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CHUSID, Eileen Daly, 1942-
MECHANISMS UNDERLYING STEROID ACTION ON
ERYTHROPOIESIS.

City University of New York, Ph.D., 1977
Physiology

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1977

MECHANISMS UNDERLYING STEROID ACTION ON ERYTHROPOIESIS

by

Eileen Daly Chusid

A dissertation submitted to the Graduate
Faculty in Biomedical Sciences in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy, The City
University of New York.

1977

This manuscript has been read and accepted for the Graduate Faculty in Physiology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

12 MAY 1977
date

16 May, 1977
date

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ABSTRACT

MECHANISMS UNDERLYING STEROID ACTION ON ERYTHROPOIESIS

by Eileen Daly Chusid

Advisor: Dr. Esmail Zanjani

The data in this thesis supports the theory that androgens affect erythropoiesis by a number of independent mechanisms. Administration of testosterone and the androgenic metabolite 5α -dihydrotestosterone to normal rodents has been shown to significantly increase erythropoiesis by increasing Ep production. The elevated level of plasma Ep was accompanied by increased red cell production and increased kidney weight. There was no increase in the weight of the liver or spleen. Incubation of plasma from TP-treated rats prior to bioassay abolished erythropoietic stimulatory activity.

A direct effect of TP and 5α -DHT on erythroid precursor stem cells has also been demonstrated. Bone marrow from transfusion induced polycythemic rats exhibited increased erythroid growth and differentiation when cultured in the plasma clot with Ep. The number of colonies per 6×10^5 cells was 284 ± 29 for vehicle-treated hypertransfused rats and 444 ± 63 for TP-treated rats. Incubation of bone marrow

from normal untreated mice with testosterone metabolites, androsterone, and 5 α -DHT, resulted in increased erythroid colony formation (402 ± 57 and 347 ± 38 vs. 172 ± 11 for controls). Testosterone was without effect in vitro. In order to determine whether this effect is mediated via androgen receptors similar to those present in male accessory sex tissues, the influence of 5 α -DHT on erythropoiesis was studied in normal and androgen insensitive (Tfm) mice, known to lack androgen receptor protein. In the in vitro plasma clot culture system, which permits the proliferation and differentiation of erythroid stem cells in the presence of Ep, 5 α -DHT markedly enhanced the erythroid colony forming ability of the bone marrow from not only the normal but also Tfm mice. Thus, at 10^{-7} M, the number of colonies were: normal mice: 358 ± 2 ; Tfm mice: $379 \pm 38/6 \times 10^5$ bone marrow cells. Administration of 5 α -DHT to normal mice resulted in a significant increase ($p < 0.001$) in Ep formation; Tfm mice receiving similar concentrations of the steroid failed to exhibit enhanced Ep formation. These results demonstrate that the direct effect of androgens on erythropoiesis either does not require the presence of an androgen receptor or that the erythropoietic action of the 5 α -DHT is mediated by

receptors which are different from those present in male sex tissues. By contrast, the Ep response to androgens appears to depend on the presence of androgen receptors.

ACKNOWLEDGMENTS

So much effort on the part of so many goes into the training of a graduate student that individual acknowledgments are difficult. Special thanks, however, are due the following:

To Dr. Esmail Zanjani, my thesis advisor, for his expert guidance during my research and thesis preparation. His enthusiasm was contagious and his generosity with his time and facilities was greatly appreciated.

To Dr. Tibor Barka, my department chairman, for setting a standard for graduate education and seeing to it that his students fulfilled his expectations. His critical reading of this manuscript was especially appreciated.

To Dr. Ralph Zalusky for the time he spent reading this manuscript and for his constructive comments.

To Dr. Joyce Shriver, my departmental advisor, who was "with me on St. Crispen's Day," a special thanks for smoothing out the rough spots. Dr. Shriver more than anyone else is responsible for the establishment of a graduate program in the Department of Anatomy. She set it up, then taught us all what good graduate education really was.

To Dr. Irving Schwartz, Dean of the Graduate School,
for his encouragement and many kindnesses over the years.

To Drs. John Lutton and Ron Hoffman for their patient
assistance during my research.

To Ms. Gertrude Lurinsky for teaching me laboratory
techniques and especially for her help with the DPG's.

To all my professors at Mount Sinai, especially those
in the Department of Anatomy. They always did their best
in teaching and they were splendid!

To Rona Weinberg--my friend--who was always there to
lend a hand.

To Danny, Robert, and Ann Marie Daly, my brothers and
sister, for their constant love and encouragement.

To my mother for always saying "you can."

To my husband Les for his constant love and encourage-
ment, for being a rock when I needed one, and for sharing
the good times. His valid and valued scientific criticism
was greatly appreciated as was the fact that, in addition
to his other areas of expertise, he found the time to add
erythropoiesis.

To those I didn't mention by name a collective thank
you.

I will try very hard to "do right" by all who had a hand in my training by continually trying to advance scientific knowledge and by trying to bring to my students the knowledge and devotion the Biomedical Sciences Faculty brought to me.

DEDICATION

This thesis is dedicated to

my father

my mother

and especially to my husband

for their love and

encouragement and without

whom none of this would

have been worthwhile.

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LIST OF ABBREVIATIONS

TP	Testosterone Propionate
5 α -DHT	5 α -dihydrotestosterone
ETIO	Etiocholanolone
Ep	Erythropoietin
ESF	Erythropoiesis Stimulatory Factor
Eg	Erythroenin
REF	Renal Erythropoietic Factor
2,3-DPG	2,3-diphosphoglycerate
P ₅₀	Partial pressure of oxygen at which hemoglobin is 50% saturated
CFU-S	Colony Forming Unit-Stem Cell
CFU-C	Colony Forming Unit-Granulopoietic
Tfm	Testicular Feminization
Anti-Ep	Anti-erythropoietin
GARGG	Goat Anti-Rabbit Gamma Globulin

INTRODUCTION

(ERYTHROPOIETIN)

The number of red cells remains remarkably constant, although red cells, having an average life-span only about 120 days, are destroyed continuously.

These events indicate a feedback control triggered by the requirements of the organism for new cells. Major mechanisms that most likely operate in this feedback regulation are either humoral in nature or involve direct cellular carriage of information.

It is now established that the four principal forms of hypoxia--anemia, hypoxic, histotoxic, and stagnant--stimulate the production of red cells in the higher organism (Gordon, 1959). This response is mediated by erythropoietin, a glycoprotein hormone which is now considered to be the prime regulator of erythropoiesis (Gordon, 1959; Krantz and Jacobson, 1970). Erythropoietin is a constituent of normal plasma as determined by in vivo assay procedures (Mirand et al., 1965) and by in vitro immunologic methods (Lange et al., 1969; Fisher et al., 1971). Further evidence that erythropoietin plays a role in day-to-day replacement of red cells is provided by the ability of anti-erythropoietin serum to

practically eliminate erythropoiesis in intact mice (Schooley, 1962).

The first indication that erythropoiesis was controlled by a humoral substance was reported by Carnot and de Flandre (Carnot and de Flandre, 1906), who noted that serum from anemic animals of the same species resulted in erythropoietic and reticulocytosis-promoting action in rabbits. Until 1950, the theory of a humoral erythropoietic factor, while viable, was difficult to confirm, and attempts to do so were only partially successful (Bonsdorf and Jalavisto, 1948; Gordon, 1959).

In 1950 this concept received support from the experiments of Reissmann. When one member of a pair of parabiotic rats was exposed to hypoxia, its bone marrow as well as the marrow of its partner displayed a marked erythroid cell hyperplasia. This hyperplasia was interpreted to be the result of a humoral substance elaborated in the hypoxic rat that was transferred to the circulation of the other parabiont, where it exerted its erythropoietic effect on the bone marrow (Reissmann, 1950). Later, Erslev demonstrated conclusively the erythropoiesis stimulating properties of plasma obtained from rabbits previously bled. Repeated injections of this plasma (200 ml total) resulted

in a marked reticulocytosis in recipient rabbits. Additionally, significant increases in red cell count, hematocrit, and cellularity of the bone marrow were noted in the recipient rabbits.

Stohlman et al. (1954) described a patient with regional tissue hypoxia as manifested by cyanosis below the waist. This patient with documented patent ductus arteriosus had a normal pO_2 and O_2 saturation in the brachial artery but subnormal values in the femoral arteries. Biopsy of the sternal marrow revealed hypercellularity and normoblastic hyperplasia, suggesting that a humoral substance produced by an organ (or organs) below the waist in response to hypoxia was exerting an effect on the entire bone marrow.

The aforementioned data support the view that erythropoietin (Ep) plays a significant role in erythropoiesis and that the relation between tissue O_2 supply and demand of the animal is a determining factor in the production of Ep (Fried et al., 1957). Thus increased levels of circulating Ep are produced in response to hemorrhage, hemolytic anemia, hypoxia, and/or cobaltous chloride (Gordon, 1959). Conversely, Ep production decreases when the O_2 supply to the responsive tissues exceeds the demand.

In rodents a reduction in the number of circulating

red cells and hemoglobin level occurs after hypophysectomy, thyroidectomy, and adrenalectomy; the anemia is attributable largely to a decrease in red cell production (Krantz and Jacobson, 1970; Gordon and Zanjani, 1970). The anemia may be prevented or overcome by the administration of the lacking hormone(s). Among the more potent of the erythropoietic hormones are androgens, thyroid hormone, and adrenal cortical steroids, as well as the adenohypophyseal hormones governing target endocrine organ functions (Gordon and Zanjani, 1970; Peschle et al., 1971).

The primary site of Ep production 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 as well as other investigators (Jacobson et al., 1957b; Goldwasser et al., 1958) confirmed the role of the kidney in the formation of Ep in many species, including man (Gurney et al., 1957; Gallagher et al., 1959; Nathan et al., 1964; Mirand et al., 1968). That the decreased production of Ep following nephrectomy is not associated with the inevitable build up of nitrogenous waste was demonstrated by Jacobson et al., 1957. In rats and rabbits, in which both ureters were ligated, Ep production in response to hypoxia was

unimpaired. Additional support for the role of the kidney in Ep production is derived from the fact that perfusates of both intact and isolated hypoxic kidneys contain Ep (Kuratowska et al., 1964; Pavalvic-Kentera et al., 1965; Halvorsen et al., 1968; Erslev, 1974).

Although the kidney is the major site of Ep production, the mechanism of Ep formation is not known. Attempts to extract Ep from this organ have been unsuccessful (Naets, 1960; Rambach et al., 1961; Zangheri et al., 1962; Goldfarb and Tobian, 1963).

A factor that is capable of generating Ep when incubated with normal plasma has been isolated from the kidneys of cobalt-treated or anemic rabbits and rats (Kuratowska and Kopec, 1969; Kuratowska, 1968; Gordon et al., 1967; Gordon, 1968; Gordon et al., 1968). This factor, originally designated REF (renal erythropoietic factor) and since renamed erythrogin (Eg), is confined to the light mitochondrial fractions of the kidney. Subcellular fractionation studies revealed an equal distribution of Eg in the renal cortex and medulla (Zanjani et al., 1967). The rate of Ep production when Eg acts on a serum substrate provided further support that the reaction was an enzymatic conversion.

An alternative theory for the mechanism of Ep production has been presented by Erslev and Kazal (1968) and Erslev et al. (1971-1972), who showed that the kidney contains high concentrations of a lipid inhibitor of Ep. The lipid-Ep complex is biologically inactive. It was suggested that during periods of hypoxic stress an increase in the synthesis and/or release of a plasma protein leads to the release of Ep from the lipid-Ep complex. In 1974, Erslev provided further evidence to support his theory by demonstrating that kidneys from hypoxic rabbits, perfused in vitro, released Ep in the absence of plasma proteins. These so called "programmed" kidneys were first described by Zanjani et al. (1971).

The kidney is the major, but not the sole, source of Ep. The major extra renal site of Ep production is thought to be the liver (Reissmann and Normura, 1962; Katz et al., 1968; Fried et al., 1969; Fried, 1972; Kaplan et al., 1973, 1974). Nephrectomized rats can still respond to hypoxia (Goldwasser et al., 1958), and nephrectomized mice respond to phenyl hydrazine-induced anemia by Ep production (Jacobson et al., 1958). However, a greater degree of hypoxic stimulus is necessary for extra-renal Ep formation (Schooley and Mahlmann, 1974).

Nephrectomized rats with 80% of their liver removed barely respond to hypoxia by increased Ep production (Fried, 1972).

Purification of Ep

A considerable degree of purification of Ep has been achieved. Espada and Gutnisky (1970) have obtained from the urine of highly anemic human subjects Ep fractions with specific activities as high as 8000 units/mg of protein. Goldwasser and Kung (1971a) have reported on preparations of Ep extracted from anemic sheep plasma with activities in the vicinity of 8000-9000 units/mg protein. These latter preparations are almost completely pure and the major contaminant present is desialated Ep. Plasma Ep is inactivated by trypsin and sialidase indicating that it is a sialic acid-containing protein. Studies by Lowry (1970) have indicated that some residues of tryptophan, lysine, and/or arginine, and possibly tyrosine residues are essential for activity. On the other hand, free sulfhydryl and hydroxyl groups do not appear to be required for activity. The molecular weight of sheep plasma Ep is 45,800 (Goldwasser and Kung, 1971b). It contains approximately 30% carbohydrate, of which 10.8% is sialic acid. Desialated Ep is inactive when administered to the animal but retains its ability to stimulate heme synthesis

by isolated marrow cells (Goldwasser and Kung, 1968). It is conjectured that sialic acid may serve as an attachment site of Ep to a carrier substance or may aid in preventing the destruction of Ep within the organism. A possibility worthy of exploration is that desialation of Ep at its target site (blood-forming tissues) is a prelude to the exertion of its physiological action.

The apparent paradox between in vivo and in vitro requirements for sialic acid may be explained by the findings of Morell et al. (1968, 1971) who showed that asialo-glycoproteins generally were cleared from the circulation at a much greater rate than the native forms. This rapid clearance was effected by hepatic cells that specifically interacted with the terminal galactose residues exposed by desialation. The desialated Ep is also sensitive to digestion by trypsin and more susceptible to heat inactivation than the intact Ep.

Methods of Assay

Both in vivo and in vitro procedures are currently employed for the measurement of Ep activity. In the in vivo assay most commonly used the increase induced by Ep in ⁵⁹Fe incorporation into the red cells of mice made plethoric by transfusion of homologous red cells or

polycythemic by exposure to reduced oxygen tensions followed by return of the mice to normal pressures for several days (Camiscoli and Gordon, 1970) is measured. With these methods, a suppression of erythropoiesis and probably Ep production eventually occurs, and, in this state, the mice are more sensitive to Ep. Radioiron incorporation values may be converted into equivalent Ep units by reference to the standard curve for the International Reference Preparation (IRP) of Ep (Cotes, 1971). These methods permit the measurement of Ep as little as 0.05-0.10 IRP units per sample. The in vitro methods presently available include: measurement of radioiron incorporation into heme of isolated bone marrow cells (Krantz and Jacobson, 1970) or of fetal liver cells (Stephenson and Axelrad, 1971); hemagglutination inhibition (Lange et al., 1969); and radioimmune assay (Fisher and Roh, 1971). As is the case for other hormones, when the specificity of the radioimmune assay is improved, this will be the method of choice. In vivo and in vitro assays of a number of Ep preparations did not always give the same values, and, therefore, estimates with the two types of assay cannot necessarily be compared (Lange et al., 1969; Dukes et al., 1970).

Site of Action of Ep

Studies utilizing Ep have revealed the existence in

the body of undifferentiated cells which upon stimulation by Ep give rise to hemoglobin-synthesizing cells (Stohlman et al., 1968). The Ep-responsive cell (ERC) is believed to originate from a primitive cell (pluripotential stem cell) which, in addition to the ERC, can give rise, upon appropriate stimulation, to precursors of different cellular elements of the blood (McCulloch, 1970). The transition from stem cells to ERC (or other "sensitive" cells) may be dependent upon the microenvironment of the stem cell (Trentin, 1970). Recent techniques permit the separation of ERC populations for studies of their proliferative and differentiative characteristics.

Cell Separation

Clinical studies revealed that not all patients with aplastic anemia respond to androgen therapy (Shahidi, 1959). The question arises as to whether these patients are lacking a specific erythroid precursor and are therefore not able to respond to testosterone. In this as in other problems in cell biology, it is useful to fractionate a complex population of cells into subpopulations which differ in function. Since cell function is often correlated with physical parameters, such as cell size, useful separations can often be obtained by fractionating

on the basis of such parameters (Miller, 1970). Isolation of relatively homogeneous populations of cells from the bone marrow enables both further analytical experiments and the study of a particular subpopulation under varying conditions.

