REGULATION OF ERYTHROPOIESIS IN THE FETUS

by

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ABSTRACT

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The data in this thesis supports the following observations on the site of production of erythropoietin (Ep) in the fetus and the role of Ep in the fetus:

1) These studies revealed that the liver, rather than the kidney, is the major site of erythropoietin production in the fetus. Only nephrectomized, partially hepatectomized sheep fetuses exhibited significantly reduced production of Ep in response to bleeding. Bilateral nephrectomy and/or splenectomy failed to affect Ep production.

In situ organ perfusion studies also support the role of the liver in Ep production. Perfusion of the kidney after hypoxic stress did not yield Ep, whereas perfusates from livers of hypoxic fetuses contained significant quantities of Ep.

Studies on erythrogenin in the sheep fetus suggest that Ep is not elaborated by the same mechanism as in the adult. Hypotonic extracts of the light mitochondrial fractions of many fetal organs including the liver did not produce Ep when incubated with normal serum, a procedure which resulted in the generation of significant quantities of Ep when hypotonic extracts from adult kidneys were used.

2) The data suggest that, as in the adult, erythropoiesis in fetal sheep is regulated by Ep. Injection of anti-Ep into fetuses suppressed their production of red blood cells and this inhibition resulted from the neutralization of circulating Ep in the fetus. In vitro studies support this view and further indicate that cells responsive to Ep migrate from the liver to the spleen and then into the bone marrow. The response to Ep in vitro seems more sensitive in the plasma clot technique than in the cell suspension technique but both techniques support the responsiveness to Ep of all erythroid tissues during fetal life.

These findings provide evidence for the contention that erythropoiesis in the mammalian fetus is regulated by Ep during the last two thirds of the gestation period.

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DEDICATION

To Dennis,

To Merrie and Cindy,

To my Nanny

..... for all their help.

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INTRODUCTION

Erythropoiesis in the adult mammal is known to be under the control of the hormone erythropoietin (Ep) (Gordon, 1973). Thus, hypoxia, the fundamental erythropoietic stimulus, has been shown to exert its influence through Ep (Fried *et al.*, 1957; Jacobson *et al.*, 1957a&b, 1959; Mirand and Prentice, 1957; Gordon, 1959; Mirand *et al.*, 1959). In this regard, the development of an increased circulating red cell mass in animals following exposure to reduced atmospheric pressures or bleeding is associated with increased production of Ep (Cotes and Bangham, 1961; Necas and Neuwirt, 1970).

Although the relationship between hypoxia and erythropoiesis had been known as early as 1878 (Bert, 1878), the mechanism underlying the association became known only during the past twenty years. Following the rediscovery by Erslev (1958) that plasma from anemic rabbits contained a factor capable of stimulating production of red cells in normal rabbits, significant advances in our understanding of the mechanisms underlying the regulation of red cell production in adult mammals were made. These include the site of production and biogenesis of erythropoietin (Jacobson *et al.*, 1957a&b; Goldwasser *et al.*, 1958) its purification (Hodgson *et al.*, 1960; Lowy *et al.*, 1960; Goldwasser and Kung, 1968), its site and mode of action (Jacobson *et al.*, 1957a&b; Bruce and McCulloch, 1964), its role in the control of erythropoiesis in the neonate (Carmena *et al.*, 1968; Lucarelli *et al.*, 1964, 1968; Matoth and Zaizov, 1971)

and the fetus (Zanjani et al., 1969; Stohlman, 1970; Zanjani and Gordon, 1971).

Biochemical separation and purification studies have established that erythropoietin is a glycoprotein with a molecular weight of approximately 46,000 (Goldwasser and Kung, 1972). The most purified preparations (9000-12,000 IU/mg. protein) which have been extracted from anemic sheep plasma, have been shown to contain hexoses, hexoseamines and sialic acid in addition to several other amino acids (Goldwasser and Kung, 1972). The sialic acid appears to be necessary for the biological actions of the hormone in vivo (Schooley and Garcia, 1971; Lukowsky and Painter, 1972) but not in vitro (Goldwasser et al., 1973). Thus desialation of erythropoietin by treatment with neuraminidase (Lowy et al., 1960; Schooley and Garcia, 1971) or even the injection of bacterial neuraminidase in vivo (Schooley and Mahlmann, 1971) destroys the circulating Ep. On the other hand, neuraminidase treated Ep retains its capacity to stimulate hemoglobin synthesis in vitro (Krantz et al., 1963; Goldwasser, 1966). It has been suggested that the desialated form of the hormone does not have sufficient opportunity to act on the hematopoietic tissue since it is removed more rapidly from the circulation than native Ep (Goldwasser, 1966; Goldwasser et al., 1973). In the absence of such a rapid disappearance, however, as in the case of in vitro studies, desialated erythropoietin is capable of stimulating red cell production with no apparent loss of activity (Goldwasser et al., 1973). This is in agreement with the observations of Morell et al. (1968) that desialated glycoproteins in general are removed from the circulation by the liver more rapidly.

The primary site of production of erythropoietin in the adult is the kidney. Jacobson et al. (1957a) demonstrated that bilaterally nephrectomized rats or rabbits produced significantly less Ep than intact animals when subjected to bleeding or cobalt treatment. Subsequent studies by these and other investigators (Jacobson et al., 1957b; Goldwasser et al., 1958) confirmed the role of the kidney in the formation of Ep in many animal species including man (Gurney et al., 1957; Gallagher et al., 1959; Nathan et al., 1964; Mirand, 1968). It was also established that the reduced ability of nephrectomized animals to produce Ep was not the result of the build up of waste products but rather the absence of renal tissue. In vitro studies have also confirmed the kidney as the major site of Ep production. Erythropoietin has also been found in fluid released from perfused kidneys both in situ or from isolated hypoxic kidneys (Kuratowska et al., 1964; Pavolvic-Kentera et al., 1965; Halvorsen et al., 1968; Erslev, 1974).

Although the kidney is recognized as the major site of Ep production, repeated attempts to extract Ep from this organ have not been successful (Naets, 1960; Rambach et al., 1961; Zangheri et al., 1962; Goldfarb and Tobian, 1963). The studies of Kuratowska and Kopec (1969) and Kuratowska (1968) provided the first insight into the mechanism by which the kidney participates in Ep formation. They extracted a factor from cobalt treated or anemic rabbit kidneys which was capable of generating erythropoietic stimulatory activity upon incubation with normal plasma. The factor was localized in the acidic nuclear protein fraction of kidneys and was named the renal erythropoietic factor (REF). These findings were confirmed

and further extended by Gordon and his associates (Gordon et al., 1967; Gordon, 1968; Gordon et al., 1968) who were able to extract a similar factor from kidneys of hypoxic rats which only produced Ep when incubated with normal serum. This factor, also termed REF, has since been renamed erythrogenin (Eg). Subcellular fractionation procedures were employed to localize the site of production and/or the distribution of erythrogenin in renal tissues. In six species including rat, sheep, and humans, an equal distribution of Eg was found in renal cortical and medullary tissue (Zanjani et al., 1967b). Only the light mitochondrial and microsomal extracts were capable of producing Ep when incubated with serum (Gordon, 1968). In kidneys from hypoxic rats, the light mitochondrial fraction was most active in producing erythropoietin after incubation with normal serum (Contrera and Gordon, 1966; Contrera et al., 1966). Subsequent studies revealed that normal rat kidneys also possessed erythropoietic activity in mitochondrial fractions. Confirmation that Ep was actually produced by the interaction of Eg and normal serum was shown by the ability of anti-Ep to neutralize the product of the interaction (Zanjani et al., 1968). Evidence suggesting that the production of Ep as a result of the interaction of erythrogenin and normal serum may be enzymatic in nature was provided by Zanjani et al. (1967a) and Smith and Fisher (1973). Sequential determinations of the enzymatic activity of renal subcellular fractions during a period of exposure to hypoxia revealed that erythrogenin may be produced and transported to storage sites via the microsomal-endoplasmic reticulum systems (Cantor et al., 1969). Gordon and Zanjani (1970) have, therefore, suggested that the

production of Ep *in vivo* results from the interaction, possibly enzymatic, of the Eg and a serum-born protein substrate. Moreover, Zanjani *et al.* (1971) have shown that significant fluctuations in the circulating levels of the substrate occur during hypoxic stress which precedes similar changes in serum Ep concentration.

Other theories have also been proposed which offer an alternative to the erythrogenin-substrate mechanism of erythropoietin production. It was observed that renal tissue contained a lipid substance which acted as an inhibitor of Ep. Although the lipid inhibitor of erythropoietin was found in several tissues, its concentration was highest in renal tissue (Erslev and Kazal, 1968). Erslev *et al.* (1971, 1972) proposed that Ep was stored in the kidney bound to a lipid substance. The lipid-Ep complex is biologically inactive. However, recovery of active Ep from this complex can be achieved in the presence of plasma protein(s) of, as yet, undefined nature. It was suggested that during periods of hypoxic stress, an increase in the synthesis and/or release of the plasma proteins leads to the enhancement of Ep release from the lipid-Ep complex.

Recently Erslev (1974) has given partial support for his theory by showing that formed erythropoietin is released from perfused kidneys even in the absence of plasma proteins. He subjected rabbits to hypoxia for three hours and then removed their kidneys and perfused them *in vitro*. These so-called "programmed" kidneys, first described by Zanjani *et al.* (1971), continued to produce Ep even when the perfusing fluid was free of protein. Of course, since the kidneys had been "programmed" to produce Ep, it

is possible that the circulating levels of the hormone had increased sufficiently so that the kidneys retained pre-formed erythropoietin which was now released.

Although the kidney is the major site of production of erythropoietin, recent studies indicate that the liver is also involved in Ep production (Reissmann and Nomura, 1962; Katz et al., 1968; Fried et al., 1969; Fried, 1972; Kaplan et al., 1973, 1974). When hypoxic livers were perfused in vitro, the perfusate contained very high erythropoietic activity (Reissmann and Nomura, 1962). When livers were removed from rats, their ability to produce Ep in response to hypoxia was greatly reduced (Katz et al., 1968). Thus, the liver was implicated in Ep production in the intact animal. When animals are nephrectomized, the liver is then capable of producing its own erythropoietin. Nephrectomized rats can still respond to hypoxic hypoxia (Goldwasser et al., 1958) and nephrectomized mice respond to phenylhydrazine-induced anemia by Ep production (Jacobson et al., 1958). If rats are nephrectomized and exposed to hypoxia, they show increased titers of Ep in their blood (Fried, 1972). Nephrectomized rats with 80% of their livers removed prior to being made hypoxic had barely detectable Ep levels (Fried, 1972). The production of extrarenal erythropoietin is related to the intensity of the hypoxic stimulus in the animal (Fried et al., 1968). If nephrectomized rats are injected with lead, which causes severe anemia and renal tubular dysfunction in the intact animal, the production of extrarenal Ep by the liver is increased after hypoxic exposure (Schooley and Mahlmann, 1974). This supports the hypothesis that the liver elaborates Ep under

particularly stressful conditions. Extrarenally-produced Ep stimulated heme synthesis *in vitro* and is neutralized by anti-Ep (Fried *et al.*, 1968); therefore it seems to be identical to renally-produced Ep. These studies demonstrate extrarenal sources of erythropoietin production which operate when animals are subjected to stress in the absence of kidneys. Whether the liver produces Ep when the kidneys are functional is unknown. It seems entirely possible that the mechanism of production of Ep in the liver is the same as in the kidney. Indeed, anephric rats show increased erythrogenin production along with increased erythropoietin production in the liver when subjected to hypoxia (Kaplan *et al.*, 1973; Zucali and Mirand, 1974).

It is generally accepted that the primary action of erythropoietin is to induce a precursor of erythroid cells to differentiate into the recognizable red blood cells. Numerous experiments have shown that Ep does not affect the pluripotent stem cell (CFU) but rather the erythropoietin responsive cell (ERC) which is derived from the CFU (Schooley, 1969). During accelerated erythropoiesis, greater numbers of ERC's are forced to differentiate but the ERC pool is also maintained. Erythropoietin maintains the ERC pool by increased self-replication of the ERC's and probably not by increasing inflow from the CFU compartment (Reissmann and Udupa, 1972). A number of studies have also demonstrated that Ep can influence the maturation of the more differentiated erythroid cells (Fisher, 1962; Hodgson and Eskuche, 1962; Gallagher *et al.*, 1963). In addition, Ep has been shown to cause early release of reticulocytes from the bone marrow into the circulation (Gallagher

and Lange, 1962; Gordon et al., 1962). Thus, within 24 hours after the administration of a dose of Ep to polycythemic rats, an increased reticulocyte count is seen in the blood; this is too short a time period to allow for the maturation of ERC's into reticulocytes, therefore indicating an early release into the circulation (Gallagher and Lange, 1962). In isolated, perfused femurs of rats and rabbits, Ep in the perfusion fluid causes a selective release of reticulocytes from the marrow within a relatively short period of time (Gordon et al., 1962; Fisher et al., 1965).

An increase in the number of divisions of erythroblasts following exposure to Ep was noted (Necheles et al., 1968), an effect which was accompanied by the increased DNA and heme synthesis (Powsner and Berman, 1967). In this regard, Ep is suggested to increase the mitotic index of erythroid cells (Matoth and Kaufman, 1962; Paul et al., 1973). When bone marrow cells were incubated with serum from anemic rabbits, the mitotic index of the erythroblasts was increased (Matoth and Kaufmann, 1962). On the other hand, Alpen and Cranmore (1959) subjected dogs to hemorrhage and found no change in the number of cell divisions or mitotic rates and no early release of reticulocytes into the circulation. It appears, therefore, that Ep is involved in nearly all aspects of erythrocyte production. The recent observations of McCuskey et al., (1972) indicating a direct influence of Ep on the splenic blood flow and sinusoid number and size in the mouse provides evidence for a role of Ep in the release of red cells into the circulation.

