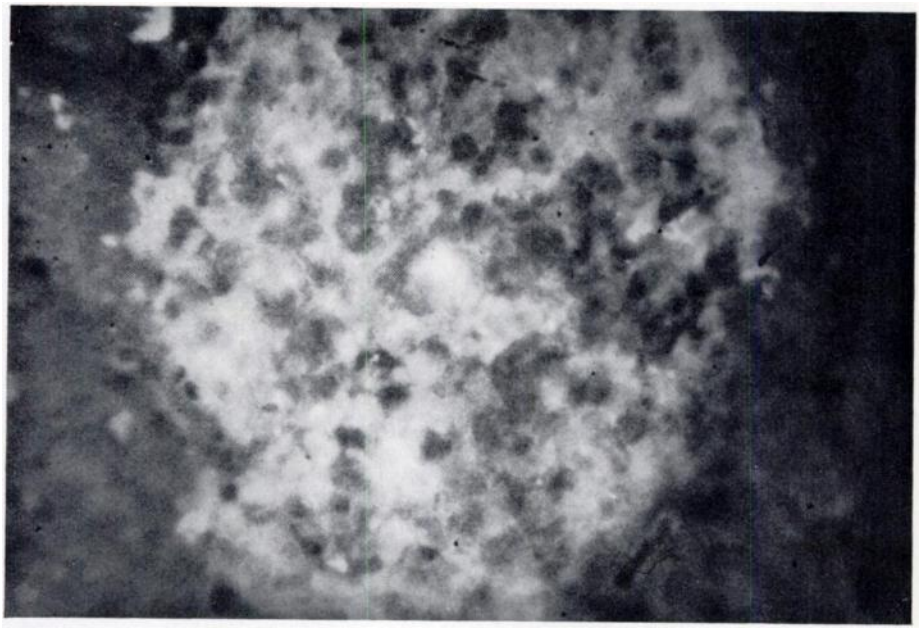

FIG. 1B. High power (440 ×) fluorescence photomicrograph of a posthypoxic dog kidney section treated with anti-erythropoietin serum. (Busuttil *et al.*, Acta Haemat., in press, 1971.)

hypoxia, and cobalt injections increased erythropoietin secretion without affecting the elaboration of renin. Mitus *et al.* (307) produced experimental hydronephrosis and polycythemia in rabbits and found a decrease in JG cell granularity in these kidneys. From these data it would appear that a correlation between juxtaglomerular cell granularity and erythropoietin levels in plasma has not been established. The alterations seen in the granularity of juxtaglomerular cells during and after exposure to hypoxia may be secondary changes which are not associated with erythropoietin production.

V. The Renal Erythropoietic Factor (REF or Erythrogenin)

The reports on recovery of erythropoietin in renal extracts or homogenates have been so variable that this has led some investigators to search for a kidney factor which in itself is not erythropoietically active but is capable of generating erythropoietin in systems *in vitro* and/or *in vivo*. Kuratowska (246, 250, 251) reported erythropoietic activity in the 600-g nuclear fraction of an anemic rabbit kidney homogenate after incubation with either plasma or an α-globulin. This factor had no significant erythropoietic activity alone but generated significant activity when incubated with a proper plasma substrate. Kuratowska (251) postulated that the kidney produced an erythropoietic factor which combined with an α-globulin in plasma to form an erythropoietically active stable complex which she proposed was erythropoietin. This renal erythropoietic factor (REF) was found to be only 50% as high in normal as in kidneys from anemic animals.

Contrera *et al.* (57) also found an erythropoietic factor in the kidneys of anemic mice which stimulated erythropoiesis in polycythemic mice after incubation with normal plasma. In further studies, Contrera *et al.* (58, 59) by using an osmotic sucrose gradient and high speed centrifugation, separated this factor in the "light mitochondrial" fraction of the hypoxic rat kidney. The light mitochondrial fraction was the most active fraction tested when compared with the microsomal, nuclear and soluble kidney fractions. The light mitochondrial fraction from normal rat kidneys when incubated with normal rat serum was also demonstrated to have erythropoietic activity (59). Further studies by Zanjani *et al.* (450) showed that prolonged incubation of the light mitochondrial fraction with normal rat serum caused a significant loss of activity which was prevented by dialysis of the normal rat serum against disodium ethylenediaminetetraacetate (EDTA) and subsequent dialysis against water to remove the EDTA. These authors postulated that the presence of cations in normal serum were necessary for the action of inhibitors of the renal erythropoietic factor. Zanjani *et al.* (451) presented evidence that the renal erythropoietic factor is an enzyme which acts on a substrate present in normal serum to produce ESF because during incubation of the REF and serum a first order kinetics reaction was seen.

In further studies Gordon *et al.* (173) and Zanjani *et al.* (452) have reported that the light mitochondrial fractions from kidney cortex, tubules, medulla and glomeruli contained this renal erythropoietic factor. Thus, it would appear that this factor is distributed throughout the kidney. The complete details of the extraction procedure and assay in polycythemic mice are included in recently

published articles by Gordon *et al.* (175, 176). It is of interest that an antiserum developed against this renal erythropoietic factor (erythrogenin) inhibits erythropoiesis in mice (279).

Malgor *et al.* (283) have presented data showing that the light mitochondrial fraction of the hypoxic dog kidney exhibits enhanced erythropoietic activity when incubated with EDTA-dialyzed dog plasma. The dog plasma alone showed no significant activity when assayed in polycythemic mice, whereas, the hypoxic light mitochondrial fraction from the hypoxic dog kidney incubated with saline showed only slight erythropoietic activity. Erslev and Kazal (86) were unable to demonstrate significant erythropoietic activity when the light mitochondrial fraction from the hypoxic rat kidney was incubated with rat serum and assayed in hypertransfused polycythemic mice. However, Schooley *et al.* (382) reported significant erythropoietic activity of these incubated samples when tested in the hypertransfused mouse. Gordon *et al.* (176) have proposed a scheme for the biogenesis of erythropoietin and a negative feedback influence of erythropoietin on substrate production. They proposed that hypoxia stimulates the kidney to produce a renal erythropoietic factor or "erythrogenin" which acts on a plasma factor, possibly from the liver, which they term "erythropoietinogen" to generate plasma "erythropoietin." High levels of erythropoietin in plasma are postulated to act as a negative feedback on production of erythropoietinogen (176). The erythropoietin in plasma acts upon an erythropoietin-responsive cell (ERC) to cause it to differentiate into proerythroblasts.

VI. Renal Control of Erythropoietin Production

The role of the kidney in the day-to-day control of erythropoietin production and feedback mechanisms for ESF are not completely understood. Jacobson *et al.* (141, 220) proposed several years ago that the ratio of oxygen supply to oxygen need or demand probably determines the level of erythropoiesis (141). We have postulated previously (110) a scheme for the role of renal cell oxygen as the primary control mechanism for erythropoietin production and a modification of this scheme is shown in figure 2. Our hypothesis states that the rate of renal erythropoietin production is inversely proportional to the concentration of oxygen in a critical renal cell. The oxygen sensor mechanism which detects small changes in renal cell oxygen and triggers the production of renal erythropoietic factor (erythrogenin) may be an enzyme system sensitive to changes in oxygen content. As described earlier, erythrogenin is probably released from a renal cell to act on a plasma substrate to generate erythropoietin.

Direct evidence for the role of renal cell oxygen in erythropoietin production is provided with the finding that hypoxemic perfusion of the isolated kidney stimulates erythropoietin production (113, 115, 116, 248, 249, 339, 357, 449). As noted in figure 2, a relative decrease in renal cell oxygen levels can be produced by either increasing oxygen demand or decreasing oxygen supply to the kidney resulting in increased production of erythropoietin. Agents which increase renal cell oxygen utilization probably stimulate erythropoietin production by creating a relative oxygen deficit in a critical renal sensor cell. Agents which are known