There are several physical methods of cell separation. These include: sedimentation, equilibrium density-gradient centrifugation (Leif, 1970), counter current distribution, electrophoresis (Shortman, 1972), and electronic separation by volume (Fulwyler, 1970).

Sedimentation and density separation are not completely independent procedures. Sedimentation separates the cells on the basis of size rather than density. However, cell density is roughly inversely proportional to cell size since the nucleus of a cell is much more dense than the cytoplasm and larger cells tend to have a smaller nucleus to cytoplasm ratio. Nonetheless, there are significant exceptions to this rule, e.g., those cells containing cytoplasmic granules have increased cell density.

Consider a sphere (volume V , radius r , density p) falling through a viscous medium under the action of the earth's gravitational field. The net gravitational force on the particle is given by $(p-p')gV$ where p' is the density of the viscous medium and g is the acceleration

due to gravity (980 cm/sec^2). The net gravitational force is apposed by a viscous drag force set up in opposition to the motion of the particle. For a sphere, the drag force is given by $6\eta r v$ (Stoke's Law) where η is the coefficient of viscosity and v is the velocity of the sedimenting particle. An equilibrium state is soon reached in which the net gravitational force is exactly balanced by the viscous drag force and the particle falls at a constant velocity, its terminal velocity, s . Equating the two forces, one finds s given by the equation:

$$s = \frac{2(p-p')gr^2}{9}$$

Under physiological conditions, the vast majority of mammalian cells have radii varying from 2.5 to 10μ and densities varying from 1.05 to 1.10 gm/cm^3 . If they are falling through an aqueous medium with a density of 1.01 gm/cm^3 , size variations can give rise to a 16-fold variation in terminal velocity whereas density variations can give rise to only a twofold variation in terminal velocity. Even for cells such as erythrocytes, which are not perfect spheres, the above expression holds with an error of less than 10% (Miller and Phillips, 1969).

The objective is to form a thin layer of cell suspension

on top of a fluid column, let the cells settle under the influence of gravity for an appropriate length of time and collect fractions containing cells which have moved different distances. The cells should be as little affected as possible by the procedure. In particular they should preserve their viability. Any changes in metabolic activity should be greatly reduced by lowering the temperature to 4°C and the fluid column should be a buffered isotonic saline, sufficiently high in molecular weight so as not to create an appreciable tonicity gradient, and it should be non-toxic to cells.

The major limitation to separation of cells by velocity sedimentation is the phenomenon of streaming. This occurs if the cell concentration (cells/ml) in the cell band loaded into the chamber exceeds a certain number called the streaming limit, or this can be overcome by using a less steep gradient (Miller, 1970; McCool et al., 1970).

Velocity sedimentation has been used in hematology to separate erythropoietin-sensitive cells from bone marrow suspensions and as a tool for studying the progressive differentiation of the Ep-sensitive cell to red blood cells. McCool et al. (1970) found that the cell population most responsive to Ep had a sedimentation velocity of

6.6 mm/hr. In the process of becoming a hemoglobin synthesizing cell, the Ep-sensitive erythroid precursor undergoes divisions and its sedimentation velocity decreases toward that of the mature red blood cell, i.e., 2.1 mm/hr (McCool et al., 1970).

Comparison of murine and human colony forming units using velocity (CFU-C) sedimentation revealed that both are large cells (Iscoe et al., 1972).

Stem Cells

At this point, I will review the concept of the pluripotent stem cell, since much of the current work with androgens hinges on this theory. Till and McCulloch in 1960 lethally irradiated mice, injected them with marrow from a normal donor, and noted survival with neither anemia nor immune deficiency. They concluded that whatever they gave these animals was sufficient to produce a whole new blood system. In the mouse the marrow and spleen participate equally in erythropoiesis. When irradiated mice are injected with hemopoietic cells, a macroscopic, tumor-like elevation on the spleen surface develops which represents a colony of cells. Each spleen colony may contain precursors of all formed elements of the blood (i.e., red blood cells, white blood cells, and platelets) or only one.

Injection of only one type of cell into a lethally irradiated recipient is sufficient to repopulate the entire bone marrow compartment.

By definition, the stem cell must be self-renewing. Furthermore, stem cells are said to be a G_0 phase of the cell cycle.

Hematopoietic Progenitors

Evidence is available that in the mouse CFU-C are not identical with the pluripotent stem cells detected by the spleen colony techniques (McCulloch and Till, 1971). By analogy, human CFU-C, like mouse CFU-C, might be predominantly committed progenitors of granulopoiesis. If so, they are not identical with the pluripotent stem cells. However, the increase in CFU-C in suspension cultures of mouse marrow separated by velocity sedimentation was maximal in fractions rich in CFU-S. It seems at least plausible that the increase in mouse CFU-C might arise from the recruitment from pluripotent stem cells. If this should prove true, it would be an important finding in the characterization of the pluripotent stem cell, a finding with wide clinical applications (Clissold, 1974).

Further progress in understanding the action of Ep requires separation of the early erythropoietic cells into

distinct classes. Cells which appear the same histologically may be functionally different in their response to Ep or other agents, such as androgens. In addition to separating erythroblasts from the more mature erythroid cells, velocity sedimentation has been used to resolve populations of early erythroid cells which differ in their response to Ep in culture. The separation method isolates two classes of early erythroid cells from anemic rabbit bone marrow, one of which appears to be largely absent from normal rabbit marrow. One fraction contains a population of cells which begins to synthesize hemoglobin later than the other and presumably represents an earlier stage of differentiation (Tepperman et al., 1974).

Early cells in the hemopoietic system may be studied using methods that depend on the capacity of such cells to give rise to clones of descendants. In the mouse, methods are available for detection of pluripotent stem cells, committed progenitors of granulopoiesis, and at least two populations of Ep-dependent erythropoietic progenitors. The first of the progenitors originally described by Stephenson and Axelrod (1971) gives rise to colonies with 8 to 30 erythroblasts after two days in culture; the cells of origin of such colonies are believed

to be separated from pluripotent stem cells by more differentiation events than the committed granulopoietic progenitors detected in culture (CFU-C) (Gregory et al., 1973).

A second erythropoietic progenitor cell is detected by its ability to form clusters of 8 to 30 cells 7 days after culture and has been given the operational name erythropoietin-dependent-burst-forming-unit (BFU-E) (Axelrod et al., 1973). The BFU-E is considered to represent an earlier stage in erythropoiesis than CFU-E (Tepperman, 1974).

In cultures of human marrow, Tepperman et al. in 1974 reported, using velocity sedimentation, that large erythropoietic colonies are derived from progenitors that may be analogous to BFU-E in the mouse. Thus, again on the basis of separation and culture two populations of cells can be described, yet neither is sufficiently mature to be identified morphologically, and each differs in its response to Ep (Heath, 1977).

Further data on the physical separation of burst-forming-unit erythroid (BFU-E) from CFU-E in bone marrow cell suspensions include evidence from fraction mixing experiments that the previously observed velocity profiles

are the true profiles of these progenitors and not the result of the presence of stimulator or suppressor cells in the bone marrow. Furthermore, hypertransfusion with red cells reduces the number of CFU-E while it has no effect on BFU-E either in number or sedimentation characteristics (Heath et al., 1975). The red blood cell peak was sharply defined, i.e., its modal sedimentation velocity ranged between 1.8 and 2.2 mm/hr. The majority of erythrocyte colonies developed from cultures of cells sedimenting at 5.7-7.0 mm/hr. By contrast the majority of erythropoietic bursts developed in cultures of more slowly sedimenting cells, i.e., these with a velocity sedimentation of 3.7-4.1 mm/hr. The velocity sedimentation data corroborate other data which also point to BFU-E and CFU-E being distinct cell populations. Other data are:

1. There are many more CFU-E than BFU-E in the normal marrow and spleen of the mouse.
2. Hypertransfusion reduces the number of CFU-E but not BFU-E. This suggests that the generation and maintenance of BFU-E in vivo is not inhibited by curtailment of Ep supply while the generation and maintenance of CFU-E is.

Singer and Adamson (1976) proposed on the basis of

separation studies that the bone marrow contains specific target cells for different classes of steroids. Incubating isolated cell fractions known to give erythroid growth in the presence of fluoxymesterone, an androgen, and ETIO, a non-androgen, it was determined that the target cell responsive to fluoxymesterone resembled Ep responsive cell populations as measured by ^3H -Tdr sensitivity, while those responsive to ETIO were not as sensitive to ^3H -Tdr and had a lower sedimentation velocity. These studies seem to indicate that the fluoxy-sensitive cells are CFU-E, while the ETIO-sensitive cells are a more primitive cell type, not in cell cycle as shown by their lack of sensitivity to ^3H -Tdr.

Mechanism of Action of Ep

The mechanism by which erythropoietin exerts its effect is the subject of much investigation, and several working models have been proposed. It is generally agreed that Ep acts on a receptor-recognition mechanism probably at the level of the erythroid-committed precursor cell to stimulate synthesis of RNA, which, in turn, leads to increased protein synthesis in these cells. Ep also appears to lead to increased DNA synthesis and mitosis (Marks and Rifkind, 1972; Bank et al., 1970; Rifkind et al., 1971;

Cantor et al., 1972; Necheles et al., 1968) in in vitro bone marrow cultures. Within five minutes after exposure of rat bone marrow cell to Ep, those cells contain small amounts of RNA (150S), which are not present in control cells (Goldwasser, 1972). The RNA is found in the nucleus and has a very short half-life of about six 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 fifteen 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 a 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 two hours. Thus, the tissue responds to Ep prior to cell replication. Recent results, while suggesting that DNA synthesis is not necessary for RNA synthesis, suggest that it is required for the stimulation of globin synthesis by Ep (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 four hours, new proteins are being synthesized which influence 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 five hours after exposure to Ep. Then, within the next hour, the induced cells begin to show increased hemoglobin synthesis (Gross and Goldwasser, 1969).

Mitosis affords the chance for genes of these cells to be reprogrammed to initiate globin mRNA transcription and subsequent globin formation. Inhibition of DNA synthesis, however, does not prevent differentiation of these cells into mature erythrocytes (Datta and Dukes, 1975), but certainly the number of such cells is markedly reduced.

An increase in the number of divisions of erythroblasts following exposure to Ep was also noted (Necheles et al., 1968), an effect which was accompanied by increased DNA and heme synthesis (Powsner and Berman, 1967). Ep is thought 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 Kaufman, 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.

In polycythemic animals, hemoglobin synthesis is not seen until ten to twelve hours after exposure to Ep. Since the polycythemic marrow is devoid of any differentiated cells, the data suggest that Ep acts on the more undifferentiated cells. Ep 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 Ep. 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 induction.

The mechanism of the early stimulation of RNA synthesis by Ep is unknown. Recent evidence by Spivak (1976) indicates that Ep stimulates incorporation of uridine into RNA species with the sedimentation coefficients of 150, 55-65, 45, 28, 18, 9, 6, and 4 with the 150S being formed first. These changes are due to the sequential activation by Ep of multiple forms of nuclear RNA polymerase (Piantadosi et al., 1976). In addition, Ep stimulates the synthesis of both histone and non-histone proteins in the spleen of the exhypoxic polycythemic mouse and synthesis of these proteins is increased before the appearance of morphologically identifiable erythroblasts in the spleen (Spivak, 1976).

In addition, Ep appears to play a role in the induction of enzymes required for the synthesis of heme. Hemoglobin is synthesized through most of the maturation process of the red blood cell, about 95% being made before the nucleus is extruded and 5% in the reticulocyte state (Labardini et al., 1973). Normal hemoglobin production is dependent on an adequate iron supply, as well as the synthesis of protoporphyrin and globin. Porphyrin synthesis begins in the mitochondria with the formation of delta-aminolevulinic acid (ALA) and continues in the cytoplasm with the combination of two molecules of ALA to form porphobilinogen (PBG).

Four molecules of PGB are converted into uroporphyrinogen and coproporphyrinogen. The final steps, carried out in the mitochondria, involve the formation of protoporphyrin and the incorporation of iron to form heme. The most important rate limiting step in this pathway is the conversion of succinate and glycine to delta-aminolevulinic acid, a reaction catalyzed by ALA synthetase (Wintrobe, 1967). The activity of this enzyme is influenced by Ep and by the presence of the cofactor pyridoxal phosphate. Ep may act to induce the enzyme delta-aminolevulinic acid synthetase (Wintrobe, 1967). An additional action of Ep appears to be a shortening of the marrow transit time of reticulocytes, thereby releasing an increased number of immature yet hemoglobin carrying cells into the circulation as a demand arises (Stohlman, 1959; Gallagher and Lange, 1962). 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, provide further evidence for a role of Ep in the release of red cells into the circulation.

Ep also appears to regulate fetal erythropoiesis (Zanjani and Poster, 1975). It has been demonstrated (Cole and Paul, 1966; Cole et al., 1968) that cells from

the livers of 10 3/4 to 14 1/2-day-old mouse fetuses synthesize hemoglobin at a low rate in tissue culture. Upon addition of Ep during these critical times, there is a marked increase in the rate of hemoglobin production. Following this initial period of Ep sensitivity in the mouse, a time occurs at which the rate of hemoglobin synthesis remains high but cannot be further increased by Ep. The response to Ep is apparently dependent on a period of early and one of relatively late RNA synthesis with an intervening phase of DNA production (Paul and Hunter, 1969). The secondary phase of RNA synthesis, which appears to be essential for hemoglobin production, is dependent on the previous short period of DNA synthesis. Morphological studies (Chui et al., 1971) suggest that a primary action of Ep on fetal liver cells is the stimulation of RNA synthesis in early erythroblasts and that Ep is required for maintaining the precursor cell population, enabling it to continue to proliferate into hemoglobin-synthesizing elements. Cultures of disaggregated cells from whole embryos (8-12 days of age) and yolk-sac tissues of the mouse respond to Ep with increased heme synthesis (Bateman and Cole, 1971). This suggests that the differentiation of primary embryonic erythroid cells in situ may be under

the influence of an Ep similar or identical to the adult type.

Evidence is unfolding that the action of Ep is not confined to a stimulation of differentiation in the ERC but that it induces other changes as well, including effects on already differentiated elements of the nucleated erythroid cell series. Thus, direct effects in vitro have been described on erythroblast morphology (Borsook et al., 1968) and on DNA and heme synthesis in erythroblasts (Powsner and Berman, 1967), as well as on erythroblast divisions (Necheles et al., 1968). Additional effects of Ep include an early release of reticulocytes (Gordon et al., 1962; Fisher et al., 1965), a switching of hemoglobin A to C in nonanemic A/A sheep (Thurmon et al., 1970) and a stimulation of progenitor cell flow into the ERC compartment or an increased rate of ERC division (Reissmann and Samorapoompichit, 1970). A distinct vasoproliferative activity in transplanted splenic tissue follows treatment of donor mice with Ep (Feleppa et al., 1971). This observation, coupled with the finding that Ep induces an increased blood flow and opening of sinusoids in the splenic microcirculation (McClugage et al., 1971), suggests that, in addition to its action on the stem cells, Ep also alters the hematopoietic

internal microenvironment (Trentin, 1970) to furnish an optimal site for erythropoiesis (McCuskey et al., 1971). These different actions may be exerted by different forms of Ep, particularly since some Ep preparations appear to contain a number of biological components with differing in vivo and in vitro activities (Dukes et al., 1970).

While the interaction between Ep and ERC remains as the determining factor in the maintenance of an adequate number of circulating erythrocytes, a variety of factors can significantly modify this interaction. Thus, nutritional deficiency, presence of humoral and cell-mediated inhibitors (Hoffman et al., 1976), and antibody to Ep (Schooley, 1966) have been shown to alter the rate of erythrocyte formation despite the presence of adequate numbers of ERC's and levels of Ep.

Endocrine Regulation of Erythropoiesis

Since hormones significantly influence a wide spectrum of biological reactions, their effects on erythropoiesis were not unexpected. Hormonal effects on erythropoiesis were indicated by early experiments which revealed that hypophysectomy in rats results in the development of anemia (Vollmer et al., 1939; Crafts, 1941). This anemia was corrected by the administration of the lacking hormonal

factors. These include anterior hypophyseal, thyroid, adrenal cortical, and testicular hormones (Meyer et al., 1940; Vollmer et al., 1942; Evans et al., 1961; Gordon, 1968). Combinations of these hormones were more effective in correcting the anemia and the bone marrow hypoplasia than single factors. Thus a mixture of thyroxine, cortisone, and growth hormone could overcome the peripheral blood and marrow changes that characterize adeno-hypophyseal deficiency (Crafts and Meineke, 1959). A combination of growth hormone, testosterone, and thyroxine also achieved this restorative effect (Gordon, 1954) thus indicating that the ameliorative actions were not restricted to one particular combination of hormones.