The biochemical mechanism through which erythropoietin exerts

its effects on erythropoiesis has been studied extensively *in vitro* employing rat bone marrow cells (Goldwasser, 1972) and fetal liver cells (Cole and Paul, 1966; Silini *et al.*, 1967; Rifkind *et al.*, 1969; Paul *et al.*, 1971; Freshney *et al.*, 1972; Nicol *et al.*, 1972; Paul *et al.*, 1973). Within 5 minutes after exposure to Ep, rat marrow cells contain small amounts of RNA (150S) which are not present in control cells. There is no comparable species of RNA seen in the fetal liver cells (Nicol *et al.*, 1972). The RNA is found in the nucleus and has a very short half life of 6 minutes. Shortly after the formation of the 150S RNA, another species of RNA (60-70S) is seen with a half life of several hours, but whether it has a role in differentiation is unknown. Within 15 minutes after Ep exposure, r-RNA is formed along with t-RNA and an "iron effector" (Gross and Goldwasser, 1970) which allows iron to affect cellular function and a 9S RNA (probably globin messenger). Stimulation of RNA synthesis after incubation with Ep is also seen with fetal liver cell cultures (Nicol *et al.*, 1972). The RNA synthesis occurs predominantly in the proerythroblast compartment (Paul *et al.*, 1973).

Synthesis of DNA in adult marrow cells starts to increase after about 2 hours. Thus the tissue responds to erythropoietin prior to cell replication. In the fetus, the situation may be different. Paul and Hunter (1969) found that DNA synthesis was a prerequisite for RNA synthesis whereas Marks and Rifkind (1972) found that Ep could stimulate RNA synthesis without prior formation of DNA. Recent results suggest that while DNA synthesis is not necessary for RNA synthesis, it is required for the stimulation of globin

synthesis by erythropoietin (Maniatis and Marks, 1974). The increased DNA synthesis in the adult or fetus could reflect an effect of Ep on either the replication of the ERC's or their differentiation into more mature cells. By 4 hours new proteins are being synthesized which influences the uptake of iron from the medium. The uptake of glucosamine, formation of a glycolipid (Dukes, 1968) and stroma (Dukes et al., 1964) do not occur until 5 hours after exposure to Ep. Then within the next hour, the induced cells begin to show increased hemoglobin synthesis (Gross and Goldwasser, 1969).

In polycythemic animals, hemoglobin synthesis is not seen until 10-12 hours after exposure to erythropoietin. Since the polycythemic marrow is devoid of any differentiated cells, the data suggests that Ep acts on the more undifferentiated cells. Erythropoietin in the fetal liver cells causes a flow of cells from the proerythroblast compartment to the basophilic erythroblast compartment (Paul et al., 1973). The primary action in both systems seems to be an early increase in RNA synthesis in sensitive cells.

Bottomley and Smithee (1969) shed some light on the possible result of DNA dependent RNA synthesis. They demonstrated that rabbit marrow cells in vitro synthesized increased amounts of delta-aminolevulinic acid synthetase (ALAS) when exposed to erythropoietin. Puromycin prevented the increased enzyme activity suggesting that it depended on previous protein synthesis. Actinomycin D also prevented the rise in activity suggesting that the RNA synthesis was also necessary for enzyme activity. Since Ep stimulated heme

and globin synthesis (Mizoguchi and Levere, 1971) and ALAS is the rate limiting enzyme for both heme and globin synthesis (Levere and Granick, 1965), the synthesis of the enzyme may represent one of the principal actions of Ep.

The activities of the heme-synthesizing enzymes have also been examined in the mouse fetal liver cell (Freshney et al., 1972). During 24 hours, although erythropoietin had induced an increase in heme synthesis, no increased activities of ALAS, aminolevulinate dehydratase (ALAD), or heme synthetase were seen. The activities of all three enzymes, however, were higher in Ep treated cultures. At different gestational stages from days 13-17, the enzyme activities fluctuated but this could not be correlated with fluctuations of any cell type or with rates of hemoglobin synthesis (Freshney and Paul, 1971). Thus the enzymes are not directly involved with increased heme synthesis in the fetus.

In the mammalian fetus, the production of red blood cells occurs in four distinct phases: the yolk sac stage, the hepatic stage, the splenic stage and the myeloid stage. The yolk sac is the first place that hematopoiesis is seen and is generally confined to the blood islands of the yolk sac mesoderm. The cells (hemangioblasts) which are the precursors of the blood cells, only form in a favorable environment consisting of both endoderm and mesoderm layers (Wilt, 1965; Miura and Wilt, 1969). Thus erythroblastic differentiation is prevented in cultures where endoderm is not present whereas endoderm acting across a cell impermeable filter can induce hematopoiesis (Miura and Wilt, 1969). Production of hemoglobins during yolk sac erythropoiesis has been studied

extensively (Shepherd et al., 1962; Huehns and Shooter, 1965). In mice, the microenvironment may prevent the expression of adult hemoglobin production. Normally three hemoglobins are formed in the murine yolk sac and conversion from embryonic to adult hemoglobin consists of the substitution of one globin chain (Fantoni et al., 1967). During hepatic erythropoiesis, the hemoglobin formed is the adult type (Marks and Rifkind, 1972). When mouse CFU from the yolk sac are transplanted into irradiated adult hosts, some of these cells can produce adult hemoglobin (Moore, unpublished). When embryonic liver tissue, surrounded by a diffusion chamber, is cultured with dispersed yolk sac cells in vitro, there is some evidence that the yolk sac cells can produce adult hemoglobin (Barker, 1968).

Most vertebrates carry on erythropoiesis, granulopoiesis and megakaryocyte production at the yolk sac stage. In mice only erythropoiesis is seen (Metcalf and Moore, 1971); a relevant observation since it appears that the yolk sac may provide the precursors for all hematopoietic cells. Cells derived from the murine yolk sac when injected in to lethally or sublethally irradiated hosts, have the capacity to repopulate the lymphoid organs (Moore and Owen, 1967a; Moore and Metcalf, 1970). In vitro they can form erythroid, granulocyte and megakaryocyte colonies (Moore and Metcalf, 1970). Once the circulation is established, all stages of developing cells that are seen in the yolk sac can be seen in the blood (Moore and Metcalf, 1971).

There are two theories to account for the derivation of blood cells. The fixed tissue theory states that blood cells

are derived from the tissues in which they are found. The other theory suggests that cellular migration from one organ to another occurs with cells differentiating in their new environment (Wintrobe, 1974). The progression from yolk sac to liver seems to depend on the passage of cells from the former organ to the latter (Moore and Metcalf, 1970) although the evidence is not conclusive. Therefore every type of cell in the developing liver has been implicated as a source of hematopoietic cells (Bloom, 1938). However, the following observations suggest that cellular migration does occur. If the yolk sac is destroyed *in vitro* or if it is separated from the embryo by a filter that does not allow the passage of cells, hepatic erythropoiesis does not occur (Moore and Metcalf, 1970). In fact, throughout the gestational period, the total population of CFU's in the blood are ten fold greater than in the adult; these possibly are the cells that seed the hematopoietic organs (Moore and Owen, 1967a; Moore and Metcalf, 1970).

Hepatic erythropoiesis in the mouse commences at day 10-11 of gestation and hemoglobin containing cells increase exponentially until day 15 of gestation at which time the population doubling time increases from 8 hours to 2 days (Paul *et al.*, 1969). Within these few days the total number of erythroid cells in the liver has increased tenfold (Matioli *et al.*, 1968). During the rapid phase, the erythroid cells are immature and poorly hemoglobinized, and comprise 55-70% of all the cells in hepatic tissue. By day 17 the percentage of proerythroblasts decreases followed by a decline in the percentage of erythroblasts 72 hours later (Silini *et al.*, 1967). Hepatic erythropoiesis has declined by

birth but some foci of erythroid producing cells are seen one week after birth (Borghese, 1959).

The number of CFU's in mouse embryonic liver is greatest at days 12-14 of gestation (Silini et al., 1969). Liver CFU's are first seen at day 10 and their population increases rapidly, so fast in fact that cells from the yolk sac are probably being accumulated (Metcalf and Moore, 1971). Total CFU's double again between days 13-17 reaching a maximum at day 18. After birth there is a decline in both CFU's and hepatic erythropoiesis.

In mammals, as exemplified in the mouse, erythropoiesis in the spleen is followed by granulopoiesis and finally lymphopoietic activity. By day 13 the splenic anlage is detectable and erythropoietic activity is seen by day 15. At 17 days granulopoiesis predominates (Borghese, 1959). Thus the erythropoietic activity of the liver and the spleen overlaps. Chromosomal analysis of parabiosed chicks show splenic chimerism with 19-74% of dividing cells being derived from the partner (Moore and Owen, 1965). Since these cells were seen as early as twelve days, stem cells must enter the splenic rudiment before the initiation of hematopoiesis in that organ. Although the onset of splenic hematopoiesis is associated with the presence of CFU's, ERC's were not detected in the mouse spleen by days 16½-18 (Cole and Paul, 1966). However, by day 18, ERC's were detected in fetal rat spleen (Cole et al., 1968). These cells were no longer present in splenic tissue after day 19 of gestation (Cole et al., 1968).

The mouse femur at 15 days is composed of cartilage. The marrow cavity forms 2 days later and then becomes populated by

granulopoietic cells (Metcalf and Moore, 1971). Erythropoiesis is not seen until after birth in rats (Lucarelli et al., 1968) or mice (Petrakis et al., 1969). In goats myeloid erythropoiesis is seen by 70 days of gestation (Buckman, 1959). In organ culture 15 day mouse femurs developed marrow cavities but Ep and thyroxin were necessary for hematopoiesis to occur (Petrakis et al., 1959). Mesenchyme cells colonize the marrow space before the onset of hematopoiesis. Although it has been assumed that those cells were the erythroid precursors, observations on chick embryos challenge this hypothesis. Sex chromosomal analysis in parabiosed chick embryos of opposite sexes showed 14-74% of dividing cells in the marrow were derived from the opposite partner at 13-20 days. At earlier times, before the onset of hematopoiesis, very low levels of chimerism are seen (Moore and Owen, 1965, 1967b).

As in the adult, the morphological appearance of the erythroid precursors is not known. In the rat primitive cells are seen during hepatic erythropoiesis (Carmena et al., 1968). These cells have a basophilic cytoplasm and leptochromatic nucleus and occur in a syncytium. Primitive cells that have a more condensed nuclear chromatin and a more basophilic cytoplasm are also seen in the fetal liver and these have been suggested as possible erythroid precursors (Lucarelli et al., 1968). With increasing maturity less syncytial cells are seen and the erythroid cells that are produced become smaller (Carmena et al., 1968). Although hepatic erythroid cells resemble those seen in the marrow, the proliferative capacity and the regulation of the stem cells in these organs differ from hepatic cells exhibiting a greater capacity

for proliferation (Micklem and Loutit, 1966). Kubanek (1969) using equal numbers of adult mice bone marrow or fetal mouse liver CFU's demonstrated that irradiated hosts receiving fetal liver CFU's showed earlier erythroid development in both marrow and spleen with extensive colonization in the marrow. Kubanek (1972) suggests that the shorter generation time of CFU's from fetal liver accounts for the quicker and better recovery of erythropoiesis. Embryonically derived cells may be able to divide more times than adult derived cells; thus in a competitive situation the fetal cells would survive longer. This would also explain the higher percentage of mixed colonies seen in fetal transplants since the shorter generation time would cause the colonies to age faster (Kubanek, 1972). The stem cells may, alternatively, be programmed to undergo a fixed number of divisions; if this is so, adult stem cells derived originally from yolk sac stem cells will have undergone many divisions already.

The regulation of stem cells in the fetus and adult also differ. Erythroid spleen colony formation was looked at in polycythemic recipient mice. With adult marrow cells, 98-100% of erythroid colonies were suppressed whereas with fetally derived cells only a 44-69% reduction in colonies occurred (Feldman and Bleiberg, 1967; Bleiberg and Feldman, 1969). Thus only the embryonic cells could differentiate without erythropoietin. Kubanek *et al.* (1970) found that Ep was necessary for fetal and adult tissues for erythroid differentiation.

CFU's are seen in the mouse and chick marrow at the stage when hematopoiesis is initiated (Metcalf and Moore, 1971). The

source of these migrating cells and their progenitors may be the liver in the mouse, since hepatic erythropoiesis declines rapidly after birth as myeloid erythropoiesis continues. After myeloid hematopoiesis is established lymphoid progenitor cells from the marrow and spleen are exchanged and then cells migrate to the lymphoid organs from the spleen.

In vitro, the fetal hepatic erythroid cells that respond to erythropoietin and therefore cause an increase in hemoglobin synthesis at a later time, are different from the pluripotent stem cell and can be separated from the CFU by velocity sedimentation (Stephenson and Axelrad, 1971). The effect of Ep on the ERC has been studied extensively in vitro. In the absence of Ep, hepatic derived cells can synthesize hemoglobin at very low rates (Cole and Paul, 1966; Cole et al., 1968; Paul and Hunter, 1969; Paul et al., 1969). Addition of Ep to comparable cultures increases hemoglobin synthesis significantly until day 15. Although heme synthesis after day 15 is high, it is no longer responsive to erythropoietin (Cole and Paul, 1966; Cole et al., 1968). It has been hypothesized that Ep is not elaborated by the fetus prior to day 15 and therefore younger fetuses are more sensitive to the hormone. After day 15, the system is maximally stimulated so that hepatic cells have become refractory to Ep (Cole and Paul, 1966; Cole et al., 1968).