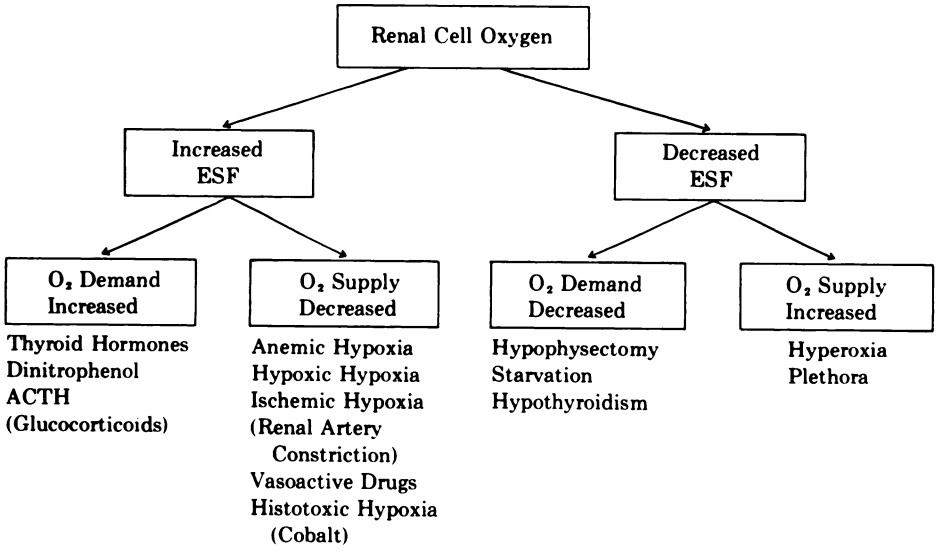

FIG. 2

to stimulate erythropoietin production and increase the demand for oxygen in the kidney as well as other tissues are thyroid hormones (98, 99, 102), dinitrophenol (99, 293) and ACTH (through the release of adrenal glucocorticords) (118). Thyroxin, 3′,5′,3-triiodothyronine, ACTH, and adrenal glucocorticoids have been found to increase metabolic rate and to elevate red cell mass in hypophysectomized rats (66, 99, 118). In addition, chronic injections of thyroxin have been reported to increase both red cell mass and metabolic rate in normal rats (99). Relative renal cell hypoxia can also be produced by decreasing the oxygen supply to the kidney (as noted in figure 2) to activate or stimulate REF production and thus ESF generation. A decrease in renal cell oxygen can be produced directly by anemia (anemic hypoxia) (202, 203, 214, 223, 319, 328, 419) and by hypoxia (hypoxic hypoxia). Studies have been reported showing a direct correlation of erythropoietin production and the degree and duration of hypoxia (51, 88, 393). Local renal vascular constriction is also known to produce hypoxia (ischemic hypoxia) and an increase in erythropoietin production or secretion. Enhanced erythropoietin production can be evoked by renal artery constriction with a clamp or ligature (119–121), and vasoactive drugs, such as angiotensin (120) and norepinephrine (122). A molecular deprivation of oxygen to the kidney (histotoxic hypoxia) can also be induced by chemical agents such as cobalt (110, 115, 116, 224) to stimulate erythropoietin production. Cobalt has been demonstrated to decrease oxygen utilization in the isolated perfused kidney (116) (see figure 4) and in kidney homogenates (269).

The negative feedback mechanism which controls erythropoietin production is also postulated to be related to renal cell oxygen tension. Elevated plasma levels of erythropoietin have been suggested previously to play a significant role in the feedback mechanism (176, 221). In addition, plasma erythropoietin levels in men

and mice exposed to high altitude reached maximum concentrations after 19 to 39 hr and then decreased rapidly with continued exposure to high altitude hypoxia (2). However, more evidence is available to support a role of renal cell oxygen tension. A relative increase in renal cell oxygen tension could occur in conditions where there is a decrease in demand for oxygen, such as after hypophysectomy (67, 98), starvation (308), and hypothyroidism (67, 98, 293). These conditions are associated with a decrease in the demand for oxygen and a decrease in erythropoiesis. A relative increase in renal cell oxygen associated with decreased erythropoietin production may also be produced by increasing the oxygen supply through hyperoxia (273) or plethora induced by transfusion or an elevation in red cell mass (192, 354). Further support for the hypothesis that decreased erythropoietin production is due to a decreased demand or an increased supply of renal oxygen is the report that hypophysectomized rats responded to severe hypoxia but not to mild hypoxia (320) as well as the observation that the hypertransfused rat responded with an increase in erythropoiesis to severe hypoxia but not to mild hypoxia (320). These conditions are probably associated with an increased oxygen carrying capacity of the animal in relation to his need.

VII. Pharmacological Agents Which Modify Production of Erythropoietin

A large number of chemical agents have been demonstrated to modify erythropoietin production and to exert their actions by either increasing or decreasing kidney ESF production. Some agents act through a renal hypoxic mechanism by: 1) decreasing renal blood flow (*e.g.*, vasoactive agents); 2) increasing renal cell oxygen utilization; and 3) interfering with kidney oxygen utilization (*e.g.*, producing histotoxic hypoxia with cobalt). Chemical agents may also stimulate kidney production of ESF directly or else decrease the level of inhibitors of ESF production.

1. Vasoactive agents

Angiotensin, norepinephrine, and vasopressin are agents which stimulate erythropoietin production probably through a reduction in renal blood flow. It has been demonstrated previously that renal artery constriction in rabbits (121, 413) and in dogs (130, 131) stimulates erythropoietin production.

a) Angiotensin. Angiotensin was first reported by Fisher and Crook (118) to increase erythropoiesis in hypophysectomized rats. Some investigators (25, 148, 285, 450) were unable to demonstrate an increase in erythropoiesis in polycythemic animals after injections of angiotensin. However, the erythropoietic effects of angiotensin were recently clarified by Fisher *et al.* (120, 122) and Nakao *et al.* (321) who have demonstrated that the effects of angiotensin on ESF production are related to the pharmacological effects of angiotensin on the renal vasculature to produce renal hypoxia by decreasing renal blood flow which results in increased erythropoietin production. In addition, Fisher *et al.* (120) have demonstrated that the pharmacological effects of angiotensin on erythropoiesis

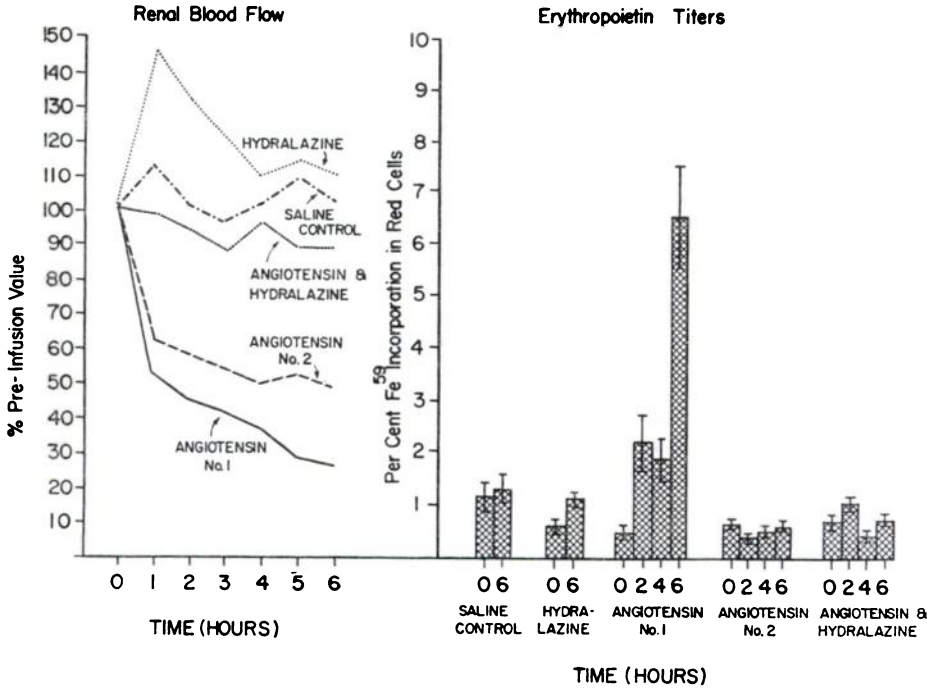

FIG. 3. Mean ^{59}Fe incorporation in red cell values in polycythemic mice during 6 hr continuous infusion of saline, angiotensin, hydralazine, or angiotensin plus hydralazine. Five to six mice were used to assay each dog plasma sample. The standard error of the mean is given at the top of each bar (five dogs in each group except the angiotensin No. 2). Asterisk (*) indicates significantly different from the respective 0-time value at the 1% level. Angiotensin No. 1 represents the mean of five dogs where renal blood flow was reduced below 1.0 ml/g/min after 4 hr. Angiotensin No. 2 includes the mean of four animals where RBF was reduced but never reached a level of 1.0 ml/g/min. (J. W. Fisher, Kidney Hormones, ed. by J. W. Fisher, p. 343, Academic Press, London and New York, 1971.)

in mildly plethoric mice can be blocked by the antibody to erythropoietin. Fisher *et al.* (122) were unable to stimulate an increase in ^{59}Fe incorporation in polycythemic mice given injections of angiotensin subcutaneously in oil. Renal blood flow must be reduced to a level below 1 ml/g of kidney per minute before sufficient hypoxia is produced to stimulate erythropoietin production (120). This explanation for the action of angiotensin is apparently related to the unique relationship between renal blood flow and oxygen extraction from renal arterial blood. In contrast to other organs, a moderate reduction in kidney blood flow results in a decrease in renal uptake of oxygen (263, 415). Only when renal blood flow is reduced to a level at which glomerular filtration ceases (less than 1 ml/g/min) and reabsorption of sodium is reduced to a very low level does the kidney extract more oxygen per milliliter of blood in response to reduction in renal blood flow thus producing renal hypoxia. It has been suggested that in the intact mammalian kidney there is a fixed relationship between sodium transport and oxygen consumption.