Hypophysectomy, adrenalectomy, and orchidectomy, or combinations of these procedures modify to some extent, but do not abolish, the enhancement of red cell production that occurs as a result of hypoxia (Feigin and Gordon, 1950; Piliero, 1959). This suggests that hormones play a facultative rather than obligatory role in the regulation of erythropoiesis (Gordon, 1957).

Although the stimulatory nature of androgenic hormones on erythropoiesis was known for many years, the mechanism by which steroids influenced red cell production remained

unclear until recently.

A resurgence of interest occurred in 1959 when Shahidi and Diamond reported the beneficial effects of androgen therapy on children with anemia due to chronic renal failure and also patients with aplastic anemia (De Gowin et al., 1970; Shahidi, 1973). This resurgence occurred at the time when the role of Ep on erythropoiesis was coming into focus, and this led to a search for a role of Ep in the mediation of the erythropoietic effects of androgenic hormones. It was not long before such an association was discovered.

The acceptance of erythropoietin as a humoral agent of prime importance has prompted reinvestigation of the mechanism by which endocrine factors influence red cell production. There is ample evidence that androgens, like testosterone, stimulate erythropoiesis in normal animals (Mirand et al., 1965; Fried and Gurney, 1965) and man (Alexanian, 1969) at least in part by increasing the production of erythropoietin. Effects on erythropoietin production have also been reported for other hormones; thus rats treated with thyroxine exhibited higher plasma erythropoietin levels than vehicle treated controls (Peschle et al., 1971). The erythropoietic effects of ACTH, triiodothyronine, and testosterone observed in mildly plethoric mice (Fisher et al., 1967) and of ACTH, cortisol,

thyroxine, and testosterone in starved or polycythemic mice (Peschle et al., 1971) could be abolished by anti-Ep. Increased amounts of erythropoietin have also been observed in the urine of normal, hypogonadal, and anemic humans treated orally with the androgen, fluoxymesterone (Alexanian, 1969).

The possibility that testosterone increases Ep production and thereby increases erythropoiesis was suggested by Fried and Gurney in 1965. They noted that administration of testosterone resulted in increased kidney mass as well as elevated red blood cell counts.

Multiple doses of testosterone result in increased erythropoiesis. In fact, even a single injection of testosterone is sufficient to increase Ep in the plasma of a normal rat within forty-eight hours.

Testosterone therapy for anemia proved to have several troublesome side effects including: masculinization of female children; unwanted hair growth; fluid retention; and, in some cases, fever. These led to a search for nonandrogenic but erythropoiesis-stimulating metabolites of testosterone, among which the 5B-OH metabolites proved most satisfactory. Gardner and Gordon, who examined a number of these metabolites, noted an increase in

erythropoiesis without an accompanying increase in Ep production. In this regard, certain of the steroids with the 5B-OH C₁₉ and C₂₁ configurations proved to be most effective (Gardner and Gordon, 1970; Zanjani et al., 1968; Gurney and Fried, 1965).

Testosterone injected into rats for seven consecutive days results in an increased Ep production by forty-eight hours persisting until day 7. A single dose of testosterone does not result in a measurable increase in hemoglobin production until 96 hours later. If the effect of testosterone is indeed to increase Ep production, a delay is expected to allow time for Ep production and for the Ep in turn to increase erythropoiesis (Mirand et al., 1965). At this point the literature becomes somewhat confusing; there is sufficient evidence to state with certainty that one of the ways in which androgens stimulate erythropoiesis is through increased Ep production. However, androgenic steroids with the 5B-OH configuration were reported by Gordon, Zanjani et al. (1970), Gardner, Gorshein, and others to increase erythropoiesis without an accompanying increase in Ep production (Levere et al., 1970).

Studies by Byron (1970) suggested a direct effect of testosterone on the stem cell.

Metabolites of testosterone were suggested to affect the progenitor cell and increase erythropoiesis without the need for Ep, i.e., a direct effect on the stem cell. This direct effect has been confirmed by Byron (1970) monitoring the sensitivity of spleen colonies to doses of high specific activity tritiated thymidine.

Schooley in 1966 was able to inhibit the erythropoietic influences of testosterone in mice by simultaneous administration of anti-Ep. However, he concluded that the only way in which androgens influence erythropoiesis is through increased Ep production.

In 1967, Levere et al. reported that hemoglobin synthesis in the chick blastoderm can be induced by certain 5B-OH steroids, probably by acting on the gene, coding for d-aminolevulinic acid synthetase, the rate limiting enzyme in heme synthesis. The mechanism of this action would not be dependent on Ep. Again it must be considered that at least a part of the androgen action on erythropoiesis is mediated by a direct bone marrow effect.

Gordon et al. (1970) examined the effect of a number of steroid compounds on erythropoiesis in normal and polycythemic mice. Testosterone and 5B-OH C₁₉ and C₂₁ nonhormonal metabolites were most effective. Anti-Ep

abolished the erythropoiesis stimulating effects of testosterone but not of its metabolites. Only testosterone, however, evoked the production of Ep in rats. Two mechanisms were postulated to underly the stimulatory action of androgens on erythropoiesis:

1. Increased Ep production.
2. A direct effect on the blood forming tissues, not associated with increased Ep levels.

Ep also stimulates DNA synthesis in the stem cell compartment--a mechanism which also enhances erythropoiesis.

Gorshein in 1972 discovered that within 16 hours Actinomycin D blocks the erythroid committed cell without affecting the stem cell pool. Coupling this with the fact that pluripotent stem cells are triggered into cell cycle by androgens and noting that the Actinomycin D blockade cannot be overcome even by massive doses of Ep, he concluded that Actinomycin D blocks G₁ erythroid precursors from entering S phase and consequently inhibits erythropoiesis (Gorshein et al., 1972). Mice receiving 19-ND along with Actinomycin D maintained higher levels of ⁵⁹Fe incorporation, and a protective role was postulated for androgens against the toxic effects of chemotherapeutic agents.

Necheles and Rai (1969) and Levere and Gidari (1974)

have suggested that ALAS induction could be the method by which 5B-OH steroids increase heme synthesis in vitro. Recent results by Singer et al. (1976) suggest that certain C₁₈, C₁₉, and C₂₁ steroids of varying configurations significantly increase the number of erythroid colonies obtained using rat bone marrow with a fixed, suboptimal dose of Ep. The results indicate that, with this system, a relative order of steroid potency may be formulated and structure-activity relationships established which might suggest compounds most suitable for in vivo uses as well as partially define the structural requirements of the steroid receptors of erythroid precursor cells.

Singer and Adamson (1976) reported the possibility that different steroids may influence in vitro erythropoiesis by different means. In fact, two active steroids, fluoxymesterone, a Δ ,4-androstane, and ETIO, a 5B-H androstane, may exert their erythropoietic effect through different target cells. Fluoxymesterone appears to act through cells already committed to respond to Ep, while ETIO appears to act on a separate, perhaps primitive, population of marrow cells. Sedimentation profiles reveal fluoxy-dependent colonies to be virtually identical to Ep-responsive cells. Certain androgenic and nonandrogenic steroids act on

different populations of marrow cells to influence the response to humoral regulation--in this case Ep. By several criteria, Fluoxymesterone appears to act upon cells already committed to respond to Ep, but the mechanism of this effect has not been elucidated. In contrast, ETIO appears to act on distinctly different cells to enhance erythroid colony formation, possibly recruiting cells otherwise insensitive to Ep in culture.

Conversely, it has been demonstrated that, in the complete absence of DNA synthesis and mitotic activity, cultures incubated with Ep do go on to differentiate (Datta et al., 1975). However, it should be noted that the in vitro effect of steroids occurred always in the presence of Ep; thus, the independence of the steroid effect from Ep is not clear.

The manner by which steroid hormones regulate growth and differentiation of target tissues has been the topic of numerous investigations. One model system which has been particularly useful in this regard is the oviduct of the immature chick (O'Malley et al., 1969, 1972; Means and O'Malley, 1972).

Administration of estrogen to these animals results in a rapid and pronounced increase in mitosis, DNA synthesis,

and consequent cytodifferentiation (Kahler et al., 1969; Socher and O'Malley, 1973). Moreover, one of the new cell types that appears will synthesize large quantities of the specific protein, ovalbumin. Thus, estrogenic steroid hormones regulate both biochemical and morphological differentiation of the oviduct in a highly ordered fashion. In the same tissue, a second hormone (progesterone) acts as a modulator of oviduct function by causing induction of another specific protein (avidin) in the absence of readily demonstrable influences on cell division or hypertrophy.

Experimental proof exists that both estrogenic and progestational steroids act in the oviduct to alter gene transcription in a manner which leads to the production of mRNA for the specific proteins, ovalbumin and avidin, respectively. Actinomycin D blocks these steroid-mediated hormone responses. Administration of these hormones results in quantitative changes in rapidly labeled nuclear RNA and RNA polymerase (McGuire and O'Malley, 1968) and qualitative changes in the populations of newly synthesized nuclear RNA (O'Malley et al., 1968). Hormone induced increase in differentiation specific oviduct proteins are temporary, and preceded by increased intracellular concentrations of the specific mRNAs coding for their proteins

(O'Malley et al., 1969). Intracellular accumulation of a specific mRNA plus repeated transcription of a single gene are necessary for the synthesis of a new gene product and steroid hormones appear to cause both events.

The general pattern of steroid hormone action is:

1. Uptake of the hormone by the target cell and binding to a specific cytoplasmic receptor protein.
2. Transport of the steroid-receptor complex to the nucleus.
3. Binding of this complex to specific "acceptor" sites on the genome, probably to DNA as well as non-histone acidic nuclear proteins.
4. Activation of the transcriptional apparatus resulting in the appearance of a new species of specific mRNAs.
5. Transport of the hormone-induced RNA to the cytoplasmic ribosomes resulting in the synthesis of new proteins.
6. The occurrence of a steroid mediated "functional response" characteristic of that particular target tissue (O'Malley et al., 1974).

It has been postulated recently that hormones control their receptor sites. Exposure of a tissue in vivo or in vitro to a hormone, e.g., insulin on pancreas, results in

a decreased number of binding sites for the hormone and may account for refractoriness to treatment observed in vivo.

It is known that the biological actions of androgens on target organs such as the prostate are mediated through their binding to a cytoplasmic receptor protein which in turn carries the androgen to the nucleus. Whether such a mechanism is involved in androgen induced erythropoiesis is unknown. The testicular feminizing mutant mouse (Tfm) which is known to have markedly reduced amounts of receptor protein provided a unique model to answer this question.

Oxygen Delivery

Recent evidence also suggested that another factor of prime importance in the overall rate of red cell production in mammalian species is the concentration of 2,3-diphosphoglycerate in circulating red cells.

The in vitro observations of Chanutin and Churnish (1967) and Benesch and Benesch (1967) that intra-erythrocytic organic phosphate compounds are potent modifiers of the oxygen affinity of hemoglobin led to the recognition that the affinity of hemoglobin for oxygen is altered in a variety of conditions and that these alterations may compensate for anemia or result in polycythemia. Changes

in the concentration of organic phosphates have implications in the preservation of blood for transfusion.

Human tissue metabolism is critically dependent on an adequate supply of oxygen. The O₂ transport system in man is the red blood cell. The erythrocyte serves the purpose because it contains the iron-protein conjugate, hemoglobin. It is thus apparent that the 2,3-DPG content of the circulating red cell may have a significant affect on the overall rate of erythrocyte formation.

Following the discovery of the influence of DPG on the O₂ affinity of hemoglobin (Benesch and Benesch, 1967; Chanutin and Churnish, 1967), increased DPG levels in patients with congenital heart disease, thyrotoxicosis, anemia, and chronic hypoxemia, as well as in normal individuals upon ascent to altitude, were described. The common denominator in all these conditions is an altered relationship between tissue oxygen demands and oxygen delivery, implying that a right shift of the dissociation curve may be an adaptive mechanism.

DPG levels are often elevated in cardiac disease, and this appears to provide an important compensatory mechanism in low output cardiac failure (Woodson et al., 1967). The DPG mediated shift of the dissociation curve is a particularly

effective compensatory mechanism in anemia (Finch et al., 1972).

The ultimate supply of oxygen to the cell is determined by a number of factors which include: the content of O_2 in the inspired air; the partial pressure of O_2 in the inspired air and alveolar air; the cardiac output; the blood volume; the hemoglobin concentration; and the passive diffusion of oxygen from the capillaries to the cells. The initial passive diffusion of oxygen from the lungs and its final release to the tissues is determined in large part by the affinity of hemoglobin for oxygen.

The oxygen-hemoglobin equilibrium curve reflects the affinity of hemoglobin for oxygen. As blood flows through the alveolar capillary bed of the normal lung, venous pO_2 of 40 mm Hg reaches the arterial level of a 110 mm Hg or an O_2 saturation of at least 95%. At this point the curve plateaus such that any further increase in O_2 tension results in only a small increase in the degree of saturation of the blood. As blood travels from the lung, the O_2 tension falls, as it is released to the tissues from hemoglobin. The P_{50} is defined as the pO_2 at which hemoglobin is 50% saturated. Normal adult hemoglobin has a P_{50} value of 27-29 mm Hg at a pH of 7.4 and a temperature of 37°C . The P_{50} , the whole blood oxygen tension at 50% saturation,

is thus stated to be 27 mm Hg. When the affinity of hemoglobin for oxygen is reduced, more oxygen is released to the tissues at a given oxygen tension. In such situations, the oxygen-hemoglobin dissociation curve is shifted to the right. It has long been recognized that changes in blood pH, CO₂ content, ionic concentration, or temperature are capable of altering the affinity of hemoglobin for oxygen and thus shift the curve to the right or left (Finch and Lenfant, 1972).

Under conditions of increased oxygen need, or a decreased blood oxygen carrying capacity, such as occurs with anemia, the body may improve oxygen delivery to the tissues either by improving oxygen transport or by decreasing the affinity of hemoglobin for oxygen. Improved oxygen transport is achieved by increasing the cardiac output or by increasing the oxygen carrying capacity of the blood by a rise in hemoglobin concentration. An increase in cardiac output is a rapid compensation but is limited in its magnitude; it requires an increase in oxygen consumption that tends to offset its compensatory advantage. The second mechanism of improving O₂ transport, that of decreasing affinity of Hb for O₂ allows more oxygen to be delivered to the tissues at an equivalent or even higher partial pressure

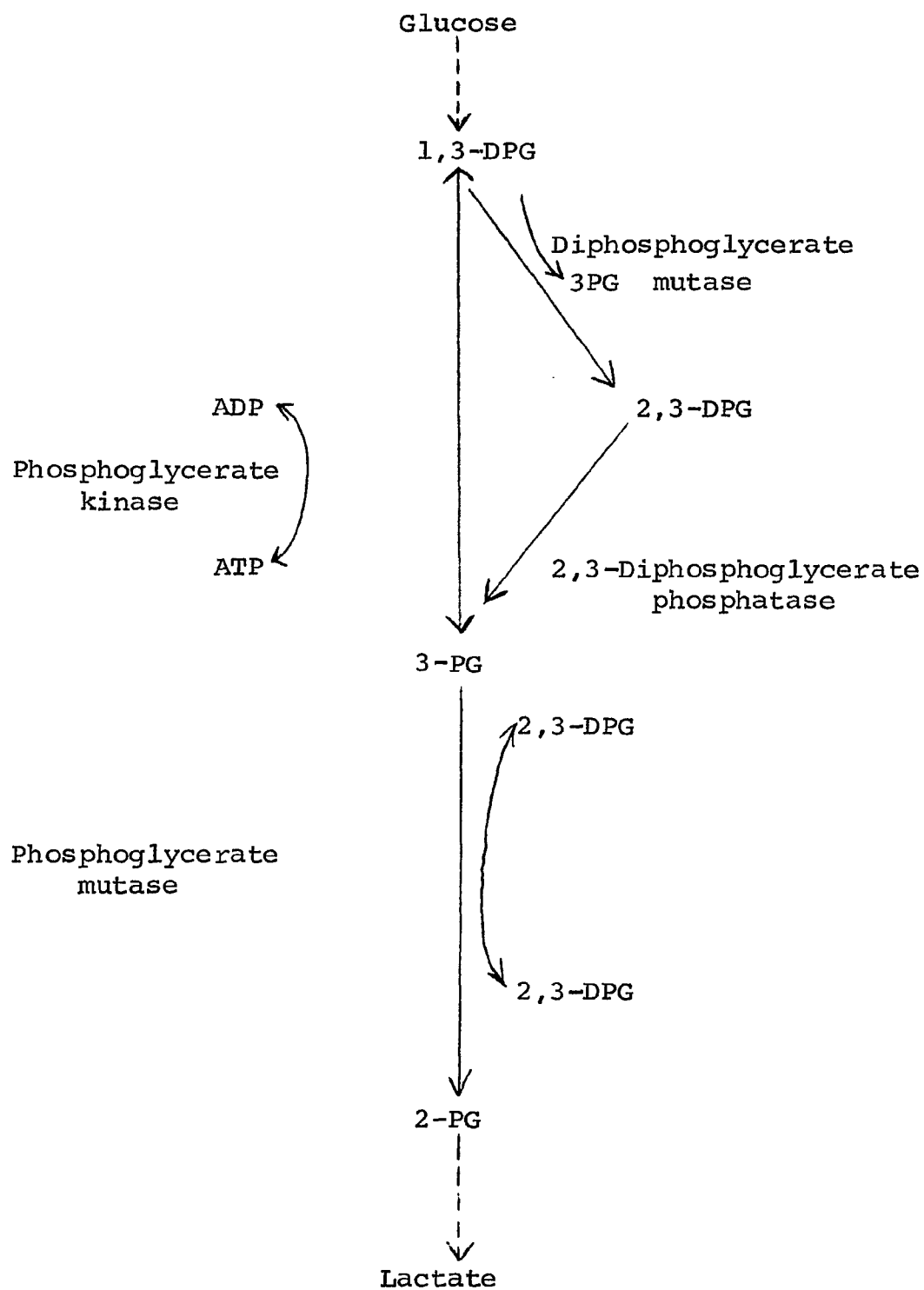
of oxygen. As a result, the end-capillary tissue oxygen gradient is maintained above the critical range despite a decrease in the degree of saturation of the blood.