While much information is available regarding the regulation of erythropoiesis in the adult, relatively little is known about the regulation of red cell production in the neonatal and fetal periods of life. In the adult, hypoxia has been shown to be the major stimulant for erythropoiesis and the response is mediated

via the hormone erythropoietin (Krantz and Jacobson, 1970). In the newborn rat, the response to hypoxia is minimal, even though a detectable amount of Ep is measurable in the one day old rat (Carmena et al., 1968). After two-three weeks, the response to hypoxia becomes more striking and parallels that seen in the adult. Polycythemic neonatal rats also exhibit slightly increased erythropoiesis following exposure to hypoxia (Zaizov and Matoth, 1971). The main difference between the adult and the neonate lies in the magnitude of the response to the given stimulus. Starvation, which suppresses erythropoiesis in the adult animal, likewise has no measurable effect on the newborn rat until day 10. The guinea pig, though, does respond to starvation with the suppression of erythropoiesis (Lucarelli et al., 1968). One of the differences noted in the above animals is the degree of maturity of the hematopoietic system at birth. Whereas the guinea pig is actively producing red blood cells in its marrow, in the rat, hepatic erythropoiesis predominates until day 5 when a tremendous surge in myeloid erythropoiesis occurs (Carmena et al., 1968). A day old rat is slightly plethoric at birth but then becomes anemic by day 5 (Scribner Seeley et al., 1971). The anemia can be characterized by a hypochromic macrocytosis (Lucarelli et al., 1964). This is unusual, since in adults microcytosis precedes the onset of hypochromia and therefore possibly suggests that the mechanisms of red blood cell production are different in the neonate and the adult. Although the level of anemia that is seen would produce a detectable amount of Ep in the adult, no Ep is seen in newborn plasma (Carmena et al., 1968). In hypoxic or anemic human neonates,

increased levels of Ep have been demonstrated (Halvorsen and Finne, 1968). Garcia (1957) suggested that the newborn is so active in producing erythrocytes to counteract the anemia that its erythropoietic system is functioning at a near maximal rate and therefore cannot be stimulated further. There are compensatory mechanisms in operation during the anemic stage. In rabbits, as hemoglobin levels decrease, there is no increase in erythropoietin (Halvorsen and Halvorsen, 1974). This may be due to the fact that 2,3 diphosphoglycerate levels are elevated after birth (Blunt et al., 1971) in some animals that have been studied. However, the neonatal rat can be stimulated to produce more red blood cells and also to respond to Ep. In one day old rats, two different responses to injected Ep can be seen (Scribner Seeley et al., 1971): a release of reticulocytes into the circulation and also an increase in stem cell differentiation. Newborns that were made polycythemic also respond to Ep (Zaizov and Matoth, 1971). The importance of erythropoietin in neonatal erythropoiesis was further shown by Schooley et al. (1968) who demonstrated that anti-Ep injected into neonatal rats suppressed their erythropoietic activity. The most effective stimulus for Ep production in the neonatal rat seems to be administration of phenylhydrazine hydrochloride which produces hemolytic anemia (Morrissey et al., 1974). Injection of phenylhydrazine to 7-10 day old rats leads to high levels of erythropoietin in their plasma at 6-12 hours after injection. Rats are poikilothermic at birth and poikilotherms respond to a decreased red cell mass at a stimulus to elaborate erythropoietin (Hirshfeld and Gordon, 1965) whereas they

do not respond well to hypoxia (Atland and Parker, 1955). Again one might consider that phenylhydrazine may provide a greater degree of hypoxia than the other methods that have been employed. In general, however, neonatal animals produce measurably less Ep in response to a given stimulus than adults. This may explain the reduced erythropoietic response of the neonate to hypoxic stimuli. Halvorsen and Halvorsen (1974), however, suggest that the decreased erythropoietic response may relate more to the absence of sufficient iron than erythropoietin responsivity.

The site of Ep production also appears to be different from that in adults. Rats bilaterally nephrectomized before exposure to hypoxia show a reduced erythropoietic activity (Carmena et al., 1968) but it is not entirely abolished. Thus extrarenal sites of erythropoietin production have been implicated in the neonate. Highly elevated amounts of erythrogenin have been found in the liver and spleen of 5 day old rats rendered hypoxic by exposure to reduced atmospheric pressures (Kaplan et al., 1974) whereas no Eg was detectable in the kidneys at this stage. This observation is interesting since neonatal rats respond only minimally to hypoxic stimuli at birth and suggests a possible change from hepatic erythropoietin production to renal erythropoietin production (Carmena et al., 1968; Wang and Fried, 1972). It also suggests that the extrarenal production of Ep is probably operating at a lower capacity than the corresponding renal system.

Recent reports indicate that erythropoietin is also the regulator of erythropoiesis in the fetus. Suppression of erythropoiesis in some fetal mice and their mothers was seen after the

administration of antierythropoietin to the mothers (Schooley et al., 1968). Enhanced erythropoiesis in fetal rats was seen after their mothers were subjected to hypoxia or bleeding (Matoth and Zaizov, 1971). Erythropoietin was also seen in the amniotic fluid of women who gave birth to erythroblastotic infants (Finne, 1964). Before direct access to the fetus was available, much research centered around manipulating the erythropoietic status of the pregnant animal and noticing what effect these procedures had on the fetus. Such indirect manipulations, however, have produced evidence suggesting that the fetus may exercise a degree of autonomy with regards to the production and use of Ep. Thus, suppression of erythropoiesis was not seen in fetal mice who had polycythemic mothers (Matoth and Zaizov, 1971). Fetal mice whose mothers' erythropoietic activity had been suppressed throughout pregnancy showed no suppression of red cell production (Jacobson et al., 1959). This was also true when maternal erythropoiesis was suppressed by starvation (Lucarelli et al., 1968). On the other hand, subjection of maternal rats to hypoxia or bleeding resulted in augmented production of red blood cells in fetal rats (Matoth and Zaizov, 1971). Matoth and Zaizov (1971) suggested that erythropoietin may cross the placenta from the mother to the fetus since maternal anemia had a significant effect on fetal erythropoiesis. However, injections of large doses of Ep to mothers produced no effect in the rat fetus (Matoth and Zaizov, 1971). In addition in goats and sheep, Ep administered to the fetus or to the mother did not appear to cross the placenta in either direction (Zanjani and Gordon, 1971). Recent studies in

fetal sheep and goats, which possess fetuses of large dimensions for gaining direct access to the fetus, have shown that the increased production of red cells in the fetus following the induction of anemia in the mother results from an increase in Ep production in the fetus. This is produced from sites in the fetus in response to maternally induced fetal hypoxia (Zanjani et al., 1974).

The production of erythropoietin in the fetus appears to be regulated by mechanisms similar to the adult system. The first direct evidence that the fetus is capable of producing Ep was obtained in the fetal lamb (Zanjani et al., 1969). Thus, induction of anemia in fetal sheep by direct administration of phenylhydrazine hydrochloride, resulted in the appearance of significant quantities of Ep in the fetal circulation (Zanjani et al., 1971). Similar results were obtained when anemia was induced by direct bleeding of fetal sheep or goats (Zanjani and Gordon, 1971). The amount of erythropoietin produced was directly related to the degree of anemia that was induced. Shortly after the development of anemia, Ep levels increased but fluctuated. This seemed to be due to variations in the amount of substrate available for erythrogenin (Zanjani et al., 1971). The response to increased circulating red cell mass is also the same in the fetus as in the adult. Thus induction of polycythemia in fetal goats by transfusion with maternal red blood cells led to decreased erythropoiesis in the fetus (Zanjani et al., 1973).

The site of production of erythropoietin differs in the fetus and the adult. If pregnant goats are bled, a rise in Ep

is seen in both the mother and the fetus. If the mother is nephrectomized prior to bleeding, increased levels of Ep are only seen in the fetus (Zanjani et al., 1974). However, if the fetuses are nephrectomized before their mothers are bled, they still show high Ep titers in their plasma, whether the mothers are nephrectomized or not. Thus the fetal erythropoietin originates within the fetus and is not produced by the kidneys, since nephrectomy of the fetus does not abolish their erythropoietic response to bleeding. The present studies deal with the site of production of erythropoietin in the fetus and the role of erythropoietin in the fetus. The studies to determine the site of production of Ep in the fetus involved:

1) Removal or perfusion of the fetal sheep liver, spleen or kidney to determine if any or all of these organs were involved in the production of Ep.

2) Investigation of the presence or absence of erythrogenin in many of the fetal tissues.

The studies on the role of Ep in the fetus included:

1) The effect of erythropoietin on the fetal liver, spleen and bone marrow utilizing two in vitro techniques.

2) The effect of injections of antierythropoietin on fetal sheep to assess the normal role of Ep in the unstressed fetus.

MATERIALS AND METHODS

I. General

1. Experimental Animals. Pregnant sheep (Dorset-Merino strain) with confirmed dates of pregnancy were obtained from the animal research facilities of Yale University, New Haven, Conn. All animals were maintained on a regular diet and given water ad libitum.

Mice (Swiss-Webster strain, 22-25 gms.) were obtained from Taconic Farms, New York.

2. Bioassay for Erythropoietin. Erythropoietic activity was determined in mice that were made polycythemic by two daily intraperitoneal injections of washed, isologous red blood cells. Five to six assay mice were used for each material tested. Each mouse received one injection of the test material intraperitoneally on day 6 after transfusion. Radioiron (0.5 μc/mouse) was injected intravenously on day 8 and the incorporation of radioiron was determined on day 10. Blood (0.5 ml.) was obtained by cardiac puncture 48 hours after the administration of radioiron. The percentage incorporation of radioiron into red blood cells (RBC's) was calculated by:

$$\text{percent RBC } {}^{59}\text{Fe incorporation} = \frac{\text{Blood Volume x CPM/ml. blood}}{\text{CPM of injected } {}^{59}\text{Fe dose}} \times 100$$

Blood volume was assumed to be 7% of body weight. Mice with hematocrits below 54% were discarded.

3. Surgical Procedures. The fetuses were prepared by Drs. Mann and Zanjani. Surgery was performed on sheep at different stages of pregnancy. The sheep were observed, acclimated, and fasted for 24 hours prior to surgery. Progesterone (lipo-Lutin, 250 mg.) was given intramuscularly one day before the surgery and daily thereafter. Spinal anesthesia with 2% lidocaine or 1% pontocaine supplemented by halothane and O_2 was used. Ringer's lactate with 10% dextrose was infused during surgery.

Laparotomy, hysterotomy, and amniotomy with delivery of the fetal leg was performed. The fetal femoral artery and vein were cannulated. The amniotic fluid that was lost during the procedure was replaced with isotonic saline containing 5 million units of penicillin G and one gm. of streptomycin. The fetal artery cannula was brought out through the uterus and maternal abdominal wall and secured to the maternal skin. The uterine and abdominal wall were closed around the cannula in layers. Heparinized normal saline was used to maintain cannula potency. Ampicillin (250 mg.) was injected directly through the cannula to prevent infection.

All nephrectomies, hepatectomies and splenectomies were performed at the time of cannulation. The animals were manipulated so that the proper organs could be removed.

4. Tissue Extraction for Erythrogenin (Eg). The liver, spleen, pancreas, testis, heart and thymus of the fetuses were removed for study of erythrogenin. The tissues were removed immediately after the sacrifice of the animal and put on ice but not permitted to freeze. The tissues were weighed, minced, and homogenized in cold 0.25 M sucrose (10ml./gm. tissue) with a loose

fitting Potter-Elvehjem homogenizer. The mixture was then rehomogenized with a standard, tight-fitting pestle until no visible chunks of tissue were present. The homogenate was then centrifuged at 6300 x g. for 15 minutes at 5°C and the precipitate was discarded. The supernatant was recentrifuged at 21000 x g. for 20 minutes at 5°C. The sediment obtained was the light mitochondrial fraction. The sediment was resuspended in cold distilled water (2 ml./gm. tissue) in the presence of one drop of detergent (Cutscum, Fisher-Scientific Co.) and briefly rehomogenized. The mixture was then allowed to freeze. It was then thawed and centrifuged at 37000 x g. for 20 minutes. The supernatant which contains erythrogenin was kept frozen until used.

5) Dialysis of Serum Against Na_2EDTA. Normal serum was dialyzed against 100 volumes of 0.005 M disodium ethyldiamine tetracetate (EDTA) solution for 24 hours at 4°C. The EDTA solution was neutralized with 1N NaOH before use. The dialysand was redialyzed immediately thereafter against 100 volumes of cold deionized water for 24 hours. The dialyzed serum was then placed in storage vessels and frozen until used. The pH of the serum after dialysis ranged from 6.8 to 7.0. A similar procedure was employed to prepare EDTA dialyzed Eg.

6) Eg-Serum Incubation Technique. The incubation system consisted of equal volumes of dialyzed or undialyzed serum and the Eg preparation. All incubation mixtures were left open to the air and were conducted in a water bath incubator, shaken at 37°C. Saline was used to bring the mixtures to the desired volume.

Reactions were stopped at the end of the incubation period

by plunging the reaction flask into ice water. Materials were injected into the assay mice immediately after incubation.

7) Preparation of Anti-erythropoietin Serum. Six white New Zealand rabbits weighing 2½ kg. were immunized against human urinary erythropoietin. The Ep was prepared by carbowax concentration of the urine from a patient with aplastic anemia. The Ep was boiled for half an hour in a water bath to denature the proteins. The potency of the original preparation was 10 U/mg. protein. After boiling, distilled water was added to bring the solution back to its original volume. The denatured Ep was then mixed with complete Freund's adjuvant in a 1:1 ratio. After complete mixing, 0.25 ml. of the solution was injected into the foot pad of the rabbits on each side; i.e., a total of 1 ml. with 10 U Ep was injected. Ten-twelve days later, Ep prepared in the same way was injected into each rabbit. This time each rabbit was injected IM with 0.25 ml. at 4 different sites.

Ten days later serum was taken from each rabbit and examined for antibody production. Two rabbits had high levels and they were then used for the preparation of the anti-Ep serum. The rabbits were given booster shots 2 weeks after the last immunization with an Ep preparation which had a potency of 250 IU/mg. protein. After the booster shots are given high potency anti-Ep titers are obtained. One ml. of the anti-Ep serum neutralized 22 IU of Step III sheep plasma Ep (Connaught Laboratories, Toronto, Canada).