In order to determine whether the effects of angiotensin on erythropoietin production were correlated with reduction in renal blood flow (RBF), Malgor and Fisher (284) antagonized the effects of angiotensin on renal blood flow and erythropoietin production by an injection of hydralazine. As indicated in figure 3, angiotensin infusion over a period of 6 hr produced a significant rise in plasma levels of erythropoietin (5 dogs indicated as angiotensin number 1 group) when RBF was depressed below 1 ml/g/min during the last 4 hr (between 2–6 hr) infusion. On the other hand, when RBF was decreased but was not sufficient to reduce renal blood flow below approximately one-third of normal RBF, which is equivalent to 1 ml/g/min, during the 6-hr infusion period with angiotensin (4 dogs indicated by angiotensin number 2) or when the effect of angiotensin on RBF was blocked by hydralazine, no elevation in plasma levels of ESF occurred. Hydralazine alone produced an increase in RBF but no change in plasma ESF levels. Infusion of angiotensin II into human subjects has also been reported to increase plasma levels of erythropoietin (31, 72, 345). In addition, angiotensin has been demonstrated to increase erythropoiesis in starved rats (340) and to cause an increase in erythropoietin extracted from rat kidneys (345). Abbrecht *et al.* (3) have shown that when renal blood flow was reduced by the injection of microspheres into the renal artery both erythropoietin and renin levels were increased in renal vein blood but the peak elevation in renin occurred at 4 hr whereas the peak ESF levels were seen at 6 hr after the injection of microspheres This different time course of appearance of renin and ESF in renal vein blood after an ischemic stimulus may indicate that even though both hormones are influenced by changes in renal blood flow ESF requires a slightly more prolonged stimulus than renin.

b) *Norepinephrine.* Intravenous infusion of low doses of norepinephrine cause an increase in renal blood flow (RBF), glomerular filtration rate (GFR) and urine output, whereas high doses produce a decrease in RBF, GFR and urine production (261). Norepinephrine in very high doses has been reported to stimulate erythropoiesis in starved rats (340). In addition, elevated plasma levels of erythropoietin and erythrocytosis have been reported in patients with a pheochromocytoma (36, 429), a catecholamine-producing tumor. Norepinephrine (24, 122) infusions have also been reported to decrease renal blood flow and to increase plasma erythropoietin levels. Fisher *et al.* (122) found that norepinephrine decreased renal blood flow and increased erythropoietin production in a manner similar to that described for angiotensin. These experiments in dogs revealed that the decline in renal blood flow with norepinephrine was not as rapid as that seen with angiotensin and the elevation in plasma levels of erythropoietin seen with norepinephrine was not as marked as with renal artery constriction with a Goldblatt clamp or angiotensin.

c) *5-Hydroxytryptamine.* Serotonin (5-hydroxytryptamine,5-HT) has been reported to stimulate radioactive iron incorporation in polycythemic mice (277, 326, 327). In an attempt to clarify the mechanism of the erythropoietic action of 5-HT, Noveck and Fisher (327) found that the antibody to erythropoietin blocked, for the most part, the effects of 5-HT on ^{59}Fe incorporation in poly-

cythemic mice, thus indicating that this action of 5-HT was erythropoietin-dependent. These workers (327) also demonstrated a dose-response relationship for 5-HT in polycythemic mice. In further studies, a rise in plasma levels of erythropoietin was seen in dogs after infusion of 5-HT into the renal arteries. Measurements of renal artery blood flow did not show a sufficient reduction in renal blood flow in dogs, based on earlier studies in which renal artery flow was reduced with a modified Goldblatt clamp, and ESF titers in plasma followed (119), to explain the elevation in erythropoietin titers. Therefore, these authors (277) postulated that the action of 5-hydroxytryptamine was either due to a more marked reduction in intrarenal blood flow than that reflected in the measurement of renal arterial flow or else 5-HT had a direct action on the kidney.

d) Vasopressin. Vasopressin is a very potent vasoconstrictor and is found in posterior pituitary extracts. This compound is well known to produce reduction in renal blood flow. An increase in radioactive iron incorporation in RBC of polycythemic mice has been reported by Jepson *et al.* (225) with vasopressin. These workers (225) also reported an elevation in erythropoietin titers in urine of a hypopituitary patient receiving vasopressin. It is not clear how vasopressin exerts its erythropoietic action but the most likely explanation would seem to relate to its effect on intrarenal vasoconstriction, renal hypoxia and kidney erythropoietin production. Vasopressin has also been reported to increase cyclic AMP levels in the kidney (20) which may also be related to its erythropoietic action.

e) Prostaglandins. Prostaglandins are potent dilators of most vascular beds and may possibly be involved with the control of renal blood flow (267) and are known to increase cyclic nucleotides in some tissues (46). Paulo *et al.* (332) and Wilkerson *et al.* (442) have reported that prostaglandins PGE_1 will stimulate radioactive iron incorporation in RBC of polycythemic mice and increase ESF titers in the perfusates of isolated perfused kidneys from dogs previously exposed to hypoxia. Schooley and Mahlmann (383) have also reported that PGE_1 and PGE_2 stimulate radioactive iron incorporation in red cells of plethoric mice, which was erythropoietin dependent, whereas $PGF_{2\alpha}$ was inactive in this system. Prostaglandins of this type have been isolated from the kidney (410) and are known to act on many systems. Prostaglandins may exert a direct action on the kidney to stimulate production of erythropoietin and its effects on ESF may be unrelated to their vasodilating properties. It would be of interest to know whether the erythropoietic action of prostaglandins is related to their effects on cyclic nucleotides (20, 46).

2. Other humoral agents which stimulate erythropoiesis

Endocrine agents have been studied extensively over the past several years and it would appear that ACTH, adrenal corticosteroids, thyroid stimulating hormone (TSH), thyroid hormones, growth hormone, prolactin and androgens have been demonstrated to stimulate erythropoiesis by increasing oxygen utilization while other endocrine agents may act through other renal mechanisms and/or on the erythroid cells in the marrow directly.

Hormones which increase renal cell utilization of oxygen probably stimulate ESF production by creating a relative oxygen deficit in a critical renal sensor cell. Increased production of some of these hormones may be related to polycythemias associated with excessive production of ESF or the anemias which occur in endocrine deficiencies. However, their significance in the physiological day-to-day control of ESF production has not been established.

a) *Thyroid hormones.* Thyroxin and 3′,5′,3-triiodothyronine have been found to increase metabolic rate and to elevate red cell mass in hypophysectomized rats (98, 99, 118). Chronic injections of thyroxin have also been demonstrated to increase red cell mass and metabolic rate in normal rats (99). An increase in plasma ESF was also reported after injections of certain doses of thyroxin in rats (344).

b) *Adrenocortical hormones and ACTH.* Adrenocortical steroids have been demonstrated to produce an increase in red cell mass in normal rats when given daily injections for 60 days (123). However, corticosteroids have been reported to exert both stimulatory (35, 123, 147) and inhibitory (35, 156, 178) effects on erythropoiesis depending upon the dose used. High dosages have been reported to suppress and low doses are known to stimulate erythropoiesis. Peschle *et al.* (343) have recently reported that cortisol induced a significant erythropoietic effect in either starved or polycythemic mice but no stimulatory effect was seen in normal mice given injections with a wide range of doses of cortisol. In addition, the erythropoietic effects of cortisol in polycythemic mice were completely abolished by anti-ESF serum (343). Dexamethasone has also been reported to stimulate erythropoiesis in normal and starved rats and in polycythemic mice (33). Furthermore, the time course of dexamethasone on ^{59}Fe incorporation in erythrocytes was similar to that produced by erythropoietin (34).

The adrenal glucocorticoids are known to increase metabolic rate in hypophysectomized animals (118) and may act similar to thyroxin in altering the ratio of oxygen supply to demand (147, 177) leading to an increase in erythropoietin secretion. Erythropoietin is apparently involved in the mechanism of action of ACTH on erythropoiesis in that the erythropoietic effect of ACTH in mildly plethoric mice was blocked by anti-ESF serum (124).

c) *Androgens.* The report that testosterone can induce remissions in patients with refractory anemias (152, 388, 389) has made this agent of great potential therapeutic value in the treatment of anemias. Elevated levels of erythropoietin have been reported in the urine of normal (8), hypogonadal and anemic human subjects after the administration of androgens (7, 9). It is interesting that the most marked elevation (15 times that of control levels) in urinary titers of ESF occurred after administration of androgens to anemic patients whose basal levels of ESF were already elevated prior to therapy (7). This finding may be related to the work of Fried and Gurney (144) who found that if mice were previously exposed to hypoxia a dosage of testosterone which alone does not produce a detectable elevation in ESF, will produce an increase in plasma ESF levels which was three times higher than that seen with hypoxia alone. This effect of hypoxia in enhancing the response to testosterone may be due to an increase in sensitivity

of the kidney to testosterone. Androgens were also found to increase the erythropoietic response of intact mice to hypoxia but failed to show this effect when the mice were nephrectomized before exposure to hypoxia (301). Fried *et al.* (145) found that ESF levels in plasma of mice injected with testosterone were no longer increased if bilateral nephrectomy was performed 8 hr prior to collecting plasma. It seems clear that at least part of the erythropoietic effects of testosterone are mediated through erythropoietin in that the effects of testosterone are blocked by the antibody to ESF (124, 381). However, the mechanism by which androgens stimulate kidney production of erythropoietin is not completely clear. Gordon *et al.* (179) have reported an increased amount of REF (erythrogenin) in kidneys from rats and rabbits which have been treated with testosterone. They have suggested that this elevation in erythrogenin in the kidney is correlated with the increased erythropoietin production seen after administration of testosterone.