The right shift in the dissociation curve produced by DPG apparently is the result of two separate mechanisms. The first involves the direct binding of the DPG to the hemoglobin molecule. DPG binds more readily to deoxyhemoglobin than to oxyhemoglobin (Benesch et al., 1968). The binding sites are located near the central cavity of the molecule, and DPG fits into this cavity when hemoglobin is in the deoxygenated state. With oxygenation, the conformation of the hemoglobin molecule changes and the central cavity closes so that DPG binding is more difficult (Perutz, 1970). Binding of DPG tends to "hold" hemoglobin in the deoxygenated conformation; the increased oxygen tension required to saturate blood is a manifestation of these events.

The second mechanism is related to the intracellular synthesis of DPG and its inability to cross the erythrocyte membrane. This produces a large transmembrane gradient of the markedly negative DPG anion. The presence of high concentrations of intracellular DPG alters the Donnan equilibrium causing a decrease in intracellular pH

(Battaglia et al., 1970). This decrease in pH shifts the dissociation curve to the right. Thus, DPG appears to decrease oxygen affinity directly by binding to hemoglobin and indirectly by its effect on intracellular pH.

The pathway of 2,3-DPG metabolism was first shown by Rapaport and Leubering (1950). It was thought that 2,3-diphosphoglycerate was formed in the red blood cell by a specific mutase and hydrolyzed by a specific phosphatase. Diphosphoglycerate mutase catalyzes the formation of 2,3-diphosphoglycerate from 1,3-diphosphoglycerate and 3-phosphoglycerate (Rose, 1969). Recent evidence suggests, however, that the phosphatase and the mutase may in fact be one enzyme. Diphosphoglycerate mutase was purified to homogeneity from outdated human erythrocytes. The native enzyme has a molecular weight of 57,000 as determined by equilibrium centrifugation and exclusion chromatography. Disc gel electrophoresis in the presence of sodium dodecyl sulfate yields a single protein band with a molecular weight of 26,500, indicating that diphosphoglycerate mutase is comprised of two subunits of similar mass. The enzyme exhibits the following intrinsic activities: diphosphoglycerate mutase; monophosphoglycerate mutase; and 2,3-diphosphoglycerate phosphatase. The latter activity is enhanced by organic or inorganic anions. Glycolate-2(B)



(Rapaport and Leubering, 1950)

has a profound activating effect. Nonspecific phosphatase and enolase activities are absent (Kappel and Hass, 1976).

Therefore, the response to hypoxia includes two adaptive features:

1. Increased red blood cell production.
2. Improved O₂ delivery via a right shift of the oxygen-hemoglobin dissociation curve.

In addition to the effect on hematopoiesis, androgens have been shown to increase DPG, with a resulting right shift of the oxy-hemoglobin dissociation curve (Molinari, 1969).

In summary, the primary target site of Ep is considered to be hematopoietic precursor elements now termed Ep responsive cells (ERC). Ep apparently induces these cells to differentiate into the earliest recognizable members of the nucleated erythron (Krantz and Jacobson, 1970). The mechanism by which Ep causes this inductive action has been studied in two types of in vitro systems: (1) adult rat bone marrow cells (Krantz et al., 1963) and (2) mouse fetal liver cells (Cole and Paul, 1966). In the marrow system, Ep triggers the early production of a specific RNA assembly (messenger, ribosomal precursor and transfer) (Gross and Goldwasser, 1969), hemoglobin production (Gallien-Lartigue and Goldwasser, 1965), and stroma

formation (Dukes et al., 1963) by the reactive hematopoietic cells. It is of interest that an enhancing effect on δ -amino levulinic acid (ALA) synthetase activity has been reported in rabbit bone marrow cultures to which Ep has been added (Necheles and Rai, 1969). As with the 5B-H steroid metabolites (Levere and Granick, 1967), Ep may function here in part as a physiological derepressor of ALA synthetase, a rate-limiting enzyme in the production of heme.

Objectives of This Study

Multiple actions of testosterone and its metabolites have been posultated. Increased erythropoiesis in response to androgen therapy may be due to a direct bone marrow effect or an indirect effect on renal Ep production. An effect on the oxygen delivery system of the red blood cells has also been suggested. However, the relationship between these phenomena remains to be determined.

My experiments were designed to study the effects of testosterone on Ep production and erythropoiesis, pluri-potent stem cell activity and oxygen delivery and the relationship, if any, between these events.

My studies involved the simultaneous measurement of Ep production, stem cell activity, and DPG production and O₂ delivery in the same host animal following androgen

treatment. Since previous studies were performed with steroids of different configurations, it became necessary to perform these simultaneous measurements not only with testosterone but with some of its metabolites as well.

MATERIALS AND METHODS

I. General

1. Experimental Animals

Normal female Sprague-Dawley rats (150-200 gm) were obtained from commercial sources (Taconic Farms, New York).

Mice (Swiss-Webster strain 22-25 gms) were obtained from Brookhaven National Laboratories, Upton, New York. Tfm mutant mice and the unaffected littermates were kindly supplied by Dr. N. T. Shahidi, University of Wisconsin School of Medicine, Madison, Wisconsin. All animals were maintained on a regular laboratory commercial diet and given water ad libitum and acclimatized to laboratory and/or animal room conditions for 5-7 days prior to use.

2. Androgens. Four Compounds

- A. androsterone (5 α -androstan-3 α -01-17-one)
- B. 5 α -dihydrotestosterone (5 α -androstan-17B-01-3-one)
- C. etiocholanolone (5B-androstan-17B-01-3-one)
- D. 5B-dihydroandrolone (5B-androstan-3 α -01-17-one)

were studied both in vivo and in vitro.

For in vitro studies, the steroid compounds were initially dissolved in ethanol in 10^{-2} M concentration as stock solutions. Subsequently, serial dilutions were made with NCTC-109 medium (Microbiological Associates, Bethesda, Maryland).

When dissolved in this manner, no visible precipitation was observed. The final concentration of steroids in the culture medium ranged from 10^{-10} to 10^{-7} M. Testosterone propionate (TP) and etiocholanolone (ETIO) were each dissolved separately in peanut oil at a concentration of 25 mg/ml. All media components (embryo extract, fetal calf serum, citrated plasma, glutamine, pyruvate, amino acids, and sodium bicarbonate) were obtained from Grand Island Biological Co. (Grand Island, New York). Bovine serum albumin (Miles Labs) was prepared according to the method of Worton et al. (1969). The androgen compounds were obtained from Sigma Chemical Co. (St. Louis, Missouri).

Sprague Dawley female rats (150-200 gm) and virgin Swiss-Webster mice (25-30 gm) were used throughout the study. They were maintained on a commercial diet and water throughout the study. Each rat received 2 mg of iron dextran intramuscularly at the beginning of this study. TP and the four previously mentioned metabolites were each dissolved separately in a mixture of 30% ethanol and 70% propylenglycol and were administered intraperitoneally in peanut oil vehicle. Control groups were given peanut oil alone.

3. Bioassay for Erythropoietin

Erythropoietic activity of all plasma samples was

determined in ex-hypoxic polycythemic mice. Mice were rendered polycythemic by exposure to 0.4 atmospheres of air 19 hours per day for a total of 219 hours. At least 5 mice were used for each test sample. Each mouse received an intraperitoneal injection of 0.5 ml/day of the rat or mouse plasma on days 5 and 6 post hypoxia. Radioiron (0.5 microcurie/mouse) was injected intravenously on day 7 and the mice were killed by exsanguination 56-72 hours later. Percent RBC- ^{59}Fe incorporation was calculated for each mouse in the group according to the following equation:

% RBC- ^{59}Fe incorporation =

$$\frac{\text{blood vol.} \times \text{cpm/ml blood}}{\text{cpm of injected } ^{59}\text{Fe}} \times 100$$

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

4. P_{50} Determination

The red blood cells which remained after the plasma had been removed were resuspended in freshly drawn plasma from normal rats of the same strain to the same Hct level and used to determine the oxyhemoglobin (Hb-O_2) dissociation curve.

The pH and $p\text{O}_2$ were determined using the IL-217 Blood Gas Laboratory, which provides direct digital measurement

of blood pH, $p\text{CO}_2$, $p\text{O}_2$, oxygen saturation, carbon monoxide saturation, and total hemoglobin concentration. Other related parameters, e.g., P_{50} , may be quickly calculated. Complete oxyhemoglobin dissociation curves and P_{50} can also be measured. The IL-208 Control Module and Monitor is used for this purpose. This equipment allows for the mixing of two gas mixtures of equal, normal arterial CO_2 tension, but of different O_2 concentration.

Changing the percentage of oxygen delivered to the tonometer permits equilibration and sampling of successive aliquots of blood at different oxygen tensions. Subsequent measurement of oxygen saturation, pH, and $p\text{O}_2$ provide the information for plotting the oxygen dissociation curve and determining the P_{50} . The function of the mechanical components is to automatically process samples for photometric measurement.

To determine P_{50} generally at least two known points of $p\text{O}_2$ and % HbO_2 are needed.

A sample of well-mixed heparinized blood is injected into a tonometer of an IL-208 Control Module. At the end of the automatic cycle, the results are read on the digital display.

The hemoglobin concentration displayed as Hb is the

total hemoglobin concentration expressed directly in grams per hundred milliliters of whole blood. The hemoglobin species which are measured are deoxygenated, oxygenated, and carboxyhemoglobin. These forms nearly exclusively constitute all of the hemoglobin present.

The co-oximeter displays a value of oxygen saturation that represents the fraction of hemoglobin combined with oxygen in relation to the total amount of hemoglobin, whether or not it is available at the time of measurement for oxygenation. This approach may be represented by the equation:

$$\% \text{ Saturation} = \frac{(\text{HbO}_2)}{(\text{Hb}_r) = (\text{HbO}_2) + (\text{HbCO})}$$

where $\% \text{ HbO}_{2t}$ = percent oxyhemoglobin

(HbO_2) = concentration oxyhemoglobin

(Hb_r) = concentration reduced hemoglobin

(HbCO) = concentration carboxyhemoglobin.

The percent carboxyhemoglobin, that is, the percentage of the hemoglobin combined with carbon monoxide (CO) compared to the total hemoglobin is displayed directly on the digital display of the co-oximeter as CO.

5. Red Cell 2,3-Diphosphoglycerate (DPG) Determination

2,3-Diphosphoglycerate content of red cells was determined according to a modification of the method of

Benesch and Benesch (1967) (Maeda et al., 1971). An enzyme suspension consisting of rabbit muscle phosphoglycerate mutase (Sigma Chemical Co., St. Louis, Missouri) 5 mg/ml was centrifuged at 8,000 r.p.m. and 4°C for 40 min. The supernatant was removed and the volume restored to 10 ml with 10 mM glycyl glycine buffer, pH 7.5. The enzyme solution thus obtained was prepared daily and stored in ice until ready to use.

Other solutions and materials were as follows: standard phosphate; 0.1 mM potassium dihydrogen-phosphate prepared daily from a 1 M stock solution; trichloroacetic acid, 20g per 100 ml of water; EDTA, 5 mM, 168 mg in 100 ml of water; phosphoglycolate, 0.5 mM, 22.6 mg of phosphoglycolic acid (tricyclohexylammonium salt, Sigma Chemical Co., St. Louis, Missouri) dissolved in 100 ml; and ammonium molybdate reagent, consisting of 1 part of 10% ascorbic acid mixed with six parts of 0.42% ammonium molybdate in 1 N sulfuric acid just before use.

Two heparized capillaries (internal diameter of 1.1 to 1.2 mm and length 74 mm) are filled with blood and sealed with Seal-ease and centrifuged in a microhematocrit apparatus for five minutes. The hematocrit is read and the clay, plasma, and white cell layers are removed with

a file. The remaining packed red cells are flushed from the capillary tubes into sterile test tubes with glycyl glycine buffer. At this point the cells hemolyze and the hemolysate can be frozen. At the time the tests are performed, the test tube is centrifuged at 8,000 r.p.m. for 40 min. and the supernatant is used for the incubation and subsequent determinations. A hemoglobin must be done on the hemolysate. For each sample, there is a blank consisting of 0.6 ml of water in a red top test tube, a standard consisting of 0.6 ml of 0.1 mM inorganic phosphate.

Into a clean, cold test tube (without anticoagulant) are placed 0.6 ml of the clear hemolysate, 0.2 ml of 5 mM EDTA, 0.2 ml 0.5 mM phosphoglycolate, and 0.04 ml of the enzyme solution. The mixture is incubated for 15 minutes at 25°C. The protein is then precipitated with 1 ml of 20% trichloroacetic acid with agitation. This is followed by centrifugation at 1,500 r.p.m. for 10 minutes and filtration through a small plug of glass wool. The clear filtrate is now ready for phosphate assay.

The estimation of inorganic phosphate in the clear filtrates is based on the method of Ames and Dubin (1960). Aliquots of 0.5 ml are mixed with 1.5 ml of ammonium molybdate and incubated for one hour at 37°C. The ammonium

molybdate reagent is prepared by adding 6 parts molybdate to 1 part ascorbic acid. The optical density is then read at a wave length of 820 nm in a Beckman Model KU2 spectrophotometer.

The hemoglobin content of the hemolysate is determined by reacting 2.9 ml Drabkin solution to 0.1 ml hemolysate for 10 minutes. The DPG concentration in the cells is calculated as follows:

2,3-DPG

O.D. Reagent Blank	0.004
O.D. Phosphate Std.	0.134
O.D. Blank Hemolysate	0.046
O.D. Incubated Hemolysate	0.267

$$\frac{0.267 - 0.046}{0.134 - 0.004} = .170 \text{ mM}$$

DPG Hemolysate = .170 mM

Hb--whole blood	2.33 mM	$\frac{\text{Hb} \times \text{mol. wt.}}{1000}$
Hct	.47	
Hb--cells	4.96 mM	
Hb--hemolysate	0.171 mM	

6. Plasma Clot Culture System: In Vitro Determination of Erythroid-Committed Stem Cell (CFU-E)

Immediately following exsanguination of rats or mice, both femurs were removed surgically and marrow was flushed from the marrow cavity with a syringe containing 0.5 ml

culture medium. A suspension of these cells was made using supplemented MEM made 2% with FCS immediately before use. Concentrations of 2×10^5 , 4×10^5 , 6×10^5 , and 8×10^5 cells were cultured in the plasma clot in the presence or absence of Step III Ep.

7. 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 nonessential amino acids (100 mM), 1% 200 mM L-glutamine, 1% Na pyruvate (100 mM), 1.25% NaHCO_3 , and 1% penicillin-streptomycin mixture giving a final concentration of 100 units/ml and 100 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$. 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 ml of medium NCTC-109 (Grand Island Biological Co., Grand Island, New York). L-asparagine (l-asp) (Calbiochem, San Diego, California) was made up in NCTC-109 to a concentration of 2 mg/ml, sterilized through a Millipore filter apparatus containing a 0.45u Millipore filter and frozen until used. In the clot, 0.5 ml of l-asparagine was mixed with 4.5 ml of NCTC-109 so that 0.1 ml contains 0.02 mg of l-asparagine. Bovine serum albumen fraction V (BSA) (Sigma Chemical Co., St. Louis, Missouri) was also used in the plasma clot. For the preparation of 10X BSA, 50 gm of BSA powder were added to 91 ml of Fe-free 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 gm resin were added and the mixture was left at 4°C for 12 hours. During the first hour, the flask was gently swirled every 15 minutes. At the end of 12 hours, the BSA was filtered through gauze and the volume measured to calculate yield. To make the solution isotonic, 1.1 ml of 1 M Dulbecco phosphate buffered saline (minus Ca^{++} and Mg^{++} ions) was added to each 15 ml 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 0.45u Millipore filter. The solution was kept frozen until used. Before use in the plasma clot system, 0.5 ml of 7% NaHCO_3 was added to the 10% BSA. Stock solutions of 0.1M α -thioglycerol (α -TG) were prepared by pipetting 0.1 ml of 10M α -TG (Aldrich, Milwaukee, Wisconsin) into 10 ml NCTC-109. Prior to use in the culture system, 0.1 ml stock solution was added to 10.0 ml NCTC-109 to yield a 10^{-3}M solution.