8) Culture Media. Supplemented Eagle's essential medium with Hank's balanced salt solution (supplemented MEM) (Grand

Island Biological Co., Grand Island, New York) was used for the plasma clot. The following components were added to a 100 ml. bottle: 1% MEM non-essential amino acids (100 mM), 1% 200 mM l-glutamine, 1% Na pyruvate (100 mM), 1.25% of 5% $NaHCO_3$ and 1% penicillin-streptomycin mixture giving a final concentration of 100 units/ml. and 100 µg/ml. respectively (all of the above are from Grand Island Biological Co., Grand Island, New York). The penicillin-streptomycin (lyophilized) was made by adding 20 ml. of bacteriostatic water to the lyophilized solution to give 10,000 units/ml. and 10,000 µg./ml. Lyophilized beef embryo extract (BEE) (Grand Island Biological Co., Grand Island, New York) was used in the plasma clot that was 50% by volume when reconstituted with the supplemented medium. This was kept frozen until used, at which time 1 ml. of the reconstituted extract was added to 5 mls. of medium NCTC-109 (Grand Island Biological Co., Grand Island, New York). L-asparagine (l-asp) (Calbiochem, San Diego, California) was made up in supplemented medium to a concentration of 2 mg./ml., sterilized through a Millipore filter apparatus containing a 0.45 u Millipore filter and frozen until used. In the clot, 0.5 ml. of asp was mixed with 4.5 ml. of NCTC-109 so that 0.1 ml. contains 0.02 mgs. of l-asp. Bovine serum albumen fraction V (BSA) (Sigma Chemical Co., St. Louis, Missouri) was also used in the plasma clot. For the preparation of 10% BSA, 50 gms. of BSA powder were added to 91 ml. of sterile distilled water and dissolved overnight at 4°C. The flask was rotated gently to insure that the powder on the sides would be dissolved. The following day 5 gms. resin were added and the mixture was left at 4°C for 2 hours. During the

first hour, the flask was gently swirled every 15 minutes. At the end of 2 hours, the BSA was decanted and the volume measured. To make the solution isotonic, 1.1 mls. of 1 M Dulbecco phosphate buffered saline (minus Ca^{++} and Mg^{++} ions) was added to each 15 mls. of BSA solution. For the final 10% stock BSA solution, the above was diluted with 0.1 M concentrated phosphate buffered saline (minus Ca^{++} and Mg^{++} ions). The solution was mixed well and filtered through a Whatman #1 filter paper and then sterilized with a Millipore filtering apparatus containing a 0.45 μ Millipore filter. The solution was kept frozen until used. Before use in the plasma clot system, 0.5 mls. of 7% $NaHCO_3$ was added to the 10% BSA.

The following media were used in both the plasma clot system and the cell suspension cultures. NCTC-109 (Microbiological Associates, Bethesda, Maryland) was also used to dilute the BEE in the clot. Otherwise, as a diluent or when it was used directly in both <u>in vitro</u> techniques, penicillin and streptomycin were added to a concentration of 100 units/ml. and 50 μg/ml. respectively and stored at $4^{\circ}C$ after use. Citrated sheep plasma was obtained from the adult sheep during our study and kept frozen until used. Fetal calf serum (FCS) which was previously heat inactivated (Grand Island Biological Co., Grand Island, New York) was kept frozen until used. Erythropoietin Step III from anemic sheep (Connaught Medical Research Laboratories, Ontario, Canada) 2.8 to 4.7 IU/mg. protein was diluted with NCTC-109 so that the final concentration was either 0.2 or 0.5 IU/.1 ml.

9) <u>Culture Methods</u>. The tissues that were utilized in the

culture techniques were removed from the fetus aseptically. Small pieces of liver and spleen were placed immediately into the collecting medium, which consisted of supplemented MEM made 2% with FCS immediately before use. Marrow specimens from the adult sheep were obtained by aspiration and placed in supplemented MEM with heparin made 2% with FCS. Bone marrow specimens from the fetal sheep were obtained by aspiration when possible and placed immediately into supplemented MEM with heparin. In the very young fetuses, the bones were not well formed enough to remove specimens by aspiration; in these cases, the femurs were cracked and the marrow cavities were scraped.

All specimens were used within 2 hours after collection. Moreover, during this time interval, the tubes containing the tissues were inverted frequently to insure that all the cells were kept in contact with the medium. The liver and the spleen cells were then spun in a Sorvall desk top centrifuge at 1000 RPM for 10 minutes at room temperature. The supernatants were removed and the cells were resuspended in supplemented MEm with FCS. The bone marrow cells were spun twice to remove the heparin and resuspended in supplemented MEM with FCS. After the second washing, the buffy coats were carefully removed and resuspended in supplemented MEM with FCS. A cell count was then made for each tissue and the proper dilution were then made from each stock of cells into the same medium. All the cells were then stored on ice until used.

The plasma clot technique used was a modification of the procedure originally described by Stephenson et al.,(1971) and

improved by McLeod et al. (1974). One tenth ml. of cell suspension containing a predetermined number of cells were added to the following mixture: 0.1 ml. BEE, 0.1 ml. BSA, 0.2 ml. FCS, 0.1 ml. asp, 0.1 ml. of Ep (step III sheep plasma) or an equivalent amount of NCTC-109 in the control cultures and 0.3 ml. NCTC-109. It has been shown that Ep in these cultures will stimulate the differentiation of erythropoietin sensitive cells (ERC) into recognizable erythroid cells.

The above suspensions were then mixed well with 0.1 ml. of citrated sheep plasma (obtained from the adult sheep) that had been warmed in the incubator immediately prior to use. After thorough mixing, achieved by repeated drawing of the materials into a one ml. syringe fitted with a 20 gauge needle, one tenth ml. was placed in each of 8 wells of a microtiter plate (disposo trays, Linbro Chemical Co., New Haven, Conn.). Four cell concentrations were used: 2×10^5, 4×10^5, 6×10^5 and 8×10^5/1.1 ml. Thus, final cell concentration in each well was 18,000, 36,000, 54,000, and 72,000 cells. As shown in Figure 1, a linear relationship exists between the number of cells plated and the number of colonies scored, indicating that each colony is derived from an individual ERC. Groups of 4 wells each were placed in a 35 x 10 mm. petri dish and two such dishes were placed in a Optilux Tissue Culture Dish (100 x 20 mm.) (Falcon Plastics, Oxnard, California), along with another petri dish containing distilled water. All but the water dish were covered. After allowing the mixture to clot, they were examined under an inverted microscope to ensure that there were no clumps of cells and also to check that the cells were

evenly dispersed. The dishes were then placed in an incubator for 2-4 days at 37°C with a humidified atmosphere consisting of 5% carbon dioxide in air. The clots were then removed from the wells and placed on a glass slide (4 clots/slide). The excess fluid around the clot was removed and then a piece of filter paper was placed on each slide to absorb the remaining fluid. The clots were then gently flattened with the side of a Pasteur pipette and fixed in a glutaraldehyde-phosphate buffer mixture. The filter paper was then removed, the clots allowed to dry and then dehydrated in absolute methanol for 30 seconds.

Staining Procedure

(1) 3,3' Dimethylbenzidine (Practical, Eastman Kodak Co., Rochester, New York). A 1% solution was made in absolute methyl alcohol. The stain was aged for several days before it was used and its quality determined by testing with a peripheral blood smear.

(2) Hydrogen peroxide 30% (Fisher Scientific Co., Fairlawn, New York). One part of hydrogen peroxide was mixed with 11 parts of 70% ethanol to give a final concentration of 3% hydrogen peroxide.

(3) Hematoxylin Stain Solution (Harris-Lillie, Fisher Scientific Co., Fairlawn, New Jersey).

The slides were placed in benzidine for 4 minutes, hydrogen peroxide for 1½ minutes, distilled water for 1 minute and hematoxylin for 5 minutes. They were then placed under running water for 10 minutes and allowed to dry. The cover slips were mounted with Permount (Fisher Scientific Co., Fairlawn, New Jersey).

Scoring of Colonies

Each clot was examined under 100 X magnification. If 8 or more cells within a group were benzidine positive, i.e. exhibited a yellow to brown cytoplasm, they were considered a colony.

Cell Suspension Culture

All of the tissues to be used for this procedure were taken from the same stock of cells that were used for the plasma clot. Therefore, they had been washed twice in 2% FCS in modified MEM. The following were added to the plastic petri dishes (35 x 10 mm.): 0.3 ml. of cells (for a final cell concentration of 1-5 x 10^6 cells), 0.7 ml. sheep plasma, 0.7 ml. FCS, 0.04 ml. pen/strep, 0.06 ml. heparin, 0.2 or 0.4 ml. 5U Ep/ml., 1.6-2.0 ml. NCTC-109, to give a final volume of 3.5 ml. The dishes were then incubated for 48 hours at 37°C in a 5% carbon dioxide-air mixture. At this time, 0.5 µc $^{59}FeCl_3$ in 0.1 ml. sheep plasma was added to each plate. The sheep plasma containing the radioiron had been incubated at 37°C for 24 hours prior to use. The plates were then gently rotated to ensure adequate mixing of the iron with the cells. After 5-6 hours incubation with the iron, the contents of each dish was transferred via a Pasteur pipette to a glass tube. Each tube was washed with 2 ml. saline and the wash was added to the cell mixture which was then centrifuged at 1000 RPM for 10 minutes in a Sorvall desk top centrifuge. The supernatant was discarded and the cells washed twice more with saline. To the final cell pellet, one ml. of distilled water and one ml. of Drabkin's reagent (Fisher Scientific Co., Fairlawn, New Jersey) was added forcefully followed by vigorous agitation. The mixture

was allowed to remain at 4°C for 24-28 hours. The samples were then centrifuged at 2500 RPM for 30 minutes and the supernatants were transferred to a new test tube. The pH of the supernatant was lowered to 2 with 1 N HCl (about 0.3 ml.) and 2.5 ml. cyclohexanone was added to each tube. After vigorous mixing the tubes were refrigerated overnight. The next day the tubes were centrifuged at 1500 RPM for 20 minutes and 1.5 ml. of the cyclohexanone layer was transferred to a counting vessel for the determination of counts.

II. Experimental: Studies on the Site of Production of Erythropoietin in the Fetus

1. Effect of Organ Removal on Erythropoietin Production. To determine the site of production of erythropoietin in the fetus, the kidneys and liver or spleen were removed and the animals were then bled to assess their erythropoietic response. A catheter was first placed in the femoral artery of the fetus and the fetus was then moved backwards so that the site for surgery was exposed at the opening of the uterus. The proper incisions were then made and the organs removed. For nephrectomies, either a single or a double retroperitoneal incision was made. In the case of a single incision, the gut was pushed aside, the capsule cut, and the kidney was popped out through the capsule. The necessary blood vessels were then ligated. For splenectomies or hepatectomies, the point of entry into the fetus was always through a longitudinal incision starting at the diaphragm. The incision was always made to one side in order to avoid the umbilical cord. In multiple organ removal studies involving the spleen and kidney, the spleen

was removed first and then the kidneys were removed. When the liver and kidney were involved, nephrectomy was performed first and then lobes of the liver were tied off and cut. Seventy five-eighty percent of the liver was removed; the smallest lobe, usually located posteriorly, was left intact. Following surgery, the fetuses were returned to the uterus.

In nephrectomy studies, the fetuses were allowed to recover for 6-7 hours and then bleeding was induced via the femoral artery catheter. Thirty-forty ml. of blood were removed, depending on the size of the fetus. The average age of these fetuses was 105 days.

In hepatectomy studies, the animals were bled as soon as the mothers came out of anesthesia. Regardless of the size of the animal, 30 ml. of blood were removed. Samples for Ep assay were removed 6 hours after bleeding in all cases, even though comparable results are obtainable from 3-19 hours after bleeding.

Assays were performed in the polycythemic mouse assay, each mouse receiving 0.5 ml. of plasma.

2. Organ Perfusion Studies. Perfusion of the liver, spleen and kidney was performed in 100 day old fetuses to determine the site of production of erythropoietin. The fetuses were cannulated as described above and then bled 24 hours later. At this time samples were taken for Ep assays to insure that the animals were anemic and that their Ep production was stimulated. The mothers were then anesthetized 21 hours after bleeding and the fetus exteriorized for the perfusion studies. The liver, spleen and kidney were not removed from the body but rather were perfused

in situ. Only one organ from each fetus was used. Four fetuses were used in each of the spleen and liver perfusion studies, whereas six animals were employed in the kidney group. All organs were washed well with Tyrode's solution before perfusion. The apparatus for perfusion was an adaptation of the one used by Gordon *et al*. (1968). In the case of the liver, the hepatic artery and vein were cannulated and blood from a normal fetus was recirculated in a closed system. For the first 35 minutes of the perfusion, Tyrode's solution was passed through the liver to remove any trapped Ep. This wash fluid was discarded. This was followed by 2 hours of perfusion with whole blood. The cannula from the vein was secured at the end of a section of 25 mm. tygon tubing which extended through a sigmamotor finger pump. After passing through the pump, the tubing was connected to the sidearm of an Erlenmeyer filtering flask. The neck of the flask was closed by a one hole stopper in which a piece of glass tubing extended from the bottom of the flask to several centimeters above the stopper. Another section of the tubing was connected to the glass tubing extending from the flask. The other end of this section of tubing was connected to the cannula that went into the hepatic artery. This section of tubing had three connections: a syringe with which to withdraw or add fluid, a connection for a temperature recording device, and a pressure register. An infrared lamp next to the flask (blood reservoir) was used to maintain the temperature of the system at 37°C. Samples were taken at 120 minutes after the start of the perfusion and were assayed for the amount of Ep present in the perfusate.

3. Erythrogenin Activity of Organs From Fetal Anemic Sheep. Ninety to one hundred and ten day old sheep fetuses were made anemic by removing 30-35ml. of blood through a catheter that had been placed previously in the femoral artery. Sixty six-seventy two hours after bleeding the following organs were removed: kidney, spleen, liver, testis, thymus, lung, pancreas, and brain. Tissues from 8 fetuses were used in this study. A hypotonic extract from the light mitochondrial fraction was prepared as described above for the Eg assays. To eliminate the possibility that the Eg extract alone had erythropoietic activity, it was necessary to test both the extract alone and the extract after it was incubated with serum. The extract alone (1 ml. extract + 1 ml. saline) was assayed by the polycythemic mouse assay and the %Fe incorporation into red blood cells was recorded. The extract was also incubated with serum (in a 1:1 ratio) and 2 ml. were injected into each assay mouse.