Considering other possibilities for the mechanism of action of androgens, it would seem appealing to postulate that androgens might provide an anabolic stimulus to increase metabolic rate, increase oxygen demand and thus create a decrease in the oxygen supply:demand ratio. However, the metabolic rate of normal human subjects (235) and normal rats (295) was not affected by testosterone. In addition, Shirakura *et al.* (391) found an increase in ^{59}Fe utilization and a very slight decrease in basal metabolic rate in normal female rats during long term administration of testosterone. On the other hand, androgens have been demonstrated to inhibit oxygen consumption in kidney slice preparations (211). Even though a change in the oxygen supply:demand ratio does not seem likely as an explanation for the mechanism of action of androgens, it is still possible that androgens could increase oxygen demand sufficient to increase erythropoietin production. The increase in oxygen consumption may be more specific for certain organs such as the kidney and a change in total oxygen consumption in the animals may not be seen after administration of testosterone. It is also possible that androgens produce some type of specific impairment in the ability of red cells to deliver oxygen to renal cells or else they may inhibit enzymes within the kidney and result in a molecular deprivation of oxygen. Even though it seems clear that androgens stimulate kidney production of erythropoietin, direct effects of testosterone *in vitro* on radioactive iron (89) and tritiated thymidine (222) incorporation in bone marrow cells as well as direct effects on increasing the poliferation of erythroblasts in culture (190, 355) have been reported.

Several 5β-H steroid metabolites have been reported by Granick and Kappas (185) and Gulinati and Salvatorelli (190) to increase hemoglobin synthesis in chick blastoderms *in vitro*. Gordon *et al.* (180) and Gorshein and Gardner (182) have also reported that certain 5β-H steroid metabolites are much more effective than testosterone in stimulating ^{59}Fe incorporation in red cells of polycythemic mice. Mizoguchi and Levere (296) have reported that certain 5β-H androgens are capable of stimulating the synthesis of both heme and globin in human bone marrow cultures. These investigators (180, 182) have postulated

that the 5β-H steroids stimulate erythropoiesis in bone marrow directly, whereas, the δ-4 and 5α-H steroids act by increasing erythropoietin production. On the other hand, Samuels and Fisher (374, 375) and Fisher *et al.* (126) found that the 5β steroids were devoid of erythropoietic activity in polycythemic mice but 19-nortestosterone produced a more marked erythropoietic effect in polycythemic mice than testosterone. More recently 5β-dihydrotestosterone has been reported to increase the sensitivity of the colony forming unit (CFU) to the cytocidal action of ^{3}H-thymidine and was postulated to trigger the CFU into cell cycle (48). It would be most useful to have a therapeutic agent for refractory anemias such as one of the 5β steroids with very little androgenic or masculinizing side effects but still maintaining a high degree of specificity for the erythropoietic system. The report by Malgor *et al.* (283) that the kidney from a dog previously exposed to hypoxia is capable of eliciting a more marked response to testosterone in producing ESF in the isolated perfused kidney system, may mean that the most optimal effect of testosterone can be seen on a background of hypoxia where there is an elevation in erythrogenin levels in the kidney.

d) Miscellaneous hormonal agents. The effects of TSH on erythropoiesis (118) are probably mediated through the release of thyroid hormones. Growth hormone has been reported to increase erythropoiesis (294) apparently without any change in oxygen consumption. The mechanism of the slight stimulatory effects of growth hormone on erythropoiesis in the isolated perfused hind limbs of the rat (174) and the dog (125) are not understood. Thyroxine and growth hormone combined restored the suppressed erythropoiesis seen in hypophysectomized monkeys (427). Fisher *et al.* (125) have postulated that growth hormone exerts direct effects on the bone marrow.

3. Cobalt

Cobalt has been known to stimulate erythropoiesis for several decades (329, 433). It was first thought that cobalt stimulated erythropoiesis by a direct action on the bone marrow. However, Barron and Barron (19) reported in 1936 that cobalt inhibited bone marrow respiration and oxygen consumption in bone marrow cultures. The finding of Laforet and Thomas (254) that cobalt depressed heme synthesis in the marrow at a concentration which was lower than that which would inhibit oxygen consumption led most workers to believe that the action of cobalt was not directly on the marrow. Goldwasser *et al.* (164) reported in 1958 that cobalt acted by increasing erythropoietin levels in plasma of rats with peak erythropoietin titers being reached 12 hr after subcutaneous injection. Other metals, such as manganese, nickel and iron, did not induce an erythropoietic effect. The erythropoietic effects of cobalt were found to be markedly reduced after bilateral nephrectomy (113, 217). In addition, Fisher and Langston (116) demonstrated that cobalt may increase erythropoietin production by acting as a metabolic inhibitor depriving the cell of oxygen by inhibiting essential cellular enzymes. Cobalt has been found to decrease oxygen utilization in the isolated perfused kidney (116), to decrease cellular respiration and oxidative phosphorylation *in vito* (269, 447) in rat kidney homogenates, and to decrease

the activity or level of a factor in homogenates of rat kidneys which inactivates ESF (136). A decrease in oxygen consumption and an increase in red cell mass have also been reported in hypophysectimized rats treated for a prolonged period of time with cobalt (118).

Cobalt has also been found to induce a change in the LDH isoenzyme pattern of kidneys which resembles that seen following tissue hypoxia (224). As seen in figure 4, the decline in oxygen saturation values in the unoxygenated blood perfusate containing cobalt from an isolated hypoxemic perfused dog kidney was found to be much less rapid and furthermore was not as low after 5 hr as that seen in the unoxygenated control group. This less rapid decline in oxygen levels in perfusates from kidneys, when cobalt is present, is probably a reflection of the inability of the kidney to extract oxygen from the blood due to an action of cobalt on the kidney. Therefore, oxygen lack through inhibition of cellular enzymes may account for the effect of cobalt on REF and renal ESF generation.

4. Cyclic nucleotides

Hormones are well known to evoke an effect on target organs by stimulating membrane adenyl cyclase to increase the intracellular concentration of cyclic

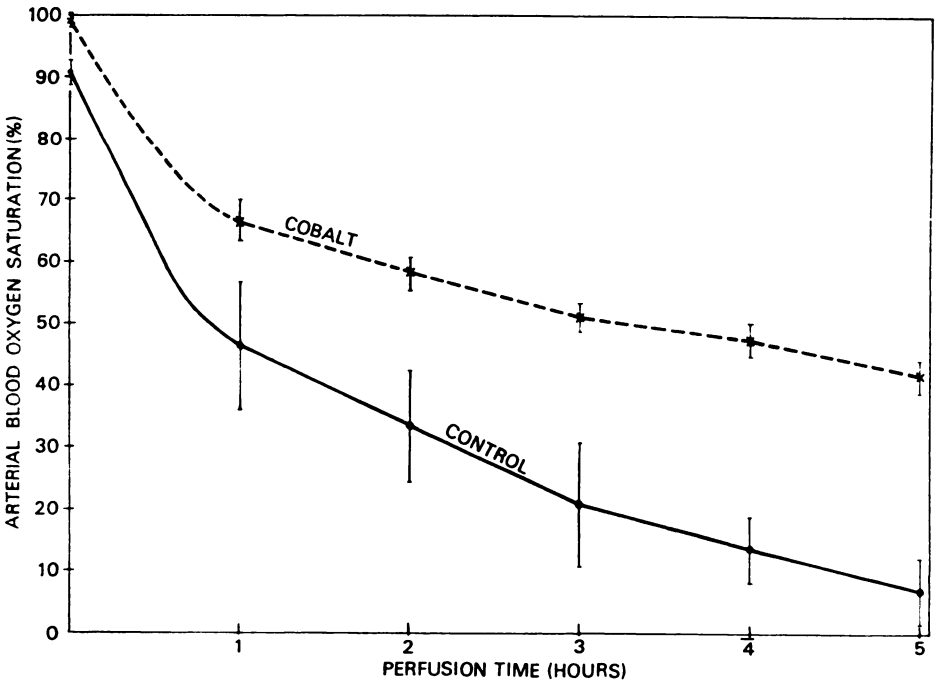

FIG. 4. Mean hourly blood oxygen saturation values on perfusates of isolated kidneys perfused with hypoxic blood alone (5 experiments) or hypoxic blood containing cobalt (5 experiments). Standard error of mean shown for each point. (Fisher and Langston, Blood 29: 114, 1967.)