The following media were used in the plasma clot culture system. 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 in vitro techniques, penicillin and streptomycin were added to a concentration of 100 units/ml and 50ug/ml respectively and stored at 4°C after use. Citrated bovine plasma was obtained from 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 and stored at -20°C 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 was added to the following mixture: 0.1 ml BEE, 0.1 ml BSA, 0.2 ml FCS, 0.1 ml l-asp, 0.1 ml α -TG, 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 bovine plasma that had been warmed in the incubator immediately prior to use. After thorough mixing, achieved by repeated drawing of the materials into a 1 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, Connecticut). Groups of 4 wells each were placed in a 35 x 10 mm Petri dish and two such dishes were placed in an 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.

8. 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 Jersey). 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 $1\frac{1}{2}$ 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).

9. Scoring of Colonies

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

10. Preparation of Anti-Erythropoietin Serum

Six white New Zealand rabbits weighing $2\frac{1}{2}$ 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 10u/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 10U Ep was injected. Ten to 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 were obtained. One ml of the anti-Ep serum neutralized 22 IU of Step III sheep plasma Ep (Connaught Laboratories, Toronto, Canada) and 90 IU of HUE.

11. Cell Separation Studies

The Sta-put apparatus of Miller and Phillips (1969) was used to separate bone marrow cells from femurs of untreated as well as testosterone-treated, young female AKR mice.

Cell concentrations of 10^8 gave the best separation

profile. A linear gradient of 1% and 2% bovine serum albumin (Grand Island Biological Co., Grand Island, New York) in phosphate buffered saline was prepared. Phosphate buffered saline was made by using 70 gm NaCl, 17.4 gm K_2HPO_4 , and 13.6 gm KH_2PO_4 diluted to 1000 ml. This 10x stock solution was stored in the refrigerator. The bovine serum albumin (BSA) was made by adding 20 gm BSA per 1000 ml PBS. All other gradient materials were made by subsequent dilutions to obtain 1%, 0.5%, and 0.3% BSA in PBS.

The above prepared media was used as the separation medium. Cells were flushed from the marrow cavities of the tibias and femurs with a 1 ml syringe containing Medium I and fitted with a 25 gauge needle. After a nucleated cell count was obtained, using the standard WBC method, the cells were pipetted into a sterile graduate cylinder and diluted to a volume of 40 ml with 0.3% bovine serum albumin made isotonic with phosphate buffered saline. A linear gradient of 1% and 2% bovine serum albumin in phosphate buffered saline was used as the separation medium. The pH of all gradient solutions was adjusted to 7.2 to 7.4.

The cells were lifted to a predetermined starting point and allowed to sediment through the gradient over a

four-hour period, at which time they were collected in 10 ml aliquots into sterile plastic tubes. The cell suspensions were centrifuged at 1,000 r.p.m. for 10 minutes, the supernatant removed and nucleated cell counts done in order to obtain a profile. Three samples were then pooled for culture in the plasma clot at cell concentrations of 2×10^5 , 4×10^5 , and 6×10^5 .

Homogeneous populations of cells can be characterized by their s values, providing that all separations are related to a standard set of conditions.

The sedimentation velocity in mm/hr. of each fraction was calculated according to the following:

1) Standard conditions

- a. Temperature = 4°C
- b. Gradient density approximately equal to 1.010 gm/cm^3 .
- c. The fractions were numbered in the order of collection, the last fraction having the number N_f and volume V_f .
- d. Define $s=0$ as the middle of the input cell band.
- e. Treat the fraction number as a continuous variable.

2) The corresponding position of each fraction, in

terms of its fraction number is given by the equation:

$$N(s=0) = \frac{N_f - (V_o + \frac{1}{2}V_{CB}/2)}{v = v_f - \frac{1}{2}}$$

where V_o is the total volume of fluid above the cell band and V_{CB} is the volume of the cell band. The final subtraction of $\frac{1}{2}$ is necessary when one treats N as a continuous variable; a particular fraction, when collected, extends from $(N-1)$ to N on a continuous scale, but we wish to consider integer values of N , and the s value associated with them, as representing the average properties of the cells in the fraction having that N value. A correction is necessary for the time the cells spend in the cone during loading and unloading, the equation becomes:

$$S(N) = \frac{(N_f - N - \frac{1}{2}) v - (V_o + \frac{1}{2} V_{CB} - v_f)}{\alpha [(t_4 - t_1) - 0.4 (t_4 + t_2 - t_3 - t_1) + N (t_5 - t_4)/N_f]}$$

where the times t_1 to t_5 (all in hours) are respectively the times at which loading started (t_1), the cells reached the top of the cone, (t_2) draining started (t_3), first fraction started (t_4), last fraction finished (t_5), and α is the chamber constant, i.e., the ml of fluid per mm of length in the cylindrical region in the chamber.

These same studies were repeated but with the androgen compounds added to the culture medium in a concentrate

10^{-7} - 10^{-6} M. In addition, the marrow of the Tfm mouse was separated using the Sta-put apparatus and cultured in the plasma clot with 5α -DHT. Also, marrow from Tfm mice treated with Tp was separated in the Sta-put. The cultures were incubated for 48-72 hours in a humidified atmosphere of 95% O_2 -5% CO_2 at $37^{\circ}C$, at which time they were removed from the culture plates, pressed onto a glass slide, fixed, stained, and the number of erythroid colonies per clot determined as described previously.

II. Experimental

1. Effect of Chronic Administration of Testosterone Propionate (TP) on Erythropoietin Production in Normal Rats and Erythropoiesis in Normal Rats

Six groups of rats (5-7 rats per group) received daily subcutaneous injections of either 2.5 mg TP dissolved in 0.2 ml peanut oil or 0.2 ml of peanut oil alone 5 days/week for 4 weeks. Groups of 3-5 animals were killed at approximately weekly intervals by bleeding under ether anesthesia through the heart using a 10 cc heparinized disposable plastic syringe fitted with a 19-gauge needle. The blood was centrifuged at 1,500 r.p.m. for 15 minutes, and the plasma was stored separately at $-20^{\circ}C$ until assayed for erythropoiesis-stimulatory

activity in ex-hypoxic polycythemic mouse as described in the section on general procedures. Hematocrit and hemoglobin values were determined for each rat at the time of killing.

2. Effects of Chronic Administration of Testosterone Propionate (TP) on the Oxygen Delivery System in Normal Rats

The effect of treatment with TP on the Hb-oxygen dissociation curve, on P_{50} , and red cell 2,3-diphosphoglycerate (2,3-DPG) was examined in the same groups of rats employed in II.1. Prior to separation of plasma, 2 ml of whole blood was removed from the blood obtained from each rat and used to determine the levels of red cell 2,3-DPG as described earlier.

Following centrifugation and removal of plasma for bioassay, the remaining cells were resuspended in a similar volume of plasma obtained by centrifugation from freshly drawn blood of untreated rats of the same strain. The newly reconstituted blood was used for the determination of the Hb- O_2 dissociation curve and P_{50} .

3. Effect of Chronic Administration of Testosterone Propionate (TP) Treatment on Erythroid Colony Formation by Normal Rat Bone Marrow Cells In Vitro

Groups of rats treated as given in I.1. were also

employed to determine the effect of TP on bone marrow erythroid colony forming ability. Immediately after exsanguination, both femurs from each rat were removed by making a longitudinal incision with surgical scissors along the length of the thigh. The forelimb was bent backward at the knee to expose the condyles of the femur. The upper end of the femur (head, neck, greater and lesser trochanters) were dissected away from the surrounding musculature. The upper and lower ends of the femurs were cut away and the marrow collected from the marrow cavity aseptically by flushing the cavity with MEM supplemented with FCS. The cells from each group of rats were pooled and a single cell suspension was made with a sterile Pasteur pipette. The cells were kept cold and used for the plasma clot. The mixture was centrifuged at 1,000 r.p.m. for 10 minutes and the cells resuspended in Medium II, the numbers of nucleated cells were determined with hemocytometer and further dilutions made with Medium II. Pooled bone marrow cells (2×10^5 , 4×10^5 , 6×10^5 , and 8×10^5 cells) from each group were cultured in plasma clots in the presence or absence of 0.2 IU of sheep plasma erythropoietin for 3 days. Eight separate clots were prepared for each cell concentration. The number of erythroid

colonies were determined after fixation and staining as outlined above. Only bone marrow cells from rats receiving TP or oil for 14 and 28 days were used in these studies.

4. The Effect of Anti-Erythropoietin Antiserum on the Erythropoiesis-Stimulatory Activity of Plasma from Testosterone-Treated Rats

An anti-erythropoietin serum (raised in rabbits as described above) with a potency of 22 (sheep plasma Ep) to 90 IU (human urinary Ep) was used in these studies. The potency of anti-Ep and goat anti-rabbit gamma globulin (GARGG) preparations used in these studies is shown in Table 1.

To 6 ml of serum from rats which received TP for 7 days were added 0.1 ml of either anti-Ep or normal rabbit serum and the mixture was incubated at 37°C for 1 hour followed by the addition of 5 mg of goat anti-rabbit IgG fraction obtained commercially (GARGG, Antibodies, Inc., Davis, California), mixed thoroughly and allowed to stand for 2 hours at 4°C. The mixture was spun at 10,000 x g for 20 minutes, the precipitate discarded, and the supernatant was assayed for erythropoiesis-stimulator activity in polycythemic mice. Controls included: Oil and TP-treated rat plasma except that no rabbit serum

or GARGG was added and a standard preparation of Ep (sheep plasma) with and without antibody treatment.

5. Effect of Chronic Administration of Testosterone

Propionate on Erythropoietin Production in Transfusion-Induced Polycythemic Rats

Rats were made polycythemic by daily intraperitoneal injections of washed red blood cells. The red blood cells were obtained via cardiac puncture from normal rats of the same strain. Blood from several rats was pooled, centrifuged at 1,000 r.p.m. for 10 min. and the plasma removed. The cells were washed three times with normal saline. After the final wash, the volume of packed cells was measured, and enough saline was added to give an 80% red blood cell suspension. Eight ml of the cell suspension were injected intraperitoneally per rat/day on two successive days. Hematocrits in recipient animals were determined from tail blood both before and on a daily basis for the duration of the study. Beginning on Day 5 after the first injection the hematocrits stabilized at $58\% \pm 3$. Animals with hematocrits less than 58% were not included in this study. These polycythemic rats were divided into two groups. Rats in one group received subcutaneous injections of 2.5 mg testosterone propionate

dissolved in 0.2 ml peanut oil/day, 5 days/week or for a total of 14 injections, while the remaining animals received 0.2 ml of peanut oil following the same schedule. Twenty-four hours after the last TP injection, rats in both groups were killed by exsanguination via cardiac puncture. Ten to 12 ml of blood was obtained from each rat. Blood from each rat was centrifuged at 1,500 r.p.m. for 15 minutes and the plasma harvested and stored at -20°C until assayed for erythropoietic activity in ex-hypoxic polycythemic mice.

6. Effect of Chronic Administration of Testosterone

Propionate on Erythropoiesis in Transfusion-Induced Polycythemic Rats

Rats in Group 1 received subcutaneous injections of 0.2 ml saline/day/rat for a total of 14 injections. Each rat in Group 2 received a similar volume of peanut oil/day for 14 days, while the animals in Group 3 were given 2.5 mg TP/day/rat following the same schedule. The additional groups of normal rats (not transfused) were also established. These groups were treated in a similar manner and served as normal controls.

Each rat in all six groups received 1 μCi ^{59}Fe Cl_3 intravenously into the tail vein 18 hours after the last TP injection, and the percent incorporation of radioiron

into red blood cells was determined. At the time of the study, each rat was weighed, anesthetized with ether, and bled through the heart using a 3 ml heparinized syringe fitted with a 19 gauge needle. Triplicate hematocrits were determined from this blood. One ml of this blood was placed in a 10 ml glass test tube and the radioactivity (CPM) was determined in a scintillation counter. Blood volume was estimated to be 5% of body weight in normal and 7% in polycythemic rats. The following formula was used to calculate the % RBC-⁵⁹Fe incorporation value for each rat.

$$\% \text{ RBC-}^{59}\text{Fe incorporation} = \frac{\text{CPM/ml blood} \times \text{blood volume} \times 100}{\text{SD}}$$

where SD represents the CPM of the injected dose of radio-iron.

7. Effect of Chronic Administration of Testosterone Propionate (TP) on the Oxygen Delivery System in Transfusion-Induced Polycythemic Rats

The effect of treatment with TP on the Hb-O₂ dissociation curve, on P₅₀, and red cell 2,3-diphosphoglycerate (2,3-DPG) was examined in the same group of rats employed in II.1. Two ml of whole blood were removed from each rat for determination of red cell 2,3-DPG as previously described. The remaining blood was

centrifuged and the plasma removed for bioassay. The cells were resuspended in an equal volume of plasma of untreated rats of the same strain. The newly constituted blood was used for the determination of the Hb-O₂ dissociation curve and P₅₀.

8. Effect of Chronic Administration of Testosterone

Propionate on Erythroid Colony Formation by Transfusion-Induced Polycythemic Rat Bone Marrow In Vitro

Groups of rats treated in II.1. were also employed to determine the effect of TP on bone marrow erythroid colony forming ability. Immediately following exsanguination, both femurs from each rat were removed and the marrow flushed out with MEM. The cells from each group of rats were pooled and a single cell suspension was made. The suspension was centrifuged at 1,000 r.p.m. for ten minutes, the supernatant discarded, and the cells resuspended in Medium II. Nucleated cell counts were obtained and further dilutions were made using Medium II. Pooled bone marrow cells (2,4,6, and 8×10^5 cells) from each group were cultured in the plasma clot in the presence or absence of 0.2 IU sheep plasma erythropoietin for 3 days.

Eight separate clots were prepared for each cell concentration. The number of erythroid colonies was

determined as outlined above. Bone marrow from rats chronically treated for 14-28 days was used in these studies.

9. Effect of Testosterone Propionate (TP), 5 α -dihydrotestosterone (5 α -DHT), 5 β -dihydrotestosterone (5 β -DHT), and Etiocholanalone on Erythropoietin (Ep) Production and Erythropoiesis in Normal Mice

Five groups of 3-4 mice each received intraperitoneal injections of 2.5 mg in peanut oil vehicle of TP; 5 α -DHT, 5 β -DHT, ETIO, or oil alone for a period of 28 days. On Day 29 of the study, the mice were killed by exsanguination via cardiac puncture. Enough blood was removed for hemoglobin and hematocrit determinations. The plasma from each mouse was assayed for Ep in the ex-hypoxic polycythemic mouse bioassay and the kidneys, liver, and spleen removed from each animal and weighed separately.

10. Effect of Testosterone Propionate and 5 α -dihydrotestosterone (5 α -DHT) on Erythropoietin Production and Erythropoiesis in Normal and Tfm Mutant Mice

Six groups (5 mice/group) were established. Groups 1-3 were composed of Tfm mutant mice, whereas unaffected littermates formed Groups 4-6. Mice in Groups 1 and 4 received daily intraperitoneal injections of 0.3 ml propyleneglycol for 3 consecutive days. Animals in

Groups 2 and 5 were administered intraperitoneal injections of 1.5 mg TP/day dissolved in 0.3 ml propyleneglycol for 3 days, while mice comprising Groups 3 and 6 received 1.5 mg 5 α -DHT/day for 3 days intraperitoneally. Twenty-four hours after the last injection, each mouse received 0.5 uCi ⁵⁹FeCl₃ in 0.2 ml saline via the tail vein, and the percent RBC-⁵⁹Fe incorporation was determined 66 hours later. Blood volume was assumed to be 5% of body weight for all mice. The procedure outlined in the bioassay procedure was used to calculate this value.