4. The Effect of Erythropoietin on the Liver, Spleen, and Bone Marrow In Vitro. Hepatic and splenic tissues were removed from the 60 day old fetus aseptically and suspended into supplemented MEM with FCS. The tissues were minced into small pieces with a scissors and then drawn through a syringe with a 19 gauge needle to disperse the cells. A smaller bore needle was then employed when possible. Marrow specimens were not obtainable from the fetus this young because the bones are still cartilagenous at this stage of development and the marrow cavity has not formed. In cases where not enough cells were available for both techniques, we utilized only one or two cell concentrations

in the clot. Within 2 hours after the cells were collected, they were washed twice with supplemented MEM with FCS and a cell count was made. This stock of cells was then kept cold and used for both the cell suspension technique and the plasma clot. Cell dilutions were made up in supplemented MEM with FCS, immediately before they were added to the tubes containing the culture media for the clot. Eight clots were made for each cell concentration for each tissue and the clots were examined 3 days later.

In both the cell suspension technique and the plasma clot technique, 2 concentrations of Ep were used. In the cell suspension technique, 0.29 and 0.58 IU Ep/10^6 cells were used and 0.25 and 0.50 IU Ep/1.1 ml. of media in the plasma clot were used. Preliminary experiments had determined that we were using the optimal Ep concentrations in both systems. Control cultures did not contain Ep but were made up to the same volume with NCTC-109 as the Ep containing samples.

The same procedure was repeated on fetuses at day 29, 34, 38, 50, 60, 105, 121, 128, and 135 days of gestation. The first time that a marrow specimen was obtainable was at 70 days. Maternal samples of bone marrow were taken on 5 occasions and cultured in the same manner as fetal tissues except that the specimens are initially collected into supplemented MEM with heparin to prevent clotting.

5. <u>The Effect of Anti-Erythropoietin Serum on Erythropoiesis in Normal Sheep Fetuses</u>. Before the fetuses were injected with antibody, it was necessary to determine the potency

of the anti-Ep serum. One milliliter of the immune serum was found to neutralize approximately 22 IU of sheep plasma Ep (Step III). To neutralize the rabbit anti-Ep, 0.45 ml. of goat anti-rabbit gamma globulin (GARGG) (Antibodies Inc., Davis, California) were incubated with 1 ml. of anti-Ep serum for 30 minutes at 37°C in a water bath with constant shaking. The resulting precipitate was removed by centrifugation and the supernatant was removed. The supernatant was found to be lacking in anti-Ep activity. All the other samples that were assayed were also incubated at 37°C for 1 hour before injection.

The following materials were assayed for erythropoietic activity in the polycythemic mouse assay and the percentage ^{59}Fe into RBC's recorded (Table 10):

saline (1 ml.)

1 IU Step III Ep (0.5 ml. + 0.5 ml. saline)

1 IU Ep (0.5 ml.) + normal rabbit serum (0.045 ml. serum + 0.455 ml. saline)

1 IU Ep (0.5 ml.) + anti-Ep serum (0.045 ml. serum + 0.455 ml. saline)

1 IU Ep (0.5 ml.) + anti-Ep serum (0.045 ml.) + GARGG (0.02 ml.) + saline (0.453 ml.)

Twelve pregnant ewes with confirmed dates of conception were used in these studies. The fetuses ranged in age from 105-120 days. Each fetus was prepared for the study by inserting a catheter into each of its two femoral arteries as described above.

Twelve ml. of blood, obtained from each fetus and mother at 15-22 hours after the completion of the surgery, was used to

obtain baseline peripheral blood data including Ep levels in the plasma. In general, materials injected into the fetus were administered via one catheter, and blood samples were withdrawn from the fetus through the second catheter. However, when flow was interrupted in one catheter the remaining one was used for both procedures.

Four fetuses (group 1) were given daily injections of one ml. of normal rabbit serum for 3 days. Five other fetuses (group 2) received three daily injections of one ml. of serum from rabbits immunized against human urinary Ep. Erythropoietin used to immunize the rabbits was prepared from urine of a patient with aplastic anemia. The remaining 3 fetuses (group 3) were given the same immune serum, anti-Ep activity of which had been neutralized by previous treatment with a preparation of goat anti-rabbit gamma globulin antibody (GARGG) (Antibodies Inc., Davis, California), prior to injection. One and four tenths ml. was injected daily for 3 days to each fetus in group 3. Peripheral blood reticulocyte levels were determined in all fetuses at daily intervals beginning at 24 hours after the first injection of the serum. Six hours after the last injection of the serum, 12 ml. of blood was obtained from each fetus and the plasma separated and used for determining Ep levels. One day after the last serum injection, each fetus (in all groups) received 30 μCi $^{59}FeCl_3$ which had been preincubated with 2 ml. of the fetus' plasma for 30 minutes at 37°C. Plasma used for this purpose was separated from 5 ml. of blood obtained from each fetus immediately prior to use. At the end of the incubation, the plasma containing the isotope was added to their

respective blood cells and the entire mixture injected into the femoral artery of the fetus. Percent incorporation of radioiron into fetal red blood cells was determined 36 hours later. Blood volumes of the fetus was assumed to be 5% of the body weight.

Three ewes from groups 1 and 2 and all the animals in group 3 were sacrificed 2 hours after samples of peripheral blood were obtained from each fetus for determination of red blood cell-radioiron incorporation values. Portions of liver, spleen, and bone marrow were taken from each fetus for isotope counting. Morphological examination of the bone marrow was also performed. The number of erythroblasts per milligram of marrow was calculated using the method of LoBue et al. (1963). The remaining ewes were subsequently used in experiments designed to determine the site(s) of Ep production in the fetus. Plasma Ep levels were determined in mice rendered polycythemic by hypertransfusion.

RESULTS

1. Effect of Organ Removal on Ep Production. In order to determine the site of production of erythropoietin in the fetus, the liver, spleen or kidneys were removed from the animals. The fetuses were then bled to assess their ability to respond to a stimulus for Ep production. Fetuses which had splenectomies or hepatectomies also had their kidneys removed at the time of surgery. Plasma samples were taken and then assayed in the polycythemic mouse and the percentage of incorporation of radioiron into RBC's was recorded. As shown in Table 1, only the hepatectomized nephrectomized fetuses showed a decline in the erythropoietic activity. There is no significant difference in the erythropoietic activity seen in plasma from anemic normal (non-nephrectomized) fetuses, nephrectomized fetuses or splenectomized nephrectomized fetuses. The fetuses which were not made anemic showed negligible amounts of erythropoietin in their plasma.

It should be noted that although the erythropoietin levels were not significantly different in splenectomized nephrectomized fetuses compared with nephrectomized controls, the former values were always lower than those found in either nephrectomized fetuses or the non-nephrectomized anemic group.

2. Effect of Organ Perfusal on Ep Production. In an effort to determine the organ(s) involved in the production of Ep in the fetus, individual organs were perfused *in situ* after the fetus had been bled to stimulate erythropoietin production. The results

of these experiments, which are shown in Table 2, indicate that only the perfusate from the liver contained a significant level of Ep after 2 hours of perfusion. There was no erythropoietic activity in the fluid which perfused the kidney of the fetus after 2 hours. Although the range shown for the fetal spleen are 0-1 IU Ep/2 hours, it should be noted that there was activity in one of the four spleens that was perfused. Since 50 ml. of whole blood were used to perfuse each organ, about 25 ml. of plasma were obtainable for the assay. This assay method detects levels of Ep as low as .05 IU/ml. so that one unit of Ep represents the lowest possible amount of Ep that is measurable by the assay.

3. Erythrogenin Activity in Organs From Fetal Anemic Sheep. To determine whether hypotonic extracts of the light mitochondrial fractions from various fetal tissues possessed any erythropoietic generating capacity, extracts were prepared from 9 organs in the fetus. The tissues employed were the kidney, liver, spleen, pancreas, lung, testis, thymus and brain. Light mitochondrial extracts from anemic sheep were prepared and these were incubated alone and with EDTA dialyzed normal sheep serum for 60 minutes and the activity of the mixture was determined. The results, as shown in Table 3, revealed that there was a minimal amount of erythropoietic activity in the extracts from fetal liver, pancreas, thymus and brain when they were incubated with normal sheep serum. These values though were comparable to values obtained upon incubation of sheep serum alone and therefore no significance was attributed to this activity.

4. Effect of Erythropoietin on Fetal and Adult Liver Cells In Vitro. Cells derived from fetal liver were put into

cell suspension culture at days 38, 50, 60, 105, and 135 of gestation. Incubation of nucleated cells obtained from fetal liver homogenates with transferrin bound radioiron resulted in significant incorporation of the label into the extractable heme. As shown in Table 4 cells derived from the liver of the 38 day old fetus demonstrated a high rate of heme synthesis in this suspension culture. The rate of heme synthesis was significantly greater ($p < 0.005$) in cells obtained from the 50 day old fetus than cells taken at 38 days of gestation. After day 50, the rate of incorporation of radioiron into heme decreases significantly reaching negligible levels by day 135. Cells capable of synthesizing heme were not detectable in preparations derived from livers of fetuses older than 105 days. This was also true of cells obtained from adult sheep liver.

When erythropoietin was added to suspension cultures of cells from fetal livers, the rate of incorporation of radioiron into heme was also greatest at 50 days of gestation. There was no detectable stimulation by Ep in cultures of liver cells from 38 day old fetuses. At 50, 60, and 105 days of gestation, the incorporation of radioiron into heme was greater ($p < 0.005$) than in control cultures of liver cells that were incubated without Ep. The magnitude of the response of fetal liver cells in the presence of Ep was essentially the same at 50, 60, and 105 days of gestation. However, the rate of heme synthesis in liver cells from fetuses after 50 days is much lower than in liver cells from 50 day old fetuses. By 135 days cells were not synthesizing heme even when Ep was present. The cells derived from adult liver did not respond

to Ep in culture, which is comparable to the results seen in the near term fetal liver cells.

In all the liver cell cultures that were studied, the addition of .29 IU Ep/1-5 x 10^6 cells provided the maximal increase of heme synthesis over the control cultures lacking Ep. Addition of .58 IU Ep/10^6 cells also stimulated the rate of heme synthesis in liver cells compared with control cultures, but the rate was lower than with the addition of .29 IU Ep. Thus the higher amounts of Ep were somewhat inhibitory.

Table 5 shows that liver cells derived from fetuses 29, 34, 50, 60, 70, 105, and 135 days of gestation were incubated in plasma clot cultures for 3-5 days. The optimal results were obtained in 3 day old cultures; by 5 days the morphological appearance of the colonies indicated that the cells were beginning to deteriorate. The membranes of the cells became crenated prior to cell lysis. A linear relationship existed between the number of cells plated and the number of colonies that were counted. The optimal dose of Ep was .25 IU Ep/1.1 ml. medium for all cell concentrations. Without Ep, there were only one or two colonies present at the higher cell concentrations except in the case of the 34 day old fetus, as shown in Table 5. In this fetus there were 4.0 $\pm$ 1.5 endogenous colonies/clot as compared with 57.3 $\pm$ 2.2 colonies/clot with Ep for 54,000 cells plated/clot.

Erythropoietin stimulated the growth of erythroid colonies at 29, 34, 50, 60, and 105 days. The maximal response to Ep occurred between 50-70 days and started to decline after 105 days (Table 5). The sensitivity of the cells to Ep in the clot was

much greater than in the cell suspension cultures at this time. For instance, at 60 days of gestation, with 72,000 cells/clot, 1.5 colonies were present without Ep compared with 88.25 colonies when Ep was present in the culture medium. At 135 days of gestation there was no growth of erythroid cells, even in the presence of erythropoietin. Adult liver tissue was also unable to develop erythroid colonies either with or without Ep in the plasma clot.

It was noted throughout these studies that when good growth of erythroid colonies occurred, there was excellent preservation of both mature red blood cells in the clot and also development of white cell colonies.

Effect of Erythropoietin on Fetal Spleen Cells In Vitro

Cells derived from fetal spleen were studied in cell suspension cultures at 105, 121, 129, and 135 days of gestation. As seen in Table 6 the incorporation of radioiron into cells derived from the fetal spleen was highest at 105 days and declined rapidly within the following 2 weeks. By 121 days of gestation, cells derived from the fetal spleen were no longer synthesizing heme at detectable levels. At gestational ages close to term, no heme synthesis was occurring in splenic-derived cells since the uptake of radioiron was negligible (Table 6). When erythropoietin was added to the culture media, a significant stimulation of heme synthesis was seen by splenic cells from fetuses at 105 days of gestation. Thereafter, the presence of Ep increased the rate of hemoglobin synthesis but not significantly, as shown in Table 6. At term, the splenic cells failed to respond to Ep in vitro (Table 6).

The degree of stimulation of heme synthesis was not dependent on the concentration of Ep used in this study since the optimal doses of Ep were employed with all the tissues that we utilized (Tables 4, 6, 8). For example, in splenic cells derived from 105 day old fetuses, a five fold increase in the uptake of radioiron occurred with either .29 or .58 IU Ep/1-5 x 10^6 cells (Table 6).

Spleen cells from fetuses that were 29, 60, 70, 105, 121, and 135 days old were incubated in the plasma clot. As shown in Table 7, the spleen derived cells were unable to produce erythroid colonies in the clot at any gestational age in the absence of erythropoietin. However, when splenic cells derived from fetuses 60-105 days old were incubated with Ep in the clot, large numbers of erythroid colonies were produced. For instance, Table 7 shows that when 4 x 10^5 cells from 70 day old fetuses were incubated in the clot with Ep, 191 $\pm$ 51.3 erythroid colonies were formed as compared to 0 colonies for cultures devoid of added Ep.

To look at the progression of erythropoiesis in the fetal hematopoietic tissues, <u>in vitro</u>, it was necessary to compare the results obtained from cell suspension cultures with the results from the plasma clot studies. Cells derived from the fetal liver are incorporating radioiron into heme at a maximal rate with erythropoietin in the culture at about 50 days of gestation (Table 4). Thereafter, the number of heme synthesizing cells decreases tremendously indicating that the liver is becoming less active as an erythropoietic organ. Using spleen cells from 105 day old fetuses (Table 6), one sees that the heme synthetic activity is very high and drops off rapidly. Spleen cells were not studied from fetuses younger than 105 days old in the cell

suspension culture since the spleens of these fetuses are too small to permit an adequate number of cells. It is evident though that the erythropoietic activity in the spleen is high when the activity in the liver is waning. These results support the observations of Buckman (1959) that the progression of erythropoiesis from the liver to the spleen in caprine embryos occurs around 70 days of gestation.