adenosine-3′,5′-monophosphate (cyclic AMP) (47). Cyclic AMP may influence membrane permeability as well as the activities of specific intracellular enzymes (36). Bottomley *et al.* (29) recently reported that cyclic AMP stimulated Δ-aminolevulinic acid (ALA) synthetase activity in rabbit bone marrow cultures. This effect was prevented by inhibitors of protein synthesis. Erythropoietin and cyclic AMP were found to be synergistic in this system. These workers (29) found an additive effect of dibutyryl cyclic AMP and erythropoietin when they were administered simultaneously to polycythemic mice. Winkert *et al.* (443, 444) have also reported an increase in ^{59}Fe incorporation in RBC in polycythemic mice injected with dibutyryl cyclic AMP. Gorshein and Gardner (183) reported that dibutyryl cyclic AMP was effective in stimulating heme synthesis in a human bone marrow culture system. Theophylline increased the uptake of ^{59}Fe in the culture further and this increase was attributed to inactivation of phosphodiesterase which permitted a longer duration of action for the dibutyryl cyclic AMP. Byron (48) recently reported that dibutyryl cyclic AMP increased the sensitivity of the colony forming unit (CFU) to the cytocidal action of ^{3}H-thymidine and may trigger CFU into cell cycle. Byron (49) also reported that isoproterenol, a beta-adrenergic stimulant, triggered the hemopoietic stem cells into cell cycle. This effect was blocked by propranolol, a compound which specifically blocks *beta*-adrenergic receptors. The adenyl cyclase system was suggested to regulate the cell cycle of stem cells. On the other hand, Morley *et al.* (311) reported that cyclic AMP inhibited the growth of marrow colonies *in vitro*. Rodgers *et al.* (364) recently reported a linear increase in ^{59}Fe incorporation in RBC of polycythemic mice with several doses of dibutyryl (db) cyclic 3′,5′-AMP. Antiserum to ESF partially inhibited the erythropoietic response to db-cyclic AMP in polycythemic mice indicating that this effect was, at least in part, erythropoietin dependent. Schooley and Mahlmann (384) found that anti-erythropoietin completely prevented the erythropoietic stimulation induced by cyclic-AMP in plethoric mice. An interesting relationship between erythropoietin production and changes in cyclic AMP in kidneys of rats has recently been reported after cobalt injections (365). Rodgers *et al.* (363, 365) reported a sequential increase in cyclic AMP and erythrogenin in rat kidneys which was followed by an increase in plasma ESF levels after the administration of cobalt. These workers (365) postulated that cobalt stimulated kidney adenyl cyclase to increase cyclic AMP which triggered a kinase enzyme to stimulate the production of erythrogenin. The erythrogenin which was produced caused enhanced generation of plasma ESF. More recently, Gidari *et al.* (154) reported that the erythropoietic effect of cyclic AMP in polycythemic mice was potentiated by prior incubation of cyclic AMP with dialyzed serum and was partially blocked by anti-erythropoietin serum *in vivo* but not *in vitro*.

5. Inhibitors of erythropoietin production

Inhibitors of erythropoietin can be divided into chemical agents which inhibit transport systems or enzymes within the kidney and pathological inhibitors which have been described in plasma or tissue fractions. In addition, inhibitors

of erythropoiesis have been described in plasma of animals following transfusion plethora (242, 243, 438) and in plasma of human subjects when they return to sea level after residing at high altitude (360).

a) Chemical agents which inhibit erythropoietin production. We reported several years ago that organic mercurial diuretics (meralluride and mercaptomerin), benzydroflumethiazide and triamterene were potent inhibitors of cobalt-induced increases in plasma erythropoietin in rats (127). Both meralluride and mercaptomerin produced a significant inhibitory effect on the effects of cobalt induced erythropoietin production. In addition, infusion of alkylating agents, such as chlorambucil and thio-TEPA in the isolated perfused dog kidney *in situ*, have been demonstrated to exert an inhibitory effect on erythropoietin production following an injection of cobalt (128). These drugs either antagonize the attachment of cobalt at a renal erythropoietic receptor site or else inhibit some essential step in the synthesis of erythropoietin.

In addition, Fisher *et al.* (129) have shown previously that the elevation in plasma levels of erythropoietin after administration of cobalt to unilaterally nephrectomized dogs could be inhibited by exposing the kidney to a dose of 3000 rads of ionizing radiation. It is also of interest that dimethylnitrosamine, an agent which induces kidney tumors in rats, was found to increase erythropoietin production (302, 313). This increase in erythropoietin production may have been due to the renal ischemia produced by the tumor compressing the renal parenchyma. Giger (155) recently demonstrated that rats treated with actinomycin D, to depress messenger RNA, prevented the increase in erythropoietin titers in plasma after exposure to hypoxia.

b) Other inhibitors of erythropoiesis. Inhibitors of erythropoiesis have been demonstrated in plasma from hypertransfused rabbits (242) and sheep (243, 438). Reynafarje *et al.* (360) demonstrated an inhibitor in plasma of human subjects with high altitude polycythemia after their return to sea level. The inhibitory activity has been demonstrated with radioactive iron incorporation in RBC as a measure of erythropoiesis in normal rats (243), in rats after exposure to hypoxia (360), in normal mice (438) and in polycythemic mice injected with erythropoietin or after exposure to hypoxia (438, 439). Whitcomb (439) and Whitcomb and Moore (438) have postulated that this substance may act to inhibit erythropoietin directly by some type of interaction with the hormone or else may interfere with the action of erythropoietin on erythroid cells. Erslev and Thorling (90) failed to confirm the presence of an ESF inhibitor in mice or rats made plethoric. The mechanism by which plethoric serum inhibits erythropoietin production and/or erythropoiesis and its significance in the physiological control of erythropoiesis are not understood.

Fisher *et al.* (136) reported the presence of an inhibitor of erythropoietin or an enzyme which inactivates erythropoietin in kidney homogenates from normal rats. Addition of cobalt to the kidney homogenates antagonized the inhibitory effects of the kidney homogenates on erythropoietin (136). Further studies with normal human kidneys and kidneys from patients with anemia associated with chronic renal disease indicated that the soluble kidney fraction was the most active kidney subcellular fraction in inactivating erythropoietin (131, 268).

Erslev and Kazal (93) and Kazal *et al.* (231) confirmed the presence of an inhibitor to erythropoietin in rabbit and human kidneys but their most active fraction was in the tissue sediment or insoluble fraction of the kidney. The inhibitor of ESF in the studies of Kazal *et al.* (231) appeared to have lipid properties. This inhibitor was subsequently demonstrated to be primarily in the neutral lipid fraction of the kidney and was neutralized by the addition of normal serum (87). A protein separated from the urine of normal and anemic nonuremic human subjects which inhibits ESF has also been reported (270). This urinary erythropoiesis inhibiting factor (EIF) was found in the Sephadex G-100 fractions with a smaller molecular weight (271).

A pathophysiological inhibitor in uremic serum was also demonstrated in 1956 by Markson and Rennie (288) to inhibit maturation of human bone marrow cells in culture. Several investigators (252, 253, 373) have found a suppression of radioactive iron incorporation in red cells, a decrease in tritiated thymidine incorporation into DNA of bone marrow cells, a shortening of the reticulocyte life-span and a decrease in normoblast maturation in rats with uremic serum. An inhibitor of erythropoietin in uremic human plasma and an inactivator of erythropoietin in kidney homogenates has also been reported (131, 309, 310). The relationship of the erythropoietin inhibitor found in plasma to that seen in the kidney is not known. Kuratowska (251) has reported that the kidney cytoplasmic inhibitor was not stable to heat, not affected by a trypsin inhibitor and was probably neuraminidase. Further work is necessary to characterize these inhibitors before their significance in the anemia associated with renal disease can be established.

6. The nervous system and erythropoiesis

Several investigators have presented evidence for a role of the central nervous system in the regulation of erythropoiesis. Most of this evidence is based on the results obtained with electrical stimulation of the hypothalamus. Seip *et al.* (387) reported that electrical stimulation of the posterior hypothalamus was accompanied by a rise in reticulocytes and blood volume in about one-third of the stimulated rabbits. Since this time a number of investigators have demonstrated that electrical stimulation of the hypothalamus in rabbits, rats and monkeys increased erythropoiesis (101, 199, 291, 303, 304, 333, 334, 414). Hypothalamic stimulation in rabbits and monkeys has also been demonstrated to produce an increase in plasma levels of erythropoietin (101, 199, 303, 304). This rise in plasma levels of erythropoietin following hypothalamic stimulation was not abolished by hypophysectomy (198, 303, 304). Halvorsen (200) found that 15 of 26 rabbits with lesions in the posterior hypothalamus showed a reduced reticulocyte response to hypoxia. On the other hand, Piliero *et al.* (348) were unable to find any difference in the reticulocytes, peripheral red cells and marrow nucleated red cell precursors in rats with lesions in the anterior, middle and posterior hypothalamus when compared to that of the sham-operated controls.