11. Effect of 5 α -dihydrotestosterone (5 α -DHT) on
Erythroid Colony Formation by Bone Marrow Cells
from Tfm Mutant and Normal Littermate Mice In Vitro

Four Tfm mutant mice were killed by cervical dislocation, both femurs removed, and the bone marrow was flushed out and prepared as detailed in II.3. Bone marrow cells (4×10^5 cells) were cultured in the presence or absence of 0.4 IU Ep (Step III sheep plasma Ep) in media containing 10^{-9} M 5 α -DHT, 10^{-7} M 5 α -DHT, or no 5 α -DHT. The cells were then cultured for 2-3 days in a humidified atmosphere of 5% CO₂ in air at 37°C. Processing of the clots and scoring of colonies were performed as described. Bone marrow cells obtained from 4 normal

littermates were cultured under similar conditions and served as controls.

12. Effect of Testosterone Propionate (TP), Androsterone (A), Etiocholanalone (B), 5B-dihydrotestosterone (C), and ~~5~~-dihydrotestosterone (D) on Erythroid Colony Formation by Normal Mouse Bone Marrow Cells In Vitro

Five normal mice (CF-1 strain) were killed by cervical dislocation, both femurs removed and the bone marrow flushed out by means of a 1 ml syringe fitted with a 25 gauge needle. Medium II was used in this process. A single cell suspension of the bone marrow was prepared with a sterile Pasteur pipette, spun at 1,000 r.p.m. for 10 minutes, and resuspended in fresh Medium II. Total number of nucleated cells were determined using a hemocytometer, and the volume was adjusted (with Medium II) to a nucleated cell concentration of 4×10^6 cell/ml.

Bone marrow cells (4×10^5 cells) were cultured in the presence or absence of 0.4 IU Ep (Step III sheep plasma Ep) in the plasma clot. The effect of TP, A, B, C, and D on erythroid colony formation was examined by the addition of these compounds in concentrations ranging from 10^{-10} to 10^{-4} M in 0.1 ml of diluting medium. Stock solutions of steroids (10^{-2}) in absolute ethanol were diluted with

NCTC-109. Cultures were separated into two groups. To one set, 0.1 ml of steroid was added, while the remaining set received 0.1 ml of NCTC-109 alone.

All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 2-3 days, processed as outlined above, and the number of erythroid colonies determined.

13. Separation of Erythropoietin and/or Androgen-Responsive Cell Population from Bone Marrow of Normal Mice

Bone marrow cells were obtained from femurs of 15 normal mice of Swiss-Webster strain as described. An aliquot of pooled marrow was set aside for culture studies in the plasma clot as outlined below. The remainder of cells (100×10^6 cells) were subjected to separation by unit gravity sedimentation as indicated in the section on general procedures. Every 3 consecutive fractions were pooled (total/36 fractions), resuspended in Medium II, and the nucleated cells determined for each pooled fraction. The unseparated marrow as well as each pooled fraction were then cultured in the plasma clot at concentrations of 2×10^5 , 4×10^5 , and 6×10^5 cells in the presence or absence

of 0.4 IU Ep (Step III sheep plasma Ep). Each cell concentration was cultured in the presence or absence of 0.4 IU Ep (Step III sheep plasma Ep); 10^{-7} M 5 α -dihydrotestosterone or 10^{-7} M etiocholanalone. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air for 2-3 days. The number of erythroid colonies formed/culture was correlated to the number of cells/fractions and the percentage of erythroid precursors determined for each fraction.

14. Separation of Erythropoietin and/or Androgen-Responsive Cell Population from Marrow of Tfm Mutant Mice and Normal Littermates

Bone marrow cells from femurs of 6 untreated Tfm mutant or normal littermates were pooled separately and subjected to sedimentation following the schedule described in I.11. However, a total of 21 fractions were employed in the pooling process resulting in 7 pooled fractions. Both unseparated and separated fractions from each group of mice were cultured in the presence or absence of Ep, 5 α -dihydrotestosterone and etiocholanalone as outlined in I.11.

RESULTS

1. Effect of Testosterone Propionate Administration on Erythropoiesis and Erythropoietin (Ep) Production in Normal Rats (Fig. 1)

Administration of TP resulted in the appearance of a significant erythropoiesis-stimulatory activity in the plasma of normal rats. This activity was first detected after 7 injections of the hormone and persisted throughout the study period. The plasma of non-injected rats or of vehicle-treated animals failed to exhibit erythropoiesis-stimulatory activity (Fig. 1).

The erythropoiesis-stimulatory effects of plasmas from TP-treated rats was due to the presence of Ep, since it could be eliminated by treatment of the plasma with anti-Ep prior to injection into assay mice (Table 2). The anti-Ep-plasma mixture was treated with goat-anti-rabbit gamma globulin (GARGG) prior to injection, excluding the possibility that this inhibition was not due to carry-over of excess anti-Ep to assay mice. The efficacy of such treatment in neutralizing anti-Ep activity is detailed in Table 1.

The increase in Ep formation in TP-treated rats was accompanied by an appreciable rise in red cell production

(Fig. 2). Blood hematocrit and hemoglobin levels were significantly higher at all time intervals after TP administration than the pre-treatment levels. No change in these parameters was noted in rats receiving peanut oil alone.

2. Effect of Testosterone Propionate Administration
on Oxygen Delivery in Normal Rats (Fig. 3)

Treatment with TP caused a significant increase in the red blood cell 2,3-diphosphoglycerate (2,3-DPG) content. This increase was accompanied by a right shift of the oxy-hemoglobin dissociation curve as manifested by elevated P_{50} values. Both 2,3-DPG values and P_{50} values were elevated by Day 3 and remained elevated for the duration of the study.

3. Effect of Chronic Administration of Testosterone
Propionate on Erythroid Colony Formation by
Normal Rat Bone Marrow Cells In Vitro (Fig. 4)

TP treatment significantly increased the number of erythroid colonies. Approximately twice as many erythroid colonies formed in cultures of bone marrow from TP-treated rats as compared to that of oil treated rats. However, in cultures lacking Ep, no growth of erythroid colonies was noted from the bone marrow of TP-treated rats or oil-treated rats.

4. Effect of Testosterone Propionate Administration on
Ep Production in Transfusion Induced Polycythemic Rats

Plasma from TP-treated rats previously rendered polycythemic by hypertransfusion did not exhibit enhanced erythropoietic stimulatory activity as compared with both vehicle-treated hypertransfused and vehicle-treated normal rats (Fig. 5). The hemoglobin and hematocrit levels, already markedly elevated in such rats, failed to exhibit any further increase in response to the TP treatment.

5. Effect of Chronic Administration of Testosterone
Propionate on Erythropoiesis in Transfusion-Induced
Polycythemic Rats (Table 3)

Administration of TP to normal rats caused a significant increase in the incorporation of radioiron into red cells. No such increase occurred in transfusion-induced polycythemic rats.

6. Effect of Testosterone Propionate Administration
on the Oxygen Delivery System of Hypertransfused Rats

TP treatment significantly increased the red blood cell 2,3-diphosphoglycerate content in hypertransfused rats. This increase was accompanied by a right shifted oxy-hemoglobin dissociation curve as manifested by increased P_{50} values increased by the third day and continued to

rise for the duration (28 days) of the experiment (Figs. 5 & 6)

7. Effect of Chronic Administration of Testosterone
Propionate on Erythroid Colony Formation by
Hypertransfused Rat Bone Marrow Cells In Vitro

In order to determine if TP treatment influenced the stem cell pool of hypertransfused rats even in the absence of demonstrable erythropoietic stimulatory activity, bone marrow from these animals was cultured in the plasma clot in the presence or absence of 0.4u Ep. No erythroid colonies were formed in cultures lacking Ep in either oil-treated or TP-treated animals. However, a linear increase in colony formation occurred when increasing numbers of bone marrow cells from vehicle-treated hypertransfused rats were cultured in the presence of 0.4 IU Ep (Table 4). At all cell concentrations examined, significantly greater numbers of erythroid colonies were formed when bone marrow from TP-treated hypertransfused rats was cultured in the presence of Ep ($P > 0.05$).

8. Effect of the Administration of Testosterone
Propionate, Androsterone, Etiocholanalone, 5B-
dihydrotestosterone, and 5 α -dihydrotestosterone
on Erythroid Colony Formation by Normal Mouse Bone
Marrow Cells In Vitro

Neither TP nor any of the four metabolites tested stimulated erythroid colony growth in the absence of

erythropoietin. In the presence of 0.4u erythropoietin, a significant ($P > 0.01$) increase in colony formation occurred when $10^{-7}M$ of androsterone or 5α -dihydrotestosterone were added to the cultures. However, the 5B-configuration of DHT failed to affect colony formation and for the same concentration Compound B proved inhibitory (Table 5).

9. Effect of the Administration of Testosterone

Propionate, 5α -dihydrotestosterone, 5B-dihydrotestosterone, and Etiocholanalone on Erythropoietin Production and Erythropoiesis in Normal Rats

In Table 6 the effects of TP, 5α -DHT, 5B-DHT, and ETIO on red cell volume, kidney weight, and plasma Ep levels are presented. Administration of TP and 5α -DHT resulted in a significant increase in kidney weight without an effect on the weights of the liver or spleen. This was accompanied by a significant increase in plasma Ep levels. Treatment with TP and 5α -DHT resulted in an increase in red cell mass in normal rats.

10. Effect of Testosterone and 5α -dihydrotestosterone on Erythropoietin Production and Erythropoiesis in Tfm Mutant and Normal Littermate Mice (Table 7)

Administration of 5α -DHT to mice resulted in a significant increase ($p > 0.001$) in Ep formation; Tfm mice

receiving similar amounts of the steroid failed to exhibit enhanced Ep formation. Administration of testosterone and 5α -DHT both resulted in an increased erythropoiesis as measured by ^{59}Fe uptake in littermate mice; neither compound increased erythropoiesis in the Tfm mutant.

11. Effect of Testosterone Propionate and 5α -DHT on
Erythroid Colony Formation by Bone Marrow Cells
from Tfm and Normal Mice In Vitro

5α -Dihydrotestosterone had no effect on erythroid colony formation of the Tfm or littermate mouse bone marrow in the absence of Ep. However, in the presence of 0.2 units of Ep, 5α -DHT exerted a significant effect on erythroid colony formation in both Tfm mutant and normal littermate mice (Fig. 8).

Figure 9 also demonstrates a dose response study with the two most active of the metabolites (androsterone and 5α -DHT). Once again TP was without an appreciable effect in this system. For both compounds the greatest degree of stimulation occurred at a concentration of 10^{-7}M with no further increase occurring when the concentration was increased to 10^{-4}M .

12. Effect of Steroids on Separated Bone Marrow Cells

Cultures of unseparated marrow incubated with 10^{-7}M

5 α -DHT exhibited markedly enhanced (300%) erythroid colony formation (Figs. 8 and 9).

The largest number of erythroid colonies was formed by Fractions 1-3 from bone marrow of normal mice separated by unit gravity sedimentation (Fig. 10). This indicates that these fractions, although lowest in cell count ($.8 \times 10^5$), contained a large number of erythroid precursors. Fig. 10 illustrates that more than 90% of nucleated cells were erythroid precursors. These cells had a sedimentation velocity of 5-7 mm/hr.

Separation of bone marrow from normal mice was repeated and 5 α -DHT added to the culture media in a concentration of 10^{-7} M with and without 0.2 U Ep. No erythroid growth occurred in those cultures lacking Ep. The greatest amount of erythroid growth occurred in Fractions 1-3 (Fig. 11).

13. Separation of Ep and/or Androgen Responsive Cell

Populations from Marrow of Tfm Mutant and Normal Littermate Mice

Incubation of 6×10^5 unseparated bone marrow cells with 10^{-7} 5 α -dihydrotestosterone (5 α -DHT) or etiocholanalone (ETIO) resulted in enhanced growth of erythroid colonies in normal mice (379 ± 38 and 248 ± 20 respectively) (Fig. 12). 5 α -Dihydrotestosterone also resulted in

enhanced growth of erythroid colonies from marrow of Tfm mice (Fig. 8).

Seven cultures, representing three successively pooled fractions from the Sta-put apparatus, cultured in the plasma clot with 10^{-7} M 5α -DHT with and without Ep showed erythroid growth only in Fractions 1-3 for normal mice and 13-16 for Tfm mutant mice (Fig. 13).

DISCUSSION

It is now firmly established that sex steroids exert significant effects on the erythron and blood-forming organs of endocrine-deficient and intact animals. The mechanism by which these hormones influence erythropoiesis is not known. The hormonal action on erythropoiesis was originally thought to be secondary to effects on general body metabolism which, in turn, determined the oxygen requirements of the animal, and, consequently, the need for red cells (Gordon, 1954; Crafts and Meinecke, 1959; Jacobson et al., 1957). This led Gordon to suggest that the role of androgens in erythropoiesis was facultative and that the fundamental action of the endocrine system, in general, was to support rather than to exercise a primary control over red cell formation (Gordon, 1957).

Mechanisms by which androgens influence the processes of erythropoiesis include: 1) a direct action on the responsive cells of the blood-forming organs, 2) a stimulation of the production of the Ep, 3) a potentiation of the effect of the Ep, and 4) an effect on oxygen delivery by red blood cells.

Recent evidence supports one or another of these

mechanisms. Thus, Gardner and Gorshein (1970) and Gordon et al. (1971) have demonstrated that certain 5 β -OH metabolites of testosterone stimulated erythropoiesis in the absence of a demonstrable increase in Ep formation, suggesting a direct influence of these compounds on the erythroid precursor cell compartment. This concept received additional support from the results of Byron (1970) who showed an increased proliferative activity of the hemopoietic stem cells by androgens. Singer and Adamson supported Byron's findings and described that increased erythroid proliferation induced by androgen occurred only in the presence of Ep (Singer and Adamson, 1976).

The effect of androgen agents on Ep production is well established (Fried and Gurney, 1965; Gordon et al., 1968; Naets and Wittek, 1966). Similar findings have been reported by Alexanian in humans (1969). The most convincing evidence was provided by Schooley (1966) who showed that the stimulatory effect of testosterone on erythropoiesis can be abolished by the administration of anti-Ep to mice. This, however, did not exclude the possibility of an effect of androgens on the cellular components of the system.

The mechanisms by which androgens stimulate Ep production are not known. There is little doubt, however,

that this effect is mediated through the kidneys (Mirand, 1965). Thus, bilateral nephrectomy completely abolished the increase in Ep formation in rats treated with testosterone (Mirand, 1965). Androgens may influence Ep formation by a direct action on renal cells responsible for the synthesis and/or release of the renal erythropoietic factor (REF). Gordon et al. (1968) demonstrated that the appearance of increased amounts of Ep in the sera of rats following treatment with androgen was accompanied by an augmented production of the REF. It was suggested that this increase in REF production and/or activity may be mediated through a mechanism similar to that involved in the effects of hypoxia on Ep formation. This suggestion was based, in part, on the observation that greater amounts of the REF were generated by the combination of androgen and hypoxia treatment than by either stimulus alone (Gordon et al., 1968).

Recent evidence has shed some light on the mechanism by which hypoxia affects Ep formation. Miller et al. (1974) demonstrated that soon after the administration of cobaltous chloride (CoCl_2) to normal rats, a dose-related respiratory alkalosis, associated with an increase in the affinity of hemoglobin for oxygen occurred. This was followed by an increase in the production of Ep. Similar findings were

reported when exposure to hypoxia was used as the stimulus (Miller et al., 1976). Moreover, bilateral nephrectomy altered the acid-base balance of the rat such that a profound acidosis occurred after the administration of CoCl_2 with an associated decrease in the affinity of hemoglobin for oxygen. Erythropoietin could not be detected in nephrectomized rats given CoCl_2 (Miller et al., 1974). These and other studies (Miller et al., 1974a) led these authors to suggest that the production of Ep in response to hypoxia-inducing stimuli is caused in significant measure by changes in the acid-base balance with its subsequent effect on the affinity of hemoglobin for oxygen (Miller et al., 1974). Androgens also cause an improvement in oxygen delivery to tissues by increasing the red blood cell 2,3-diphosphoglycerate level which results in a right shift of the Hb-O_2 dissociation curve (Gorshein et al., 1972).

However, the above mentioned studies did not take into consideration the possibility that the beneficial influence of androgens on erythropoiesis may in fact be mediated through a combination of these mechanisms. Our studies showed a combination of effects of androgens on red cell production and oxygen delivery by a number of separate but interdependent phenomena. In addition, the

effect of androgens on erythroid precursor cells may not require specific androgen receptors similar to those necessary for reproductive functions.

By simultaneously examining the effect of androgens on these different parameters we found that increased red cell production in response to androgen administration occurs only where it is accompanied by increased Ep formation. The normal rat has been shown to respond to both testosterone and hypoxia by increased production of Ep (Gordon et al., 1968). Normal rats showed a significant increase in Ep production, as measured by bioassay, following testosterone administration. This activity was totally abolished by prior treatment of the plasma with anti-Ep. That this inhibition was not caused by an excess antibody present in the plasma-anti-Ep mixture was ruled out by the fact that treatment of the mixture with GARGG did not alter the results.