Support for this observation can be found in the plasma clot studies with cells derived from either the fetal liver (Table 5) or spleen (Table 7). Cells derived from the fetal spleen are maximally stimulated by erythropoietin in the clot at 70-105 days, whereas at 30 days of gestation, there are no colonies formed from spleen tissue. At 60 days of gestation, Ep stimulates the fetal spleen cells to produce erythroid colonies (Table 7). Liver cells of 29 and 34 day old fetuses were both stimulated by Ep in the plasma clot, indicating that the liver is erythropoietically active before the spleen becomes active.

Effect of Erythropoietin on Fetal and Adult Bone Marrow Cells In Vitro

Bone marrow cells were aspirated from fetuses at 70, 105, 121, 128, 135, and 138 days of gestation. The washed cells were then placed in the cell suspension culture or in the plasma clot. As seen in Table 8, in the cell suspension culture, the incorporation of radioiron into heme by these cells was negligible in the absence of erythropoietin at all the time periods that were studied except at 138 days (Table 8). At all gestational ages addition of Ep, however, resulted in significant stimulation of heme

synthesis. For instance, in marrow cells from fetuses at 128 days of gestation, Ep increased heme synthesis six fold over cultures that were incubated without Ep (Table 8). Bone marrow cells from adult sheep synthesize hemoglobin in the cell suspension culture without Ep but a four to five fold increase in synthesis is seen upon addition of .29 IU Ep to the medium. A greater stimulation of heme synthesis in adult sheep bone marrow cells occurs when .58 IU Ep are present in the culture (Table 8). In the near term fetus (128-138 days old) the stimulation by Ep of heme synthesis reached a maximal level and then leveled off (Table 8). Although the level of heme synthesis oscillated in the control cultures, the increased heme synthesis in the presence of Ep as compared to the control cultures remained constant in the later time periods (Table 8). A similar pattern was observed in the plasma clot cultures (Table 9). Unlike the results obtained in the cell suspension cultures, no colonies were formed in the absence of erythropoietin in the plasma clot except from fetal bone marrow cells from 138 day old fetuses. At this stage of gestation, several colonies were formed without Ep in the clot (Table 9); nevertheless with Ep there was a significant increase in the number of colonies. In bone marrow cells from 70 day old fetuses, the number of erythroid colonies was large and there were also many 4-6 cell colonies in the clot, suggesting that the bone marrow at this stage of gestation was just beginning to become a hematopoietic tissue.

In the adult marrow cells, there is a thirty fold increase in erythroid colony formation when the cells were incubated with

Ep in the clot (Table 9). Erythropoietin stimulated colony formation to a greater extent in the near term fetus than in the adult, a feature which was also noted in the cell suspension technique.

5. Potency of the Anti-Ep Serum and the Effect of Anti-Ep Serum on Erythropoiesis in Normal Sheep Fetuses. Table 10 lists the data pertaining to the potency of the anti-Ep serum used in this study and the effect of GARGG on the neutralizing ability of the antiserum. One milliliter of the immune serum was found to neutralize approximately 22 IU of sheep plasma Ep.

Table 11 shows the results of Ep assays on fetal plasma samples before and after three injections of rabbit serum. Plasmas from one fetus (b) in group 1 (treated with normal rabbit serum) and 2 fetuses (a and d) in group 2 (treated with anti-Ep serum) exhibited detectable erythropoietic activity before the injections of sera. Erythropoietic activity was still demonstrable in the plasma sample obtained from the fetus in group 1 at 6 hours after the last injection of normal rabbit serum. However, plasmas obtained from the 2 fetuses in group 2 at 6 hours after the last injection of anti-Ep serum were devoid of any detectable activity (Table 11). Plasmas from the other fetuses in the 3 groups did not exhibit erythropoietic activity either before or after treatment (Table 11).

Table 12 demonstrates that a significant depression of erythropoiesis occurred in fetuses receiving anti-Ep serum. This was evident from the reduced uptake of radioiron by the red blood cells, spleen, and bone marrow cells in anti-Ep treated fetuses compared to fetusus receiving normal rabbit serum or GARGG-treated

immune serum (Table 12). The rate of incorporation of radioiron by hepatic tissue from fetuses receiving anti-Ep serum was not affected.

Suppression of erythropoiesis in anti-Ep treated fetuses is further documented in Table 13. A sharp decrease in the numbers of circulating reticulocytes occurred in these fetuses following injections of anti-Ep. The values for percent reticulocytes after treatment shown in Table 13 were obtained at 24 hours after the third and last injection of serum into the fetus. However, a significant decrease in the numbers of circulating reticulocytes was also noted in this group following the second injection of the immune serum. The decline in the numbers of circulating reticulocytes was accompanied by a significant decrease in the numbers of nucleated erythroid cells (which are indicated in Table 13 as erythroblasts) in the bone marrows of these fetuses.

DISCUSSION

It is well established that the kidney is the major site of erythropoietin production in the adult mammal (Jacobson et al., 1957a&b). Intact animals exposed to hypoxia or treated with hemolytic agents respond to these erythropoietic stimuli by producing increased quantities of Ep. However, it has been demonstrated that bilateral nephrectomy significantly suppressed the production of Ep in animals exposed to hypoxic stimuli. Further studies have shown that isolated kidneys perfused with hypoxic blood released Ep into the perfusate suggesting a renal origin of the hormone (Kuratowska et al., 1961).

The site of erythropoietin production in the fetus, however, is unknown. It has recently been shown that the kidney is not the primary site of Ep formation in the fetus (Zanjani et al., 1974). Unlike the adult, Ep production during fetal life is not affected by bilateral nephrectomy. Thus, bilaterally nephrectomized fetal goats produced significant amounts of Ep in response to bleeding (Zanjani et al., 1973). Available evidence indicates that Ep found in the nephrectomized fetuses originated from sites within the fetus. In this regard, Zanjani et al. (1974) have demonstrated that bled, nephrectomized fetuses produced Ep regardless of whether or not their mothers had been nephrectomized. In fact, nephrectomized fetuses with either normal or nephrectomized mothers had comparable levels of Ep activity (Zanjani et al., 1974). This occurred despite the fact that removal of maternal kidneys totally

suppressed erythropoietin production in the mothers. Moreover, it is highly unlikely that Ep in the fetus originated from extrarenal sites in the mother since the maternal plasma did not exhibit erythropoietic activity at the time activity was detected in the fetal plasma.

Initial observations suggesting the independent production of erythropoietin in the fetus came from the studies of Jacobson et al. (1959) who found that transfusion of pregnant mice resulted in suppression of maternal but not fetal erythropoiesis. More recently, large doses of Ep injected into pregnant rats stimulated maternal erythropoiesis but had no demonstrable effect on erythropoiesis in the fetus (Matoth and Zaizov, 1971). In addition, Zanjani and Gordon (1971) have demonstrated that Ep does not cross the placenta from the fetus to the mother. They injected large doses of Ep directly into the fetal circulation and plasma samples were taken periodically from both the fetus and the mother. No Ep was detected in the plasma of the mothers at any time during the study period. These studies suggested that no placental transfer of Ep occurs from the mother to the fetus or vice versa. Whereas the above data suggest that Ep is produced by both the fetus and the mother but does not traverse the placental barrier, it has also been shown that the erythropoietic status of the mother can have a significant effect on fetal erythropoiesis. Matoth and Zaizov (1971) demonstrated that increasing maternal erythropoietin levels of rats by bleeding or subjection to hypoxia resulted in augmented production of red blood cells in fetal rats, an effect not observed when mothers were injected with high doses of Ep.

The mechanism by which the altered maternal erythropoietic status exerts an influence on erythropoiesis in the fetus was investigated by Zanjani et al. (1974) in goats. The increased production of red cells in the fetuses of bled mothers was found to result from enhanced formation of Ep by the fetus in response to maternally induced hypoxia.

It would appear, therefore, that the increase in the rate of fetal erythrocyte production in response to maternal anemia is due to an increased production of Ep from sources within the fetus. The fetus appears to be more sensitive to reductions in maternal blood cell levels than the mothers, since the oxygen dissociation curve for fetal blood is normally shifted to the left of that for the adult (Behrman, 1968). This suggests that in the goat in particular, the fetus (at about 120 days) is normally on the brink of hypoxia. Superimposition of maternal anemia would exacerbate the oxygen deficiency in the fetus, resulting in the prompt production of larger amounts of Ep by the fetus.

Therefore, changes in fetal erythropoiesis which reflect a change in maternal erythropoiesis do not imply that the hormone is coming from the mother but rather show that the fetus produces its own Ep in response to its own needs. In this particular case, the need stems from changes in the maternal environment.

The results presented here provide further support for the relative independence of the fetus and, in addition, localize the site of production of erythropoietin in the fetus. The data show that the liver is the primary site of Ep production in the fetus.

The evidence supporting this conclusion are:

1) Fetal sheep in whom 75-80% of the hepatic tissue was removed, in addition to both kidneys, produced significantly less Ep in response to bleeding than both nephrectomized and/or splenectomized fetuses; and

2) In situ perfusion of the fetal liver in anemic sheep resulted in the appearance of highly significant quantities of Ep in the perfusate. Similarly prepared kidneys or spleens had no measurable Ep activity.

Our discovery that the liver is the primary site of erythropoietin formation in the fetus is compatible with the structural and functional aspects of the fetal circulation. Oxygenated blood returning to the fetus from the maternal side of the placenta passes through the ductus venosus and partially bypasses the liver (Guyton, 1971). Therefore, the blood which is most fully oxygenated does not go to the liver, but directly to the heart.

The results presented here also provide support for the recent observation that the liver can assume a significant role in the production of extrarenal erythropoietin. It was initially demonstrated that nephrectomized rats (Goldwasser et al., 1958; Fried et al., 1969) or mice (Jacobson et al., 1958) could still respond slightly to a hypoxic stimulus even though both kidneys were absent, suggesting that there was an extrarenal site of Ep production. Elevated erythropoietin titers were also seen in bilaterally nephrectomized humans (Mirand, 1968; Mirand et al., 1968; Naets and Wittek, 1968). A number of studies have suggested

that the liver is the extrarenal site of Ep production (Fried et al., 1969; Fried, 1972; Schooley and Mahlmann, 1974). Although the liver assumes a significant role in Ep formation in the absence of renal tissues, it is not known whether this organ participates in the manufacture of the hormone when the kidneys are functional. The kidneys assume the primary role of Ep production after birth. It is likely, however, that the liver also retains a limited capacity to produce Ep throughout all stages of development. Whether nephrectomy serves as the triggering mechanism to reactivate the hepatic mechanism or simply permits the detection of such activity remains to be clarified. Thus, hepatectomy did not decrease plasma Ep levels in hypoxic rats that had intact kidneys (Fried, 1972) suggesting that the liver is not functional when the kidneys are present. The studies of Katz et al. (1968), however, demonstrate the necessity of the liver for full Ep production in intact animals. They found that the capacity to form erythropoietin was reduced in hypoxic hepatectomized animals as compared with non-hepatectomized controls. However, these results may not be incompatible since the rats in the latter studies were exposed to shorter periods of hypoxia which might have allowed for the changes in Ep production that were observed. Even if the liver is relatively dormant in the adult, reactivation of its function to participate in Ep formation is not a unique situation. In man, certain clinical states such as myeloid metaplasia are accompanied by the involvement of both the liver and the spleen in the production of red blood cells (Pollycove et al., 1966), a function ascribable to these organs only during

early fetal life (Borghese, 1959; Buckman, 1959; Cole and Paul, 1966; Cole et al., 1968; Lucarelli et al., 1964; Lucarelli et al., 1968).

Our studies also indicate that the mechanism of production of erythropoietin in the fetus is different from that seen in the adult mammal. It has been suggested that Ep production in the adult involves the interaction of erythrogenin with a factor found in normal serum (Gordon et al., 1967; Gordon, 1968; Gordon et al., 1968). Erythrogenin had been localized in hypotonic extracts of the light mitochondrial fraction of hypoxic kidneys (Gordon, 1968). Our data show that similar extracts prepared from a variety of fetal sheep organs failed to yield any detectable erythropoietic activity upon incubation with normal sheep serum. This suggests that fetal production of Ep does not involve the Eg-serum mechanism. This is in contrast with the reported observation that Eg is present in the livers of nephrectomized adult rats (Zucali and Mirand, 1974) and the livers and spleens of 5 day old rats (Kaplan et al., 1974). Several explanations for the lack of Eg activity in the fetus may be considered. The fetus may, of course, produce Ep by a completely different mechanism. Support for this concept comes from a study in which a uterine fibromyoma was found to produce Ep directly and not by the interaction of Eg with serum (Ossias et al., 1973). Perhaps certain tumors, which can revert back to a more primitive dedifferentiated state and produce a variety of hormones, produce Ep by the same mechanism as the fetus. It is possible, however, that the fetus may form Ep by a mechanism different not only from that seen in the

adult but also unrelated to the mechanism seen in the tumor. In regard to this hypothesis, Erslev et al. (1971, 1972) have suggested that the kidney can produce Ep directly without Eg participation. He believes that a lipid inhibitor binds Ep in the kidney and keeps it inactive until cleaved by a plasma protein when the animal is subjected to a hypoxic stress. Perhaps the liver, which has been shown to contain inhibitors of Ep (Erslev and Kazal, 1968), contains a lipid which binds Eg in the fetus. This theory could explain the absence of Eg activity in the organs that were studied. Alternately our inability to find Eg may reflect the possibility, though highly unlikely, that it may be present in fractions other than the light mitochondrial fraction or it may be present in other organs. By whatever mechanism, however, the fetus is capable of producing the hormone in amounts sufficient for the production of red blood cells.