Baciu (16) reported an increase in the erythropoietic activity of plasma from an isolated hypoxemic perfused dog head. When the trunk alone was perfused in the same manner no erythropoietic activity was found in the perfusate. He

postulated a central nervous system control of erythropoiesis. Splanchnic nerve section was reported by Takaku *et al.* (414) to reduce the erythropoietic response to hypoxia. It is of interest that atropine was found to abolish the reticulocyte response seen following hypothalamic stimulation in rats (291, 386) and in rabbits (331, 333, 334). Atropine has also been reported (335, 336) to inhibit the rise in plasma erythropoietin levels in rabbits after exposure to hypoxia.

Latner (264, 265) demonstrated in 1937 and 1938 that removal of the carotid body and denervation of the aortic arch produced a hyperchromic macrocytic anemia and a marked reticulocytosis in rabbits which was followed later by normalization of the blood picture. This author suggested a brain center for the control of erythropoiesis. The carotid bodies of animals exposed to high altitude have also been reported to be enlarged and hyperplastic (80). Grant (189) reported that denervation of the carotid and aortic areas in rabbits produced a more marked increase in hematocrit, red cell counts and reticulocytes after prolonged exposure to hypoxia when compared to that of control animals. Badger (15) reported a more pronounced polycythemic response in dogs exposed to 3800-meters altitude for 8 months after splenectomy and carotid body resection when compared with control dogs. They suggested that this response was due to the more pronounced hypoxemia seen in dogs after carotid body ablation resulting from the reduced ventillatory response to hypoxia (15). Tramezzani *et al.* (416) recently reported an increase in erythropoietic activity in the efferent blood from the carotid body in cats after a bleeding stimulus. They also reported that carotid body ablation in cats prevented the reticulocytosis usually seen after phlebotomy (416). Erythropoietic activity was also demonstrated in a carotid body extract. These authors (416) postulated that the carotid body releases two factors, one which causes an early reticulocyte release and a second which is similar to erythropoietin in stimulating erythropoiesis. However, Paulo *et al.* (334, 337, 338) found that erythropoietin levels were significantly higher in plasma of carotid body ablated rabbits after exposure to 18 hr of hypoxia than that of sham-operated controls. These workers (337, 338) also reported a marked reduction in blood PO_2 and an increase in reticulocytes in peripheral blood of rabbits during the first 14 days after carotid body ablation. Lugliani *et al.* (278) were unable to find any change in blood reticulocytes and hematocrit in human subjects after bilateral carotid body resection. On the other hand, six of their patients had secondary polycythemia which required phlebotomy after removal of the carotid bodies (278). It seems clear that the central nervous system can cause increased erythropoietin elaboration but much further work is necessary to clarify the mechanism of this action. It is quite possible that the hypothalamus may cause the release of a humoral agent that stimulates kidney production of erythropoietin and/or reticulocyte release from the bone marrow but until such an agent is found this attractive hypothesis lacks support.

7. Estrogens and erythropoiesis

Estrogens have been known for several years to produce a decrease in red cell count in castrated males to levels which are normal for females when physio-

logical amounts of estrogen are administered (347, 396, 428). When pharmacological doses of estrogens were given to experimental animals, a marked anemia was seen (68, 347, 420). When large doses of estradiol cyclopentylpropionate were administered to male tamarins, mice, or rats before exposure to hypoxia, production of erythropoietin was inhibited (305). Blood from the estrogen treated mice in these studies failed to show an inhibitor to erythropoietin or erythropoiesis (305). Physiological amounts of estradiol-17β were reported by Dukes and Goldwasser (76) to depress erythropoiesis in male rats. Jepson and Lowenstein (226) found that estradiol valerate inhibited the erythropoietic response to hypoxia but no change in the levels of erythropoietin in plasma occurred. It has been suggested (226) that estrogens inhibit the effects of erythropoietin on the erythropoietin responsive stem cell but do not affect maturation of the late erythroid cells in marrow. It would appear from the studies reported that high doses of estrogens are capable of depressing erythropoietin production while small doses may have an effect directly to depress bone marrow.

VIII. Metabolism of Erythropoietin

Renal excretion studies on erythropoietin have been hampered because of the lack of an assay method sufficiently sensitive to detect the low levels of erythropoietin found in urine. The normal human daily urinary excretion of erythropoietin has been reported to be between 0.9 to 4.0 units (4, 10). An interesting experiment was reported by Rosse and Waldmann (371) in which a decrease in erythropoietin titers in serum and urine was seen in anemic patients after transfusion. They found that the erythropoietin levels in urine were proportional to that of the serum which suggested that the hormone was derived from plasma. The amount of erythropoietin found in urine was approximately 10% of the daily loss. They calculated the renal clearance of erythropoietin to be 0.06 to 0.67 ml/min. Weintraub *et al.* (436) injected sheep erythropoietin into dogs. They found a 70% reduction in plasma levels of erythropoietin during the first 3.5 hr but were able to recover only 2 to 5% of erythropoietin in the urine. They calculated the renal clearance of erythropoietin to be between 0.1 to 0.6 ml/min which represented only a very small fraction of glomerular filtration rate.

Marver and Gurney (289) have reported that alkalinization of the urine enhanced the urinary excretion of erythropoietin in patients. They found a great variability in the ratio of the urinary to plasma concentration of erythropoietin in some anemic patients. However, when they alkalinized the urine by infusing sodium bicarbonate they found an increase in the concentration of erythropoietin in the urine. On the other hand, when they infused ammonium chloride into these patients to acidify the urine, they found a decrease in the urinary excretion of erythropoietin. These authors suggested "non-ionic diffusion" as a possible mechanism for excretion of erythropoietin. Thus, in alkaline urine it was postulated that there was more of the ionized form of erythropoietin and less renal tubular reabsorption.

The role of the liver in the metabolism of erythropoietin was suggested by

Jacobsen *et al.* in 1956 (214), when these workers found higher plasma levels of erythropoietin in rabbits made anemic by phlebotomy and injection with acetylphenylhydrazine, than in the anemic control rabbits. Marked central lobular degeneration of the livers of the phenylhydrazine treated rabbits was seen and it was suggested that the liver damage had prevented the metabolism of erythropoietin. Prentice and Mirand (350) and Mirand *et al.* (297) found higher titers of ESF in rats with acute liver damage due to carbon tetrachloride injections after exposure to hypoxia than in normal rats treated in a similar manner. Alpen (13) also showed that rabbits treated with carbon tetrachloride had a greater erythropoietic response to injections of exogenous erythropoietin than normal rabbits and suggested that this was due to a slower rate of inactivation of erythropoietin in the animals with liver damage. Burke and Morse (41) showed in their isolated perfused rat liver studies that erythropoietin levels in the perfusates of livers from normal rats were significantly less than that seen in rats treated with carbon tetrachloride and perfused with blood containing erythropoietin. These investigators interpreted this finding to mean that the liver damage induced by carbon tetrachloride resulted in a decrease in the metabolism of ESF. However, Fischer and Roheim (105) failed to show a loss of erythropoietin in a rat liver perfusion system.

Inactivation of erythropoietin by incubation with liver homogenates has also been found (77). More recently, Fisher and Roh (132) have reported a rapid loss of erythropoietin in the isolated perfused dog liver. On the other hand, when livers from dogs pretreated with SKF 525-A (367), a drug which inhibits hepatic enzymes, were perfused with blood containing erythropoietin, this inactivation of erythropoietin in the isolated perfused liver could be prevented. These authors interpreted their findings to mean that an enzymatic degradation of erythropoietin was occurring in the isolated perfused liver system.

The hypothesis has been proposed that the decline in erythropoietin levels in plasma could be due to increased bone marrow utilization of erythropoietin, especially when there is also an increase in the number of erythroid cells in the marrow (204, 402, 403). Stohlman (402) and Stohlman and Brecher (403) found a slower decline in plasma erythropoietin levels in irradiated rats than in control animals and suggested that this may be due to decreased utilization of erythropoietin by the bone marrow injured by irradiation. It would be of interest to know whether sufficient liver damage was produced by this amount of irradiation to explain this difference in the rate of decline in plasma erythropoietin. Hammond and Ishikawa (204) compared the decline in plasma erythropoietin in anemic patients with erythroid aplasia with that of patients with erythroid hyperplasia. They found a much less rapid decline in erythropoietin titers in patients with hypoplastic marrows than in patients with hyperplastic marrows. However, Van Dyke and Pollycove (421) found that erythropoietin titers in plasma were approximately the same in patients with hyperplastic or aplastic marrows. In addition, Fisher *et al.* (133) found that erythropoietin titers in the isolated perfused hind limb of rabbits were not decreased during a 6-hr perfusion period. More evidence is necessary to prove that the bone marrow plays a sig-

nificant role in the inactivation of erythropoietin. Until more definitive experiments can be designed to control such variables as production, compartment shifts in distribution and excretion of erythropoietin, this question will still remain unresolved.