The increase in Ep formation was accompanied by a significant enhancement of red blood cell production in normal rats. By contrast, no enhancement of red cell production was seen in transfusion-induced polycythemic animals following the administration of androgens. The primary reason for the absence of increased erythropoiesis was the lack of stimulation of Ep production in these animals.

The inability of testosterone to affect Ep-formation in transfusion-induced polycythemic animals was first documented by Gordon et al. (1966, 1968) and was attributed to the relative insensitivity of these animals to factors normally capable of inducing a significant increase in Ep formation (e.g., hypoxic-hypoxia, cobaltous chloride, androgens, etc.). These findings may also be indicative of a common mechanism for stimulation of Ep formation by these different stimuli. While the need for Ep in the formation of red blood cells is clearly apparent, the overall increase in the numbers of circulating red blood cells in androgen-treated animals is influenced not only by the effect of the steroid on Ep formation, but also by its effect on the population of precursor cells destined to form erythrocytes.

Additional evidence that testosterone and/or its metabolites may affect erythroid progenitor cells directly has been demonstrated in the present study. Rats and mice treated with androgens showed a significant increase in the numbers and/or sensitivity of the bone marrow erythroid-committed precursor elements. Thus, significantly greater number of in vitro erythroid colonies were formed by similar concentrations of bone marrow cells from steroid-treated animals than vehicle-treated controls in the

presence of Ep. This was also true of bone marrow cells obtained from transfusion-induced polycythemic rats following testosterone injections. Since no enhancement of Ep formation occurred in this latter group of rats, the increased formation of erythroid colonies probably resulted from a direct influence of the steroid on the precursor cell pool. No erythroid colonies were formed in the absence of exogenous Ep by bone marrow cells from either the steroid-treated or control animals, thus demonstrating once again the pivotal role of Ep in the formation of red blood cells. The similarity between in vivo and in vitro observations provided additional evidence that the in vitro plasma clot culture system is a useful method for the study of mammalian erythropoiesis; 2) addition of certain steroids (androsterone, 5 α -dihydrotestosterone, etiocholanolone) to cultures of bone marrow cells from untreated animals caused the appearance of significantly greater numbers of erythroid colonies in the presence of Ep. For example, addition of 10^{-7} M of 5 α -dihydrotestosterone to cultures of normal mouse bone marrow cells containing Ep caused approximately a 300% increase in the number of colonies formed. These compounds influenced erythroid colony formation by at least three mechanisms. Firstly, the steroids might have caused an

increase in the proliferation and differentiation of uncommitted, pluripotent stem cells into the erythroid-committed compartment. However, in vivo studies have revealed that this would require at least 72-96 hours (Reissmann, 1950). Numerous studies in our laboratory had shown that, in the plasma clot culture system, the formation of an erythroid colony from the committed stem cell of mouse bone marrow requires at least 48 hours. Since the total culture period for my studies did not exceed 72 hours, it is unlikely that such a mechanism is involved in the increased formation of erythroid colonies. Although the studies by Singer and Adamson (1976) suggest that etiochalanolone may in fact act on a more primitive component of the committed stem cell pool, no evidence has been presented supporting the view that such an effect causes increased proliferation of these cells. The second possibility is that these agents act on a population of cells already committed to the erythroid series. Such cells may exist in a form not as yet susceptible to the actions of Ep, and that the steroid may somehow "speed-up" the maturational steps required for the acquisition of their Ep-responsiveness. Finally, it is possible that the influence of steroid compounds

may be mediated through an effect on the Ep-responsive precursors by rendering these cells more sensitive to Ep. Although any of the three mechanisms may explain the erythropoietic effects of these steroids in vivo, based on the in vitro, observations, it is more likely that the latter two possibilities, either alone or in combination, may be involved. The possibility that steroids may interact with Ep and either prolong the life of the molecule or render it more active was not investigated. In this regard, Lewis et al. (1975) have suggested that the core component of Ep may be a steroid. However, analysis of "pure" Ep did not indicate the presence of steroid-like compounds (Goldwasser, personal communication). In addition, frequent attempts to demonstrate a direct effect of steroids on Ep have been unsuccessful (Gordon and Zanjani, unpublished observations).

Recently Singer and Adamson (1976) have demonstrated that androgenic steroids, and their non-androgenic metabolites, enhance the number of erythroid colonies grown from rat bone marrow in response to Ep. Moreover, their findings suggested that the target cells for the two classes of steroids were different: cells influenced by the androgenic steroid resembled cells responding to Ep, as determined by velocity sedimentation gradient

separation, whereas, cells responding to non-androgenic metabolites had significantly different sedimentation characteristics. I was unable to detect separate populations of target cells in mouse bone marrow for 5 α -dihydro-testosterone (androgenic) and etiocholanolone (non-androgenic). Only one cell fraction was found to respond to both of these steroids and Ep. These studies, however, did not include the Ep dose-response determinations reported by Singer and Adamson (1976) and were performed in mice. Nevertheless, under the experimental conditions described here, I have confirmed that androgen treatment results in an increased red cell 2,3-DPG and improves oxygen delivery. The administration of testosterone propionate to male and female rats was followed by increase of erythrocyte 2,3-DPG. Molinari et al. (1973) found that, when rat reticulocyte-rich erythrocytes were incubated with heat-deproteinized plasma of testosterone propionate-treated rats, erythrocyte glycolysis was stimulated leading to an increase in erythrocyte 2,3-DPG concentration (Molinari et al., 1973). The question arises as to whether the variation of erythrocyte glycolysis is primarily androgen dependent or erythropoietin dependent. The present studies provide evidence that the change in

red cell 2,3-DPG levels cannot be attributed to Ep. Thus a significant increase in red cell 2,3-DPG following testosterone administration occurred not only in normovolumic rats but also in rats rendered polycythemic by hypertransfusion with red cells. In the latter group of animals the increase in 2,3-DPG was not accompanied by detectable rise in Ep formation, as maintenance of a high circulating red cell mass in these rats effectively inhibited Ep formation. Moreover, recent studies have shown that no detectable increase in red cell 2,3-DPG occurred in mice and rats receiving injections of exogenous Ep.

The rise in red cell 2,3-DPG was accompanied by a shift to the right in the Hb-oxygen dissociation curve as evidenced by the elevated Hb-P₅₀ values. In general, an increase in Hb-P₅₀ is indicative of a more efficient release of oxygen by the red cells at the tissue level.

The effect of androgens on erythropoiesis is mediated through a number of independent mechanisms which combine to improve the rate of red blood cell production. The critical step in this process appears to be the influence on Ep formation. It is apparent that in situations where existing levels of Ep may not be sufficient for an adequate rate of erythropoiesis, androgen therapy, by stimulating the production of the hormone, can cause

a significant improvement in red cell production. However, frequently, circulating levels of Ep may be significantly above normal levels and yet the amount may be inadequate to provide for normal levels of erythropoiesis. Such a situation exists in the genetically anemic ww^V mice, which have elevated levels of circulating Ep (McCulloch et al., 1965). Although a stem cell defect exists in these mice (McCulloch et al., 1965), Keighly et al. (1968) have shown that increased production of Ep induced by hypoxia can result in increased formation of erythrocytes. Androgens have also been shown to improve erythropoiesis in ww^V mice. My studies suggest that the ameliorative influence of steroids in these mice, at least in part, may be mediated through an increase in Ep production. However, for Ep to be effective, an adequate supply of responsive precursor cells must also be present. We have shown that a significant increase in the size of the erythroid-committed stem cell pool occurs following androgen therapy. It is, therefore, suggested that androgens represent a more effective mode of therapy in anemias than treatments which either improve Ep formation or expand the stem cell pool. The latter affect of androgens may be the primary mechanism in cases of anemias in man, in whom the committed stem cell compartment is deficient (Shahidi, 1970, 1977).

The increased number and/or efficiency of committed-stem cells resulting from androgen administration appears to occur independently of an influence on Ep formation. This was evident from the results obtained in transfusion-induced polycythemic rats, from the in vitro effects of steroids on erythroid colony formation reported here and by Singer and Adamson (1976). However, for these cells to proliferate and differentiate into erythrocytes Ep must also be present. Another action of these steroids, which occurs independently of Ep and erythropoiesis, is the influence exerted on the oxygen delivery system in the host animal and man. The data suggest that the increase in red cell 2,3-DPG following testosterone administration occurs rapidly and well before an increase in Ep formation and erythropoiesis can be documented. Thus the immediate beneficial effect of androgen treatment may be improved oxygen delivery to tissues followed by enhanced erythropoiesis.

My studies indicate that androgens most probably effect Ep formation by a different mechanism than that suggested by Miller et al. Chronic administration of testosterone propionate, as well as several metabolites of testosterone, resulted in a marked increase in red cell 2,3-DPG which was accompanied by a simultaneous

improvement in oxygen delivery. This occurred not only in normal rats but in hyper-transfused animals as well, thereby indicating that the effect of androgens on Ep production and oxygen delivery were independent of one another. Improved oxygen delivery is mediated via a direct effect on the Leubering-Rappaport pathway resulting in an increase in 2,3-DPG, while the increase in erythropoietin production is the result of a direct renal effect as evidenced by an increase in kidney weight in androgen treated animals whose Ep levels rose demonstrably. Although androgens may affect Ep production in the kidney in a manner similar to hypoxia, it appears more likely that a hormone receptor mechanism is involved.

Androgen treatment of the Tfm mouse (a mutant strain known to be lacking the testosterone receptor protein) with testosterone failed to elicit increased Ep production or renal hypertrophy. While the presence of a receptor in the kidney is necessary for androgenic steroid to cause increased Ep production, no such receptor appears to be required in bone marrow cells, since testosterone and 5 α -DHT (an androgenic steroid) in the presence of 0.2u Ep caused a significant increase in erythroid colony formation in vitro. The biological action of androgens on target organs, such as the prostate, is mediated

through their binding to a cytoplasmic receptor protein and the complex in turn is transferred to the nucleus (Tomkins, 1970). Whether such a mechanism is involved in the stimulation of erythropoiesis by androgens remains unknown.

5 α -DHT in the presence of 0.2u Ep exerted a significant effect on erythroid colony formation in both Tfm and normal littermate mice. However, Tfm mice exhibited no increase in ⁵⁹Fe incorporation upon administration of the androgens.

This indicates a direct cellular effect of the androgens on the hemopoietic stem cells, and this effect either does not require the presence of a specific androgen receptor protein in erythroid cells or the receptors are under different genetic control. Injection of a single dose of 5 α -DHT to Tfm mice increased also the absolute number of committed granulocytes and macrophage precursors and triggered the pluri-potential stem cell from G₀ to S phase (Shahidi, 1975). This may also be a direct cellular effect. The inability of the 5 α -DHT to stimulate erythroid colony formation in the absence of Ep is in accord with the essential role of this hormone in cytodifferentiation.

Physical separation of cells is a useful tool in

elucidating mechanisms whereby various chemicals effect a given cell population. Specific populations of bone marrow cells have been successfully separated on the basis of their velocity sedimentation profiles. Such isolated populations include CFU-C and CFU-S (Worton et al., 1974), cells causing Graft-vs-Host reaction (Phillips and Miller, 1970), Ep sensitive cells (Clissold, 1974), BFU-E and CFU-E (Axelrod, 1974), and subpopulations of erythroid precursors sensitive to steroid hormones of differing configurations (Singer et al., 1976).

A prerequisite of separation of cells by physical means is that the isolated cells retain their functions. This has been ascertained by measuring responses of mixed fractions.

Iscoe et al., 1972, using velocity sedimentation, was able to confirm that the sedimentation velocity of cells forming colonies in culture is more rapid than the majority of other bone marrow cells. Furthermore, this was found to be true for both human and mouse marrow, indicating that murine marrow provides a good experimental model for hematologic research.

Evidence is available that in the mouse CFU-C are not identical with the pluripotent stem cells detected

by the spleen colony counting technique (McCulloch and Till, 1971). A direct assay of human pluripotent stem cells would be of great clinical value and physical separation by velocity sedimentation may provide the tool for this. These cells sediment at 3-4 mm/hr. Velocity sedimentation has the advantage of providing a large number of cells in each separated fraction, which retain their function and morphology. Sedimentation techniques will prove an invaluable tool in separating not only cells of differing lines but also cells committed to the same differentiation and thus enable us to, e.g. do hybridization experiments of DNA and RNA to determine exactly what mRNA is formed in response to Ep, steroids, and a variety of drugs. Separation techniques can also be used to detect pathologic changes in various subpopulations of bone marrow cells.

The results presented here indicate that there is a distinct subpopulation of bone marrow precursors which is particularly susceptible to the action of Ep and the anabolic steroid 5α -DHT. This is evidenced by the fact that cells in Fractions 1-3, i.e., yielding the rapidly sedimenting (5-7 mm/hr) cells known to yield erythroid growth in culture, 5α -DHT markedly increased the number of erythroid colonies in the presence of 0.2u Step III

Ep as compared to those cultures of Fractions 1-3 cultured with 0.2u Ep alone.

Furthermore, separated marrow from the Tfm mice responded to 5 α -DHT stimulation by exhibiting increased erythroid colony formation but a different, more slowly sedimenting cell population appeared to be involved (Fractions 13-16 showed erythroid growth). Moreover, erythroid growth was absent on day two in Tfm marrow cultures, indicating that perhaps in the Tfm mutant a more primitive cell type is stimulated to differentiate along erythroid lines. Nevertheless, these results are compatible with the contention that the effect of androgens on hemopoietic tissue occurs independently of a receptor. The lack of a significant iron uptake by erythrocytes of Tfm mice as compared to control suggests inadequate stimulation of erythropoietin production by testosterone and 5 α -DHT in Tfm mice and indicates that this aspect of androgen induced erythropoiesis is dependent on the presence of androgen receptors, most probably in the kidney.

Table 1. Erythropoietin (Ep)-neutralizing activity of anti-Ep before and after treatment with goat anti-rabbit gamma globulin (GARGG).

Material Assayed ¹	% RBC- ⁵⁹ Fe Incorporation = SEM ²
Saline	0.56 $\pm$ 0.15
1.0 IU Ep ³	24.62 $\pm$ 1.97
1.0 IU Ep + 0.025 ml normal rabbit stem	23.98 $\pm$ 1.32
1.0 IU Ep + 0.05 ml normal rabbit stem	25.14 $\pm$ 2.08
1.0 IU Ep + 0.025 ml anti-Ep	16.19 $\pm$ 1.32
1.0 IU Ep + 0.05 ml anti-Ep	0.47 $\pm$ 1.09
1.0 IU Ep + 0.025 ml GARGG	27.32 $\pm$ 3.18
1.0 IU Ep + (0.05 ml anti-Ep + 0.025 mg GARGG) ⁴	23.12 $\pm$ 1.25

1. All volumes were adjusted to 6 ml with saline.
2. Each value represents mean $\pm$ 1 standard error of results from 5 mice.
3. Step III, sheep plasma Ep (Connaught Laboratories, Toronto, Canada).
4. Anti-Ep and GARGG were incubated together for 1 hour before addition of Ep.

Table 2. Erythropoietic stimulatory activity of serum from testosterone treated rats previously inactivated with anti-Ep and GARGG as measured by ^{59}Fe uptake in assay mice.

Table 2. Neutralization of the erythropoiesis-stimulatory activity of serum from androgen-treated normal rats.¹

Materials Assayed ²	% RBC- ⁵⁹ Fe Incorporation = SEM ³
Saline	0.69 $\pm$ 0.13
0.4 IU Ep ⁴	14.31 $\pm$ 1.42
0.4 IU Ep + anti-Ep	0.42 $\pm$ 0.07
0.4 IU Ep + (anti-Ep + GARGG) ⁵	15.19 $\pm$ 2.00
Vehicle-treated rat serum	1.08 $\pm$ 0.29
Vehicle-treated rat serum + (anti-Ep + GARGG)	0.98 $\pm$ 0.13
TP-treated rat serum	11.06 $\pm$ 1.27
TP-treated rat serum + anti-Ep	0.79 $\pm$ 0.28
TP-treated rat serum + (anti-Ep + GARGG)	0.85 $\pm$ 0.34

1. See text for explanation.
2. All samples were brought to similar volumes with saline and were incubated at 37°C prior to assay.
3. Each value represents mean $\pm$ 1 standard error of the mean of results from 5 mice.
4. Step III sheep plasma Ep.
5. Steps in parenthesis were carried out prior to addition of the remaining component.

Table 3. Effect of administration of testosterone on erythropoiesis as measured by percent ^{59}Fe incorporation directly in the test rat.

Table 3. Effect of chronic administration of testosterone propionate (TP)
on erythropoiesis in normal and transfusion-induced polycythemic rats.