The day to day production of red cells in the sheep fetus during the last trimester of gestation is regulated by erythropoietin; i.e., the hormone plays a physiological role. This was evident from the results obtained in this study when fetuses were treated with anti-erythropoietin. Indirect evidence supporting the role of Ep in fetal erythropoiesis was provided by Schooley et al. (1968) who injected anti-Ep into pregnant rats and found suppression of erythropoiesis not only in the mothers but in some of the fetuses as well, suggesting that Ep was involved in the regulation of red cell production in the fetus. The work of Gordon et al. (1971) demonstrated that the induction of poly-

cythemia in goats by transfusion with maternal red blood cells led to the suppression of erythropoiesis in the fetus. In this regard, it is well known that an increased circulating red cell mass serves to decrease erythropoiesis by suppressing the formation of Ep (Gordon et al., 1966). Since the fetal suppression of erythropoiesis occurred after transfusion, this would suggest that erythropoiesis in the fetus, as in the adult, responds to the same negative feedback influence of elevated numbers of circulating red blood cells. Furthermore, it was shown (Zanjani et al., 1973) that highly significant stimulation of erythropoiesis occurred in these transfused fetuses following injections of Ep. The evidence that Ep could stimulate red cell production in the polycythemic fetus suggests that the initial suppression of erythropoiesis was caused by decreased levels of Ep. This study provided the first evidence for the role of erythropoietin in the regulation of red cell production in the fetus.

Our experiments were performed in the normal, unmanipulated fetus unlike the above mentioned studies, which involved fetuses with altered erythropoietic status. These results demonstrate that administration of sera from rabbits immunized against adult Ep completely abolished erythropoiesis in these fetuses. This was evident not only from the reduced incorporation of radioiron into circulating red blood cells, but also from the decreased numbers of circulating reticulocytes and the reduced numbers of nucleated erythroid cells in the bone marrow. In addition, treatment with anti-Ep significantly decreased the uptake of radioiron by the spleen and bone marrow of these fetuses. The site of origin of the

Ep that was suppressed, of course, was unknown but our other studies would indicate that the Ep had originated from the livers of these fetuses. That the inhibitory influence exerted by the immune serum on fetal erythropoiesis can be attributed to the neutralization of circulating Ep in the fetus and not to a nonspecific effect of the serum is demonstrated by the fact that 1) injections of sera from nonimmunized rabbits to fetal sheep failed to alter the various parameters examined; 2) factor(s), other than anti-Ep, present in the immune serum (but not in normal rabbit serum) were not responsible for the observed inhibitory effect; and 3) plasma from two fetuses in the group receiving the immune serum exhibited erythropoietic activity prior to treatment. However, plasmas from these same fetuses obtained at six hours after the last dose of the serum was given were devoid of erythropoietic activity. This did not occur in one fetus of the group receiving normal serum. Plasma from this fetus displayed erythropoietic activity both before and after treatment. The presence of Ep in the plasma of these three fetuses requires further comment. Routine examination of maternal erythropoietic status in all these preparations revealed the existence of a relative degree of anemia in the mothers of these three fetuses. In a previous study (Zanjani et al., 1974) it was shown that Ep production in fetal goats was stimulated following induction of anemia in mothers. Moreover, the fetus appears to be quite sensitive to small reductions in the maternal circulating red cell mass which induced the production of significant quantities of Ep in the fetus (Zanjani et al., 1974). Therefore the higher

than normal activity of the plasma in these fetuses can be ascribed to the stimulatory influence of maternal anemia on fetal Ep production. Thus, when the Ep-neutralizing capacity of the immune serum was eliminated by treatment with GARGG prior to administration to the fetus, no significant effect on fetal erythropoiesis was seen. These observations, combined with previous findings (Schooley et al., 1968; Zanjani et al., 1969; Matoth and Zaizov, 1971) lend support to the concept that Ep plays an important role in the regulation of erythropoiesis, not only in the mammalian adult, but in the mammalian fetus as well.

Our studies involving anti-erythropoietin utilized fetuses that were over 100 days of gestation since the operative procedures prevented us from using fetuses that were smaller. Thus, fetuses less than 100 days do not lend themselves to catheterization readily. In those cases where a catheter was successfully inserted in such fetuses, flow through these catheters could not be maintained for more than 48 hours. In addition, at 30 days we observed that the fetus is only 5-6 mm. long, thus rendering catheterization in very young fetuses unfeasible. In order to determine the role of Ep at the earlier stages of fetal development, techniques designed to assess the erythropoietic activity of hematopoietic tissues in vitro were employed. The cell suspension technique (Krantz et al., 1963) and the newer plasma clot technique (Stephenson et al., 1971; McLeod et al., 1974) were utilized since they measure different parameters of erythroid development. The plasma clot system was developed by Stephenson et al. (1971) who

found that in the presence of Ep, cells derived from fetal mouse liver tissues formed discrete colonies composed of erythroid cells within the clot that could be stained and easily quantitated. The cells which are the precursors for the erythroid colonies were called CFU-E to distinguish them from the pluripotent stem cell (CFU-S) or cells which give rise to granulocytic colonies in culture (CFU-C). The plasma clot has been used as a tool to provide more direct information about the properties of the erythropoietin sensitive cell (McLeod et al., 1974). Axelrad et al. (1974) has found two distinct populations of cells derived from mouse marrow which were responsive to Ep in the clot. With adequate amounts of Ep in the clot, erythroid colonies develop in two days which can be attributed to the proliferation of the CFU-E's. Upon longer incubation, these colonies die off and large bursts of erythroid colonies appear several days later. Whereas the formation of colonies from CFU-E's requires only low amounts of Ep, the so-called burst forming units (or BFU) require large amounts of Ep for differentiation. In an attempt to correlate the effects of Ep in vitro with the effects seen in vivo, Gregory et al. (1974) found that hypertransfused mice had decreased numbers of CFU-E in their spleens and bone marrow suggesting that there was an earlier precursor of the CFU-E. These progenitor cells were postulated to be able to develop independently of the hormone but they would be sensitive to its effects. Axelrad et al. (1974) has postulated the the BFU is the progenitor of the CFU-E since the bursts are seen several days after the CFU-E and a high concentration of Ep was necessary to stimulate this population of

cells to differentiate into CFU-E's. As would be expected, the BFU's respond to Ep but their numbers were not reduced by hypertransfusion suggesting that their population is maintained even when the supply of Ep is limited.

We used the plasma clot since it provides an environment in which erythroid precursors (CFU-E) can divide and differentiate within the clot whereas the cell suspension technique measures the incorporation of radioiron into hemoglobin synthesizing cells. Using both techniques, we confirmed the progression of erythropoiesis in the mammalian fetus from the liver to the spleen and finally to the bone marrow. The liver responded to Ep as early as 38 days of gestation and remained erythropoietically active until shortly before birth. This observation supports the histological studies of Buckman (1959) which showed the persistence of hepatic erythropoiesis from 20 days until birth. In the fetal liver tissue we noted that our two techniques gave different results which suggested that the plasma clot system was more sensitive. In cell suspension culture, the only significant stimulation of heme synthesis by Ep in hepatic derived cells occurred between 50-60 days and the number of hemoglobin synthesizing cells dropped off sharply after 60 days, both with or without Ep. The number of erythroid colonies increased with erythropoietin in the clot to reach a plateau by 80 days; however, no colonies were formed in the absence of Ep. The cell suspension technique, therefore, probably does not measure the growth of cells but rather a limited incorporation of iron into cells which are already synthesizing heme, a process which is effected by Ep. The fact

that there were no colonies in the absence of Ep in the clot but colonies are formed when Ep is present, indicates that even at the earliest stages of fetal development (38 days), Ep is involved in the production of red cells. At all stages of hepatic erythropoiesis that were studied, the cells derived from the fetal sheep liver were stimulated by Ep in the clot. This is unlike the situation seen in the mouse fetal liver cells, which are stimulated by Ep when derived from fetuses ranging from 10½-14 days but are no longer responsive at 15 days when splenic erythropoiesis commences (Paul et al., 1972). Using a technique similar to the cell suspension culture employed in our studies, Cole and Paul (1966) have found that the rate of hemoglobin synthesis in the mouse fetal liver cells increases between 14 and 15 days of gestation and then decreases sharply. However, when Ep was added to the cultures, hemoglobin synthesis could be greatly increased prior to day 15. This observation correlates well with the studies on the morphology of cells in the fetal liver during early erythropoiesis. Initially, one sees only primitive erythroblasts but, from day 15 on, more orthochromatic erythroblasts populate the liver suggesting that the earlier cells were stimulated by Ep and are maturing. However, the refractoriness to Ep after day 15 cannot be attributed to any morphological changes since pro-erythroblasts and basophilic erythroblasts are still present in culture after 15 days (Paul et al., 1971). These cells should be responsive to Ep. However, it is unknown whether these cells are functionally from the cells which responded in the liver at an earlier stage of development. In this regard, it has been

demonstrated (Fantoni et al., 1968; Djaldetti et al., 1970) that Actinomycin D inhibited hemoglobin synthesis in mouse fetal liver cultures on days 11-12 whereas by day 13, the cells were insensitive to the antibiotic. During both stages of gestation, Actinomycin D inhibited RNA synthesis (Djaldetti et al., 1970). These results suggest that hepatic cells differ in their functional characteristics at different gestational stages, which becomes manifest at least in part by their responsiveness to Ep in culture.

The fetal sheep liver may also become refractory to Ep around 110 days of gestation. This would explain our observations in the antibody studies that the splenic and myeloid erythropoiesis were suppressed following treatment with anti-Ep but hepatic erythropoiesis was not. Alternately, the technique utilized by Cole and Paul (1966) may not have been sensitive enough to show changes in hepatic erythropoiesis.

Splenic erythropoietic activity in the sheep fetus reaches its peak activity at 70 days both with and without Ep in the plasma clot when the liver is still active. Its erythropoietic activity declines rapidly afterwards but there may still be some foci of activity at birth in both the liver and the spleen. Contrary to the findings that the spleen is not sensitive to Ep at a late stage in the mouse fetus (Paul et al., 1972), we found stimulation by Ep at all periods that the spleen is producing erythroid cells.

Our in vitro studies confirm the observation that bone marrow erythropoiesis is initiated before 70 days of intrauterine life in fetal sheep and goats (Buckman, 1959). From 70 days until

birth the marrow is very active in producing red blood cells and a significant stimulation by Ep in culture is seen. The presence of myeloid erythropoiesis at birth is comparable to the situation described for the guinea pig and the human rather than for the rat and the mouse (Lucarelli et al., 1968).

Our studies have shown that the fetus responds to erythropoietin in vitro as early as 38 days of gestation. There is no way at the present time to determine whether Ep is actually present in the fetus at this early stage of development. However, if it is present, we can assume that it is stimulating the fetal hematopoietic tissues.

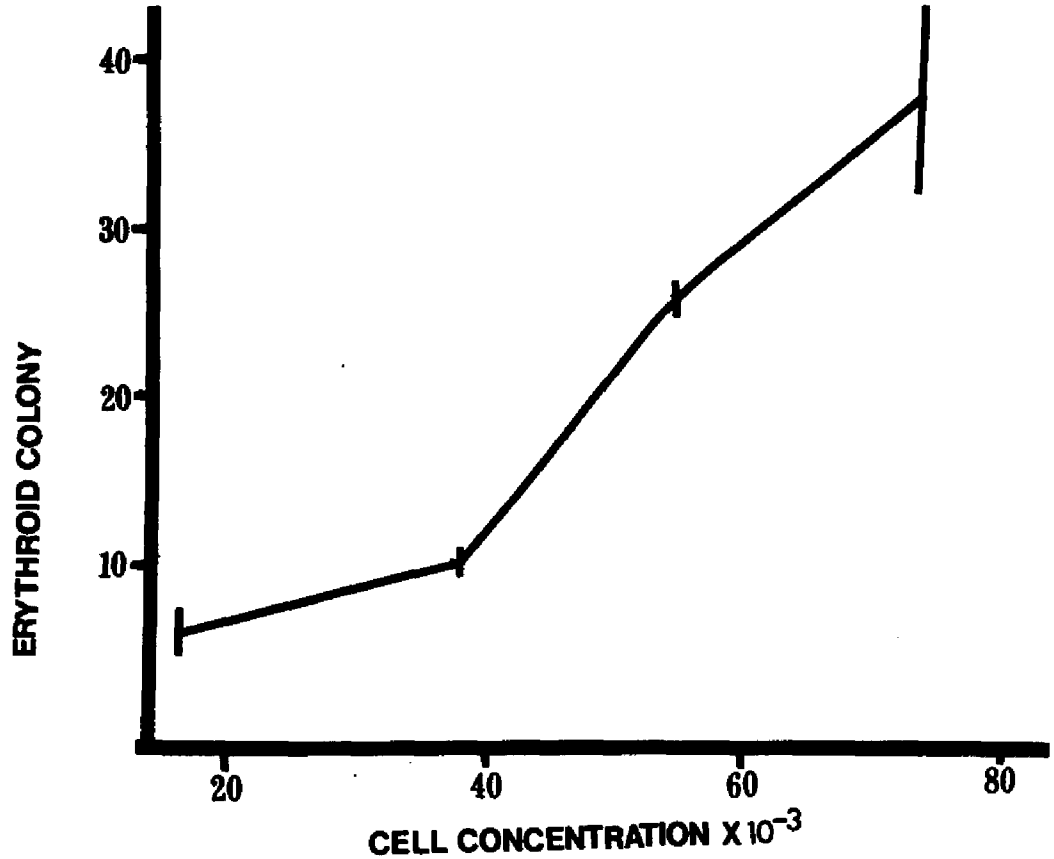
40
30
20
10
ERYTHROID COLONY
20
40
60
80
CELL CONCENTRATION X 10⁻³

Table 1

Effect of Bleeding on Erythropoietin Production by Nephrectomized, Hepatectomized and/or Splenectomized Fetal Sheep

Material Assayed[1]	% RBC-^{59}Fe Uptake SEM[2]
Plasma from Normal Fetus	0.54 ± 0.19
Plasma from Anemic Normal Fetus	15.68 ± 1.98
Plasma from Anemic Nephrectomized Fetus	13.11 ± 2.87
Plasma from Anemic Splenectomized Fetus[3]	10.46 ± 1.06
Plasma from Anemic Hepatectomized Fetus[3]	3.32 ± 0.98

Saline	0.20 ± 0.06
0.10 IU Ep	3.97 ± 0.65
0.40 IU Ep	8.73 ± 1.23

1. Each sample was assayed on two separate occasions.
2. Mean ± standard error of the mean of two separate assays.
3. These fetuses were bilaterally nephrectomized immediately prior to removal of the liver or spleen.