The plasma half-life of erythropoietin has been studied by a number of investigators (13, 134, 234, 358, 368, 402–404, 436). Keighley (234) reported that intravenously administered erythropoietin had a plasma half-time disappearance in normal rats of about 1 hr. He also found erythropoietin to be present in lymph in an amount which was proportional to that in the corresponding plasma sample. Stohlman and Howard (404) found that the plasma clearance in normal rats given injections of human urinary erythropoietin was non-exponential, indicating re-entry. Within 25 min of a single 6-unit intravenous dose of human urinary ESF, the activity had declined to 50% of the initial activity. A second phase of the curve had a slower rate of decline: the half-time disappearance was near 3 hr. Reissmann *et al.* (358) by continuous infusion of erythropoietin into normal rats calculated the distribution space for erythropoietin to be equal to twice the plasma volume. This may mean that the fast initial component of erythropoietin disappearance is due to the rapid movement of the hormone into the extravascular space. The half-time clearance of erythropoietin has also been studied in the dog by Weintraub *et al.* (436). These workers also found two components to the disappearance curve in the dog: an initial rapid phase in the range of 25 to 40 min, and a slower component of 9 to 10.5 hr. Recovery of erythropoietin from the urine in these studies was only 2 to 5% of the injected dose, even though 70% of the administered erythropoietin was lost from the plasma compartment. Rosse and Waldman (371) in studying the disappearance of ESF in patients with erythroid hypoplasia found only a single exponential disappearance curve with a mean half-time clearance of 24.9 hr (range 6.6–42.6 hr), thus indicating a more prolonged disappearance in patients with suppressed marrow function. More recently Fisher *et al.* (134) and Roh *et al.* (368) reported the plasma $T_{1/2}$ disappearance of ^{125}I-labeled human urinary erythropoietin in normal rabbits. The plasma erythropoietin disappearance curve also showed a biphasic pattern: an early rapid phase with a mean plasma half-time disappearance of 36 min and a slow phase with a mean of 10.25 hr. The doses of erythropoietin used by these authors more approached a physiological range and they suggested that this may be a more accurate estimate of the true plasma half-time disappearance of erythropoietin.

IX. Mechanisms and Sites of Action of Erythropoietin

The primary site of action of erythropoietin is on stem cells to cause differentiation of these cells into the erythroid line (405). Fried *et al.* (141) and Alpen *et al.* (14) demonstrated that erythropoietin causes differentiation of immediate precursor cells into a recognizable or differentiated erythroid cell. As outlined by Stohlman (406) the differentiated erythroid cell compartment is not self-sustaining and must be supported by an influx of cells from a precursor compartment which is identified as the committed stem cell compartment. This com-

partment is postulated to be self-sustaining under normal conditions but when there is an increased demand for cells, either due to physiological reasons or after damage by irradiation or drugs, this compartment is repopulated by a pluripotential cell. This pluripotential cell compartment is capable of differentiating into either myeloid, megakaryocytic, or erythroid elements.

Bruce and McCulloch (40) have suggested the existence of an erythropoietin sensitive precursor cell, the so-called erythropoietin responsive cell (ERC), which is already committed to the erythroid line and is separate and distinct from a colony-forming stem cell. Although the ERC is already committed to erythroid differentiation, erythropoietin is still necessary to initiate hemoglobin synthesis which begins in the erythropoietin responsive stem cell. Stohlman (406) has suggested that erythropoietin not only initiates, but also governs the rate of hemoglobin synthesis and the rate of maturation and thus bone marrow transit time. Bottomley (30) found that erythropoietin stimulated the rate-limiting enzyme Δ-aminolevulinic acid synthetase, but not heme synthetase, in rabbit bone marrow erythroid cells. The biochemical mechanism of action of erythropoietin is still not completely understood. However, it has been suggested that it may de-repress a repressor which involves gene reduction or induction. In doing this, it initiates the RNA synthesis which is necessary for hemoglobin production.

After differentiation occurs the erythroid cells mature and at the same time undergo a series of divisions. Some cells may die *in situ* before emerging from the marrow compartment (ineffective erythropoiesis), while others are postulated to mature without dividing, the so-called upper pathway of skipped divisions, and to enter the peripheral blood as macrocytes (38). The response of the polycythemic mouse to erythropoietin is certainly good evidence for a stem cell effect of erythropoietin. Filmanowicz and Gurney (103) have shown that an orderly wave of erythropoiesis sweeps through the hematopoietic tissue after a large single dose of erythropoietin. These workers found after a single injection of 12 units of erythropoietin that an orderly differentiation and maturation of erythrocyte precursors was seen. An increase in early erythroid cells (pro-erythroblasts) occurred after 1 day and a striking increase in normoblasts (late erythroid cells) was observed on the 2nd day. No change in blood reticulocytes was seen after 1 day but an increase was seen after 2 days which reached a peak 3 days after administering of erythropoietin.

In addition to a primary effect of erythropoietin on stem cells, ample evidence has been reported to show that erythropoietin affects erythroid cell proliferation, maturation, and release from the differentiated erythroid cell compartment. Erythropoietin in high sustained doses has also been reported to produce a direct effect on reticulocyte release from the bone marrow (133, 181, 244) of isolated perfused femurs and a marked increase in the appearance of ^{59}Fe-labeled red cells in the perfusate of isolated perfused rabbit hind limbs (133). In addition, Yoffey *et al.* (448) found that by the 2nd day of hypoxia in the guinea pig there was a significant rise in blood reticulocytes and a significant decrease in marrow reticulocytes, indicating an accelerated discharge of marrow reticulocytes during

the early stages of hypoxia. Stohlman *et al.* (407) found that a reticulocyte response and macrocytosis developed 24 hr earlier in normal than in hypertransfused animals after a single massive dose (35 units) of erythropoietin. An effect of erythropoietin on the already differentiated erythroid precursors was suggested as an explanation for these findings. McCuskey *et al.* (280) have found an elevated blood flow through the microvascular system of the splenic red pulp accompanied by an increase in erythropoiesis in the murine spleen after the administration of erythropoietin. These workers (280) suggest that erythropoietin, in addition to its action on stem cells, also influences the microenvironment to establish an optimal site for erythropoiesis.

X. Relation of Erythropoietin Secretion to Clinical Anemias and Polycythemias

Anemias which are related to loss of red cells either due to hemolysis or hemorrhage are usually associated with increased blood and urinary titers of erythropoietin as well as increased erythroid cellularity in bone marrow. A second type of anemia resulting from decreased production of red blood cells may be caused by protein or endocrine deficiency and is usually associated with decreased titers of erythropoietin in plasma. Another type of anemia may be seen when increased blood levels of erythropoietin occur but the erythropoietin is apparently ineffective in correcting the anemia because of some alteration in the bone marrow and/or the erythropoietin. The most common anemias which have been demonstrated to have elevated blood and urinary titers of erythropoietin are the iron deficiency anemias (196, 205), aplastic anemias (23, 203, 205, 260, 341, 361, 363) and the megaloblastic anemias (341, 362). The finding that a wave of erythropoiesis can be induced in the marrow of some patients with hypoplastic anemia by fresh plasma infusion (205) may indicate that a component of normal plasma, possibly in Cohn fraction III (78), may be required in the regulation of normal erythropoiesis.

The anemia associated with uremia and chronic renal disease in the human has been the subject of investigation by several workers. Usually most of the patients with chronic renal disease do not have elevated urinary titers of erythropoietin with use of the polycythemic mouse assay. However, recent studies with the radioimmunoassay for ESF may indicate that serum ESF titers in anemic uremic patients may be above normal (411). Krugers Dagneaux (241) used a sensitive immunoprecipitation technique for the assay of ESF and reported 0.5 to 2.0 mU ESF/ml in normal human plasma but no ESF was detectable in the plasma of patients with chronic renal disease which indicates that ESF deficiency may be a important factor in the anemia associated with chronic renal disease. Even though depressed erythropoiesis and decreased plasma levels of erythropoietin (104, 425) are important factors in most cases of uremia, in some instances inhibitors of erythropoietin may be involved. The fact that improvement in the ferrikinetics and erythropoiesis occurs after dialysis (21, 94, 286) without an increase in erythropoietin levels (21, 94) indicates that some toxic or inhibitory factor is causing a depression of marrow function and/or a decrease

in erythropoietin production. In addition, a negative correlation between the degree of anemia and the half-saturation value of the oxygen dissociation curve in the blood of patients in chronic renal failure has been reported which was considered as a compensatory change known to be helpful in ameliorating the tissue hypoxia in anemia (306).

Experimental animals with anemia and uremia show a response to erythropoietin (32, 282, 286). Although the response may be decreased (32, 286), this is sufficient reason to vigorously approach the problem of clinical therapy with erythropoietin. Van Dyke *et al.* (422, 423) studied the response to exogenous erythropoietin of normal human volunteers as well as patients with anemia associated with chronic renal disease. In these preliminary clinical trials Van Dyke *et al.* (422) obtained an erythropoietic response in a hematologically normal 50-year-old man who was receiving a total subcutaneous dose of 1940 units (147–425 units every 3 days for 7 days) of a human urinary erythropoietin concentrate. The reticulocyte count increased abruptly during treatment and the total circulating red cell mass was found to be increased by 28% after 21 days of treatment. However, the patient developed redness, swelling, and tenderness at the injection site but no signs or symptoms of a systemic reaction. The dose that was estimated to produce a reticulocytosis in human subjects was 2 units/kg/day for several days and the dose necessary to produce an unquestionable increase in total circulating red cell volume was estimated to be 5 units/kg/day for 7 to 14 days. However, Van Dyke *et al.* (423) in further studies administered erythropoietin to a patient with moderate anemia secondary to chronic glomerulonephritis and found no evidence of erythropoietic stimulation. They suggested that successful treatment of this type of anemia in man may require on the order of 200 units/kg/day. These data may indicate that an important mechanism in the anemia associated with chronic renal disease is decreased responsiveness of the marrow to erythropoietin. It is also possible that erythropoietin is inactivated in plasma by an enzyme and/or else complexed to a factor which blocks its biological activity.

A second study on the clinical use of erythropoietin in anemia was a report by Larsen *et al.* (262). The erythropoietin preparation used was plasma from patients with pernicious anemia or iron deficiency anemia. Eight patients were included in this study who had chronic renal disease with creatinine clearance rates of approximately 10 mg/100 ml. When each patient was infused intravenously with 300 ml plasma per day for 4 days, a reticulocytosis was seen in 5 of the 8 patients. An increase in red cell mass was seen in 2 of the patients. The dose of ESF was estimated to be 5 IRP units/kg/day for 4 days.

Erslev (92) also reported that treatment of a patient with chronic renal disease and severe anemia with cobaltous chloride, testosterone and one injection of erythropoietin were not beneficial in relieving the anemia. On the other hand, Gardner (153) reported that 17 patients with anemia associated with renal disease responded to cobalt with an increase in erythropoiesis. The increased peripheral red cell values declined to pretreatent levels when the cobalt was discontinued. The erythropoietic action of cobalt has been demonstrated to be

through an increase in erythropoietin production by the kidney (164) which may be related to an increase in kidney cyclic AMP (365).

Erythropoietin titers in plasma are usually elevated in aplastic anemia (203, 205, 260). The mechanism of aplastic anemia is not understood. The marrow from a patient with red cell aplasia responded to erythropoietin *in vitro* with a marked increase in heme synthesis (240). This erythropoietic response was prevented by plasma from this patient and an antibody to erythroblast nuclei was demonstrated in this patient (240). This antibody and the red cell aplasia disappeared when the patient was treated with 6-mercaptopurine (240). Patients with acute panhypoplasia (227) and erythroblastopenia (228) of the bone marrow were also found to have an inhibitor to erythropoiesis in plasma which exhibited biological activity similar to that of the antibody to ESF. A serum inhibitor of ESF has also been reported in a patient with a pure red cell aplasia and thymoma (12). The inhibitor level in plasma declined and was absent 10 days after thymectomy (12).

Polycythemias are often associated with abnormal production of erythropoietin. The term polycythemia is used when there is an increase above normal in the absolute numbers of red cells in peripheral blood. A condition called polycythemia vera or primary polycythemia receives such a designation because there is no apparent cause for the increased erythropoiesis and it usually occurs without apparent stimulation of erythropoietin production (5, 6, 238). Polycythemia may be of the secondary type when there is increased erythropoiesis with elevated titers of erythropoietin resulting from a physiological stimulus, such as hypoxia. Conditions which are associated with an increase in erythropoiesis caused by hypoxia are high altitude (51, 361, 424), pulmonary disease (390, 409), chronic heart disease (292, 409, 430) and obesity with inadequate pulmonary ventilation (43, 434). All of these conditions have been shown to have elevated blood (149, 194, 342, 440) or urinary (276, 424) titers of erythropoietin. On the other hand, a secondary type of polycythemia may be due to inappropriate production of erythropoietin where no such physiological stimulus can be demonstrated (73, 193). Usually when the oxygen supply is equal to the oxygen demand a new level of homeostasis is reached. When a sufficient increase in red cell mass has occurred, there will no longer be an increase in erythropoietin production. Polycythemia may also be due to increased inappropriate erythropoietin production due to a renal tumor or another type of neoplasm.

Erythrocytosis with increased erythropoietin production has been reported as an unusual complication of tumors or renal abnormalities (74). Gallagher and Donati (148) reported that 3 out of 4 patients with renal cysts, 2 of 3 patients with hydronephrosis, 4 out of 5 patients with cerebellar hemangioblastomas, and individual patients with hepatic carcinoma or an aldosterone-secreting adrenal adenoma had elevated plasma levels of erythropoietin in association with erythrocytosis. A summary of the data (excluding the clinical anemias) where erythropoietic activity has been reported in plasma, tumor extracts, or cyst fluid from patients with erythrocytosis is shown in table 1.

An association of high hematocrit or hemoglobin with hypertension related

TABLE 1

Diseases reported with increased erythropoietin levels

Condition	Reference No.	Erythropoietic Activity	
		Tumor extract or cyst fluid	Plasma
Hypertension (renovascular)			
Gallagher and Donati (1968)	148		+
Renal carcinoma			
Hewlett *et al.* (1960)	206	+	
Korst *et al.* (1959)	236	+	+ (preop.) − (postop.)
Donati *et al.* (1963)	74		+
Cerebellar hemangioblastoma			
Waldmann *et al.* (1961)	431	+	−
Gallagher and Donati (1968)	148	+	+
Race *et al.* (1964)	351	+	
Pheochromocytomas			
Bradley *et al.* (1961)	36	+	+
Adrenal adenoma			
Gallagher and Donati (1968)	148		+
Hepatic carcinoma			
Brownstein and Ballard (1966)	39	+	
Gallagher and Donati (1968)	148		+
Nakao *et al.* (1966)	322	+	+
Waldmann *et al.* (1968)	432	+	+
Gordon *et al.* (1970)	165	+ (erythrogenin) (substrate)	+
Pulmonary insufficiency			
Freedman and Pennington (1963)	138		+
Renal cysts			
Gurney (1960)	195	+	−
Waldmann *et al.* (1968)	432	+	+
Gallagher and Donati (1968)	148	+	+
Rosse *et al.* (1963)	372	+	
Vertel *et al.* (1967)	426	+	+ (preop.) − (postop.)
Uterine fibromyoma			
Wrigley *et al.* (1971)	446	+	−
Adenocarcinoma (1965)	266		+
Meningeal lymphoma (1965)	266		+
Carcinomatosis (1965)	266		+
Hydronephrosis			
Gallagher and Donati (1968)	148		+
Jones *et al.* (1960)	229		+
Congenital cardiac defects			
Stohlman *et al.* (1954)	397		+
Strausz *et al.* (1962)	408		+

to renal arterial disease has been reported by Frohlich and Tarazi (146). In addition, two patients with renovascular disease and hypertension were found to have elevated plasma levels of erythropoietin (148). As indicated in table 1, other conditions where erythropoietin levels have been found to be elevated in

association with erythrocytosis are renal carcinoma (74, 206, 236), cerebellar hemangioblastoma (148, 351, 431), pheochromocytoma (36), adrenal adenoma (148), hepatic carcinoma (39, 148, 165, 322, 432), pulmonary insufficiency (138), renal cysts (148, 195, 372, 426, 432), uterine fibromyoma (446), adenocarcinoma (266), meningeal lymphoma (266), carcinomatosis (266), hydronephrosis (148, 229) and congenital cardiac defects (397, 408).

XI. Summary

The purpose of this review has been to describe the pharmacological actions of erythropoietin and its role in the control of erythropoiesis. It includes: a historical introduction on the discovery of the humoral control of erythropoiesis; techniques used for the assay and standardization of erythropoietin; purification and physicochemical characterization of erythropoietin; renal and extrarenal sites of production of erythropoietin; the biogenesis of erythropoietin as it pertains to the renal erythropoietic factor (erythrogenin); and a schematic model for the renal control of erythropoietin production. Pharmacological agents included in this review which are known to increase erythropoietin production are androgens, pituitary ACTH (through an action on the release of glucocorticoids from the adrenal), angiotensin, norepinephrine, 5-hydroxytryptamine (5-HT), vasopressin, prostaglandins, dibutyryl cyclic 3′,5′-AMP and cobalt. The pharmacological agents which depress erythropoietin production are the diuretic drugs, such as mercurials, triamterene, benzydroflumethiazide, as well as chlorambucil, thio-TEPA, actinomycin D, and ionizing radiation. Other areas in the review include: the nervous system and carotid body influences on erythropoiesis; physiological and pathological inhibitors of erythropoietin and erythropoiesis; the mechanisms and sites of action of erythropoietin on the erythron; and clinical anemias and polycythemias associated with erythropoietin secretion. The recent reports on the complete isolation, purification and characterization of erythropoietin offer hope that sufficient amounts of this glycoprotein-hormone will be available in the near future for the treatment of some of the erythropoietin deficiency anemias.

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