Table 2

Erythropoietin Production by Perfused Kidney, Liver and Spleen From Anemic Fetal Sheep

Organ Perfused	Range of Units of Ep	Total Units Ep Produced/2 hr[1]
Kidney	0	0
Spleen	(0-1)	0.3 ± 0.2
Liver	(2-6)	4.5 ± 0.4

1. Mean ± standard error of the mean. The value was arrived at by considering the results of assays from all perfusates.

Table 3

Erythropoietic-Generating Activity of Hypotonic Extracts of Organs From Anemic Fetal Sheep

Material Assayed[1]	% RBC-^{59}Fe Incorporation SEM[2]	
	Extract Alone	Extract + Serum
Fetal Kidney	0.42 ± 0.10	0.96 ± 0.28
Fetal Liver	0.79 ± 0.22	1.40 ± 0.37
Fetal Spleen	0.31 ± 0.08	0.76 ± 0.21
Fetal Lung	1.18 ± 0.42	0.89 ± 0.18
Fetal Pancreas	0.97 ± 0.26	1.32 ± 0.46
Fetal Testis	0.62 ± 0.19	0.88 ± 0.32
Fetal Heart	0.33 ± 0.06	0.77 ± 0.19
Fetal Thymus	0.78 ± 0.20	1.63 ± 0.51
Fetal Brain	0.85 ± 0.29	1.10 ± 0.28
Saline	0.87 ± 0.25	
0.10 IU Ep	5.17 ± 0.92	
0.40 IU Ep	14.64 ± 2.26	
Sheep Serum	1.06 ± 0.33	

1. Each sample was assayed on three separate occasions.

2. Mean ± standard error of the mean of three separate assays.

Table 4

Effect of Erythropoietin on Heme Synthesis by Hepatic Cells From Fetal and Adult Sheep Liver *in vitro*[1]

Age of Fetus	Additions to Culture	CPM/10^7 cells[2]
38 days	none	742.8 ± 38.4
	.14 IU Ep	699.6 ± 8.9
	.29 IU Ep	778.1 ± 13.2
	.58 IU Ep	659.5 ± 29.7
50 days	none	1974.1 ± 72.0
	.29 IU EP	3020.3 ± 47.5
	.58 IU EP	1621.2 ± 30.6
60 days	none	261.2 ± 9.1
	.29 IU Ep	466.9 ± 33.3
	.58 IU Ep	468.1 ± 5.6
105 days	none	256.2 ± 19.6
	.29 IU Ep	468.1 ± 33.4
	.59 IU Ep	392.1 ± 9.4
135 days	none	19.1 ± 11.1
	.29 IU Ep	0 ± 0
	.58 IU Ep	10.3 ± 0.5

1. Cells were culture in suspension by method of Krantz *et al.* (1963).
2. Mean ± standard error of the mean of triplicate cultures. Values were normalized for 10^7 cells to allow a statistical comparison of results from different experiments.

Table 5

Effect of Erythropoietin on the Formation of Erythroid Colonies by Hepatic Cells Derived From Fetal and Adult Sheep in the Plasma Clot[1]

Age of Fetus	Addition to Culture	Number of Erythroid Colonies[2] 18000 cells	36000 cells	54000 cells	72000 cells
29 days	none			5.3± 4.9	
	.2 IU Ep		23.5±6.4	26.8±11.4	
34 days	none	0	0	4.0± 1.5	
	.2 IU Ep	20.0±5.6	32.8±9.8	57.3± 2.2	
50 days	none	0.3±0.3	1.3±0.6	2.0± 0	2.5±0.9
	.2 IU Ep	14.3±3.1	24.0±4.1	49.5±11.7	47.0±4.2
60 days	none	0.5±0.3	0.8±0.5	2.3± 1.0	1.5±0.3
	.2 IU Ep	16.5±2.0	44.8±8.2	61.8± 8.2	88.3±4.7
70 days	none			1.8± 0.9	
	.2 IU Ep		33.0±3.9	57.3±17.7	
105 days	none	0	0	0	0
	.2 IU Ep	5.0±1.4	19.5±4.7	65.0±10.9	43.8±6.3
135 days	none		No Growth		
	.2 IU Ep				
Adult	none		No Growth		
	.2 IU Ep				

1. Cells were incubated in the plasma clot by the method of McLeod *et al.* (1974).
2. Mean ± standard error of the mean of four clots.

Table 6

Effect of Erythropoietin on Heme Synthesis by Spleen Cells From Fetal Sheep *in vitro*[1]

Age of Fetus	Additions to Culture	CPM/10^7 cells[2]
105 days	none	351.4 ± 5.2
	.29 IU Ep	1644.0 ± 18.7
	.58 IU Ep	1928.0 ± 34.5
121 days	none	6.9 ± 2.1
	.29 IU Ep	22.0 ± 4.1
	.58 IU Ep	23.2 ± 2.2
129 days	none	45.4 ± 21.1
	.29 IU Ep	69.4 ± 21.2
	.58 IU Ep	34.1 ± 18.5
135 days	none	21.4 ± 21.4
	.29 IU Ep	31.0 ± 15.3
	.58 IU Ep	36.0 ± 10.0

1. Cells were cultured in suspension by the method of Krantz *et al.* (1963).
2. Mean ± standard error of the mean of triplicate cultures. Values were normalized for 10^7 cells to allow a statistical comparison of results from different experiments.

Table 7

Effect of Erythropoietin on the Formation of Erythroid Colonies by Spleen Cells From Fetal Sheep in the Plasma Clot[1]

Age of Fetus	Addition to Culture	Number of Erythroid Colonies[2] 18000 cells	36000 cells	54000 cells	72000 cells
29 days	none	0	0	0	0
	.2 IU Ep	0	0	0	0
	.4 IU Ep	0	0	0	0
60 days	none			0	
	.2 IU Ep			4.5±0.7	
70 days	none		0	0	0
	.2 IU Ep		191.0±51.3	>300	>300
105 days	none	0	0	0	0.3± 0.3
	.2 IU Ep	6.5±2.0	10.8± 1.6	26.8±0.9	38.9±11.0
121 days	none		No Growth		
	.2 IU Ep				
135 days	none		No Growth		
	.2 IU Ep				

1. Cells were incubated in the plasma clot by the method of McLeod *et al.* (1974).
2. Mean ± standard error of the mean of four clots.

Table 8

Effect of Erythropoietin on Heme Synthesis by Bone Marrow Cells From Fetal and Adult Sheep *in vitro*[1]

Age of Fetus	Additions to Culture	CPM/10^7 cells[2]
105 days	none	173.9 ± 16.1
	.29 IU Ep	1250.7 ± 46.9
	.58 IU Ep	1640.4 ±203.7
121 days	none	25.8 ± 2.3
	.29 IU Ep	174.2 ± 23.6
	.58 IU Ep	168.5 ± 9.3
128 days	none	173.8 ± 8.0
	.29 IU Ep	744.9 ± 96.3
	.58 IU Ep	724.4 ± 82.1
129 days	none	67.5 ± 3.4
	.29 IU Ep	348.6 ± 67.7
	.58 IU Ep	———
135 days	none	155.5 ± 32.2
	.29 IU Ep	927.0 ± 63.7
	.58 IU Ep	949.8 ± 95.9
138 days	none	654.7 ± 21.8
	.29 IU Ep	1873.9 ±250.9
	.58 IU Ep	3276.2 ±168.8
Adult	none	234.0 ± 62.5
	.29 IU Ep	160.8 ± 31.2
	.58 IU Ep	959.2 ± 15.3
Adult	none	114.3 ± 15.0
	.29 IU Ep	351.9 ± 18.8
	.58 IU Ep	1054.4 ±697.7

Table 8 (cont.)

Age of Fetus	Additions to Culture	CPM/10^7 cells[2]
Adult	none	351.4 ± 5.2
	.29 IU Ep	1644.0 ± 18.7
	.58 IU Ep	1928.0 ± 34.5

1. Cells were cultured in suspension by the method of Krantz et al. (1963).
2. Mean ± standard error of the mean of triplicate cultures. Values were normalized for 10^7 cells to allow a statistical comparison of results from different experiments.

Table 9

Effect of Erythropoietin on the Formation of Erythroid Colonies by Bone Marrow Cells From Fetal and Adult Sheep in the Plasma Clot[1]

Age of Fetus	Addition to Culture	Number of Erythroid Colonies[2] 18000 cells	36000 cells	54000 cells	72000 cells
70 days	none		0		
	.2 IU Ep		60.5±18.2		
105 days	none	0	0	0	0
	.2 IU Ep	4.8±0.9	11.5± 1.9	16.0± 4.5	22.7± 4.8
121 days	none	0	0	0.8± 0.8	
	.2 IU Ep	10.0±0.9		49.0± 6.1	
128 days	none	0	1.8± 0.8	1.3± 0.3	5.5± 1.3
	.2 IU Ep	32.5±7.5	62.0± 8.5	91.0± 8.2	98.8±16.8
135 days	none	0	1.3± 0.9	1.3± 0.3	1.8± 0.6
	.2 IU Ep	15.5±4.2	30.5± 3.9	65.3±10.9	30.5±10.0
138 days	none	0	2.0± 0.7	8.5± 1.0	13.0± 2.1
	.2 IU Ep	19.8±2.1	39.5± 5.2	93.0± 7.9	48.0±10.6
Adult	none	0.5±0.5	1.3± 0.5	1.0± 0.4	1.0± 0
	.2 IU Ep	11.5±3.6	25.5± 2.5	37.8± 2.2	33.0± 3.3
Adult	none	0	0	0.5± 0.3	0.5± 0.5
	.2 IU Ep	11.3±3.4	15.8± 0.9	47.8± 3.8	64.8± 7.5

1. Cells were incubated in the plasma clot by the method of McLeod *et al.* (1974).

2. Mean ± standard error of the mean of four clots.

Table 10

Erythropoietin-Neutralizing Activity of Anti-Ep Serum Before and After Treatment With Goat Anti-Rabbit Gamma Globulin Antibody (GARGG)

Material Assayed[1]	% RBC-^{59}Fe Incorporation SEM[2]
Saline	0.13 ± 0.02
1 IU Ep[3]	12.42 ± 1.32
1 IU Ep + Normal Rabbit Serum	13.56 ± 1.63
1 IU Ep + Anti-Ep Serum (0.045 ml.)	0.38 ± 0.06
1 IU Ep + (0.045 ml. Anti-Ep Serum + 0.02 ml. GARGG	12.87 ± 1.10

1. Volumes brought to 1 ml. with normal saline. All samples were incubated at 37°C for one hour prior to injection.
2. Mean ± standard error of the mean.
3. Sheep plasma Ep (Step III, Connaught Labs., Toronto, Canada).

Table 11

Erythropoietic Activity of Plasma Obtained From Fetal Sheep Before and After Treatment With Rabbit Serum

Material Assayed	% RBC-^{59}Fe Incorporation SEM[1] Before Treatment	After Treatment
Saline	0.08 ± 0.0	0.1 ± 0.02
0.10 IU Ep[2]	1.14 ± 0.27	1.32 ± 0.30
0.40 IU Ep	5.19 ± 0.73	6.12 ± 0.69
Plasma From Fetuses in Group 1		
Fetus a	0.21 ± 0.04	0.28 ± 0.06
Fetus b	1.26 ± 0.14	0.98 ± 0.19
Fetus c	0.18 ± 0.0	0.31 ± 0.08
Fetus d	0.38 ± 0.12	0.24 ± 0.1
Plasma From Fetuses in Group 2		
Fetus a	1.26 ± 0.31	0.20 ± 0.06[3]
Fetus b	0.19 ± 0.0	0.11 ± 0.04
Fetus c	0.19 ± 0.0	0.15 ± 0.06
Fetus d	0.93 ± 0.24	0.16 ± 0.07[3]
Fetus e	0.27 ± 0.04	0.11 ± 0.05
Plasma From Fetuses in Group 3		
Fetus a	0.21 ± 0.12	0.19 ± 0.06
Fetus b	0.22 ± 0.04	0.21 ± 0.05
Fetus c	0.30 ± 0.08	0.26 ± 0.06

1. Mean ± 1 standard error of the mean.
2. Human Urinary Ep (supplied by NIH).
3. $P < 0.01$ compared to pre-treatment plasma.

Table 12

Effect of Anti-Ep on the Uptake of Radioiron by RBC, Spleen, Liver and Bone Marrow in Fetal Sheep

	% ^{59}Fe Incorporation SEM[1]			
Treatment of Fetus	RBC	Spleen/ 100 gm.	Liver/ 100 gm.	Bone Marrow/ 100 gm.
Normal Rabbit Serum	28.30±3.9	1.7 ±0.3	19.9±3.4	15.8±2.9
Anti-Ep Serum	4.10±1.3[2]	0.79±0.1[3]	18.3±2.8	5.7±0.8[4]
(Anti-Ep Serum + GARGG)	32.40±3.7	1.93±0.3	19.0±4.6	14.7±1.3

1. Mean ± 1 standard error of the mean.
2. $P < 0.0005$.
3. $P < 0.025$.
4. $P < 0.005$.

Table 13

Effect of Anti-Ep Serum on Numbers of Circulating Reticulocytes and Bone Marrow Erythroblasts in Fetal Sheep

Treatment of Fetus	% Reticulocytes[1] Before Treatment	% Reticulocytes[1] After Treatment	No. of Erythroblasts/mg. marrow[2] X 10^{-3}
Normal Rabbit Serum	2.1 ± 0.3[3]	1.9 ± 0.2	142 ± 14
Anti-Ep Serum	1.8 ± 0.2	0.02 ± 0.0[4]	18 ± [4]
(Anti-Ep Serum + GARGG)	1.9 ± 0.2	1.7 ± 0.1	143 ± 23

1. Determined by counting 5000 cells for each of 2 (duplicate) slides.
2. Determined by counting 1000 nucleated cells for each of 2 (duplicate) slides in a single blind experiment. For calculations of numbers/mg. see LoBue *et al.* (1963).
3. Mean ± 1 standard error of the mean.
4. $P < 0.005$ when compared to normal rabbit serum-treated group.

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