Treatment ¹	% RBC- ⁵⁹ Fe Incorporation = SEM ²	
	Normal Rats	Polycythemic Rats
Saline	26.4 $\pm$ 2.1	4.2 $\pm$ 0.80
Vehicle	25.3 $\pm$ 1.40	4.8 $\pm$ 0.76
TP	38.9 $\pm$ 1.32	5.1 $\pm$ 1.21

1. See text for explanation.

2. Each value represents mean $\pm$ 1 standard error of results from 7 rats per group.

Table 4. Effect of testosterone treatment in vivo
on erythroid colony formation of bone
marrow from transfusion induced polycythemic
rats in vitro.

Table 4. Effect of chronic administration of testosterone propionate (TP) on erythroid colony formation by bone marrow cells from hypertransfused rats in vitro.

Number of Bone Marrow Cells Cultured	Erythroid Colonies (Mean $\pm$ SEM)			
	Vehicle Treated		TP Treated	
	No Ep	0.4 u Ep ²	No Ep	0.4 u Ep
2x10 ⁵	0	104 $\pm$ 18	0	194 $\pm$ 36
4x10 ⁵	0	197 $\pm$	0	--
6x10 ⁵	0	284 $\pm$ 29	0	444 $\pm$ 63
8x10 ⁵	0	474 $\pm$ 88		731 $\pm$ 49

1. Each value represents mean $\pm$ 1 standard deviation of the mean of three separate cultures derived from two rats.
2. Step III sheep plasma Ep.

Table 5. Effect of testosterone metabolites on
erythroid colony formation in vitro of
bone marrow from normal, untreated mice.

Table 5. The effect of steroids ($10^{-7}M$) on erythroid colony formation by bone marrow cells from normal mice in the presence or absence of erythropoietin in vitro.

Additions to Culture Number of Colonies/ 6×10^5 Bone Marrow Cells

Steroid	No Ep	+ Ep
None	0	172 $\pm$ 11
Androsterone (A)	0	402 $\pm$ 57
5 ~~α~~ -dihydrotestosterone (D)	0	347 $\pm$ 38
5 β -dihydrotestosterone (C)	0	190 $\pm$ 17
ETIO (B)	0	105 $\pm$ 20
TP	0	169 $\pm$ 14

Table 6. Effect of testosterone and testosterone metabolites on erythropoiesis and Ep production in normal mice as determined by red cell volume, bioassay for Ep, and kidney weight.

Table 6. The effect of testosterone and testosterone metabolites on plasma erythropoietin levels, red cell volume, and organ weight in normal rats.

	<u>Red Cell Volume</u> cc/kg $\pm$ SD	<u>Plasma</u> <u>Erythropoietin</u> u/ml	<u>Kidney</u> gm/100 gm.body wt.
Vehicle	24.8 $\pm$ 1.95	0	.66 $\pm$.04
TP	<u>29.82 $\pm$ 2.4</u>	<u>.3 $\pm$ 0.01</u>	<u>.79 $\pm$.05</u>
5 ~~0~~ -DHT	<u>30.9 $\pm$ 2</u>	<u>.3 $\pm$ 0.01</u>	<u>.82 $\pm$.03</u>
5 ~~0~~ -DHT	25.5 $\pm$ 9.56	0	.66 $\pm$.06
Androsterone	26.66 $\pm$ 3.96	0	.68 $\pm$.02
ETIO	24.9 $\pm$ 3.69	0	.66 $\pm$.06
No injection	23.6 $\pm$ 3.38	0	.70 $\pm$.07

Table 7. Effect of androgen treatment on erythropoiesis in normal Swiss-Webster mice, Tfm mice, and normal littermate mice as measured by percent ⁵⁹Fe incorporation directly in the test animals.

Table 7. In vivo iron uptake in normal Swiss Webster,
Tfm, and male littermates following
administration of Androgens

Type of Mice and Number	Compound Administered	Percent of ⁵⁹ Fe Uptake ($\pm$ S.E.M.)	Significance
Swiss Webster (7)	Vehicle	31 $\pm$ 1.90	
Swiss Webster (8)	Testosterone	40.67 $\pm$ 1.47	P < .001
Swiss Webster (9)	5 α -DHT	50.59 $\pm$ 1.9	P < .001
Littermate Controls (4)	Vehicle	26.76 $\pm$ 1.87	
Littermate Controls (4)	Tesosterone	46.36 $\pm$ 5	P < .001
Littermate Controls (4)	5 α -DHT	51.05 $\pm$ 2.11	P < .001
Tfm (3)	Vehicle	30.06 $\pm$ 1.61	
Tfm (3)	Testosterone	36.58 $\pm$ 0.89	NS
Tfm (3)	5 α -DHT	39.9 $\pm$ 3.96	NS

Fig. 1. Effect of administration of testosterone on erythropoietin production in normal rats as measured by percent ^{59}Fe uptake in assay mice.

EFFECT OF TESTOSTERONE PROPIONATE (TP) ON
PERCENT RBC ^{59}Fe UPTAKE

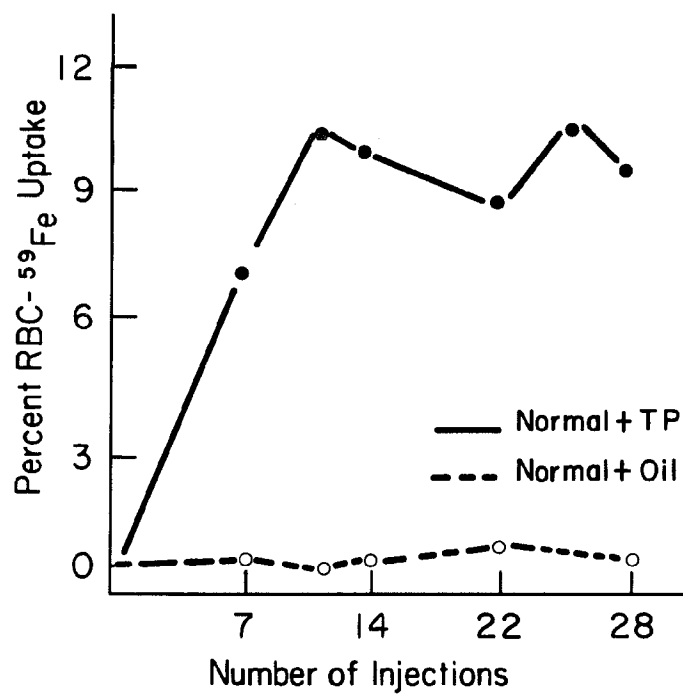


Fig. 1

Fig. 2. Hemoglobin and hematocrit values of normal female rats determined over a 28-day course of testosterone treatment.

EFFECT OF TREATMENT WITH TESTOSTERONE
ON CIRCULATING Hbg AND HCT IN FEMALE RATS

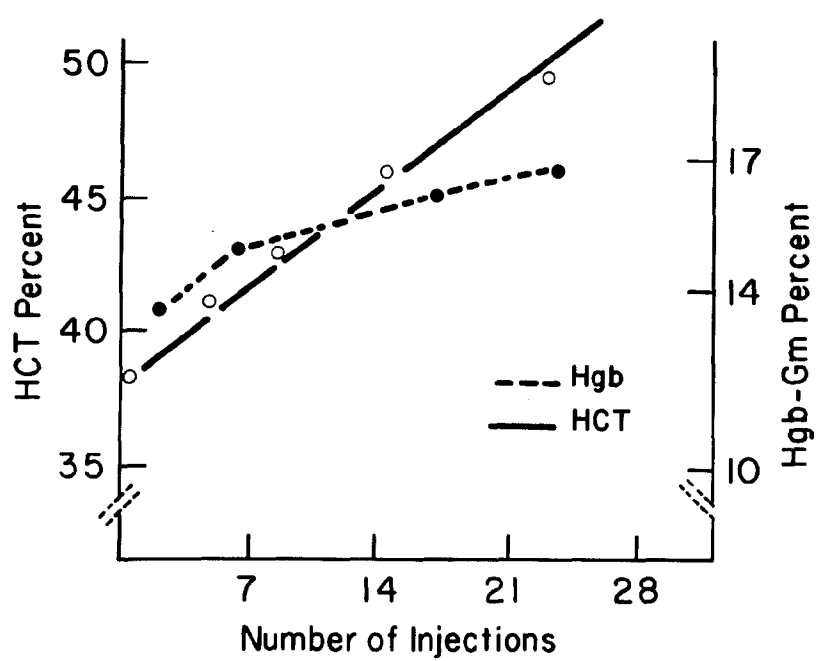


Fig. 2

Fig. 3. Effect of testosterone treatment on the oxygen delivery system of normal rats as measured by 2,3-DPG levels and P_{50} over a 28-day course of treatment.

RED CELL 2,3-DPG AND P_{50} (of Hbg)
IN RATS TREATED WITH
OIL OR TESTOSTERONE

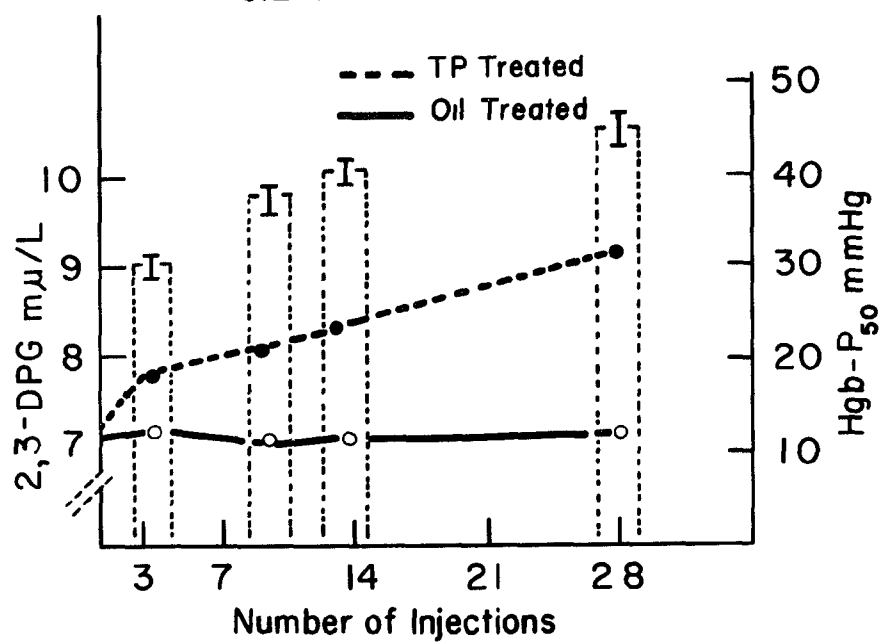


Fig. 3

Fig. 4. Effect of in vivo testosterone treatment of normal rats on erythroid colony formation in the plasma clot.

ERYTHROID COLONY FORMATION BY BONE MARROW CELLS FROM
OIL-TREATED AND TESTOSTERONE RAT BONE MARROW CELLS IN
RESPONSE TO E_p IN VITRO

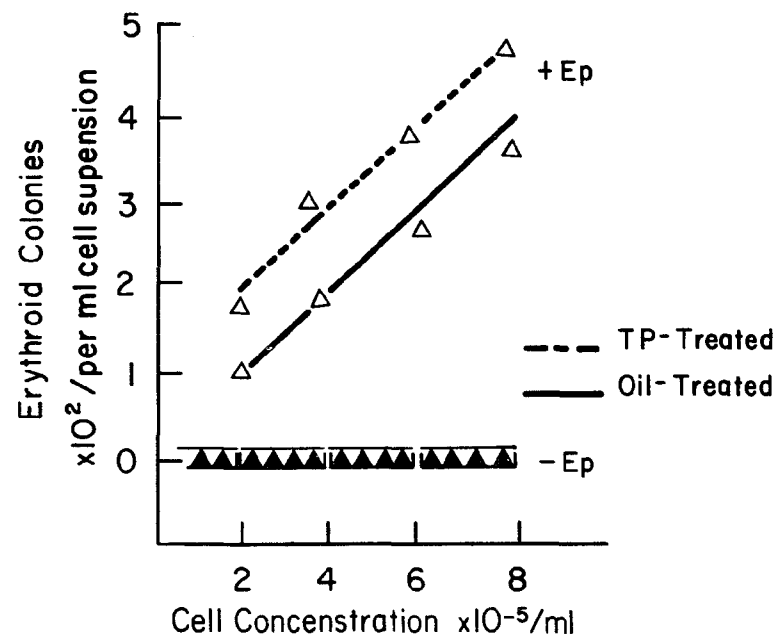


Fig. 4

Fig. 5. Effect of administration of testosterone on erythropoietic stimulatory activity of plasma from transfusion induced polycythemic rats as measured by percent ^{59}Fe uptake in assay mice.

Effect of TP on Ep Production in Transfusion
induced Polycythemic Rats

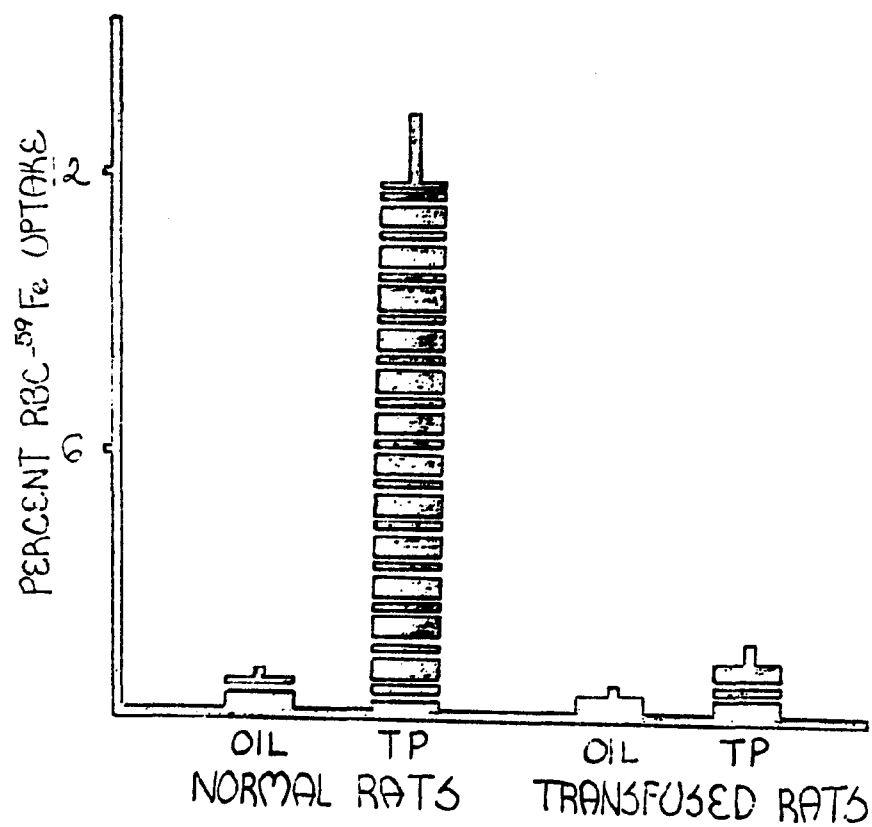


Fig. 5

Fig. 6. Effect of testosterone treatment on the oxygen delivery system of transfusion induced polycythemic rats as determined by 2,3-DPG values.

Effect of TP on RBC 2,3- DPG in Transfusion Induced
Polycythemic Rats

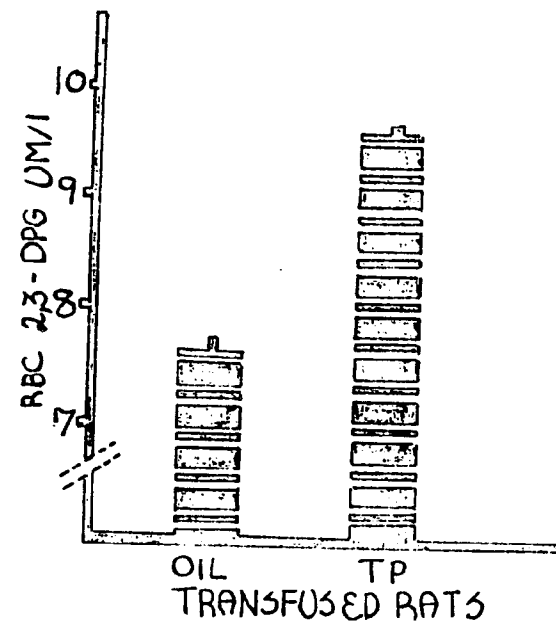


Fig. 6

Fig. 7. Effect of testosterone treatment on the oxygen delivery system of transfusion induced polycythemic rats as determined by P_{50} values.

Effect of TP on P_{50} in Transfusion
Induced Polycythemic Rats

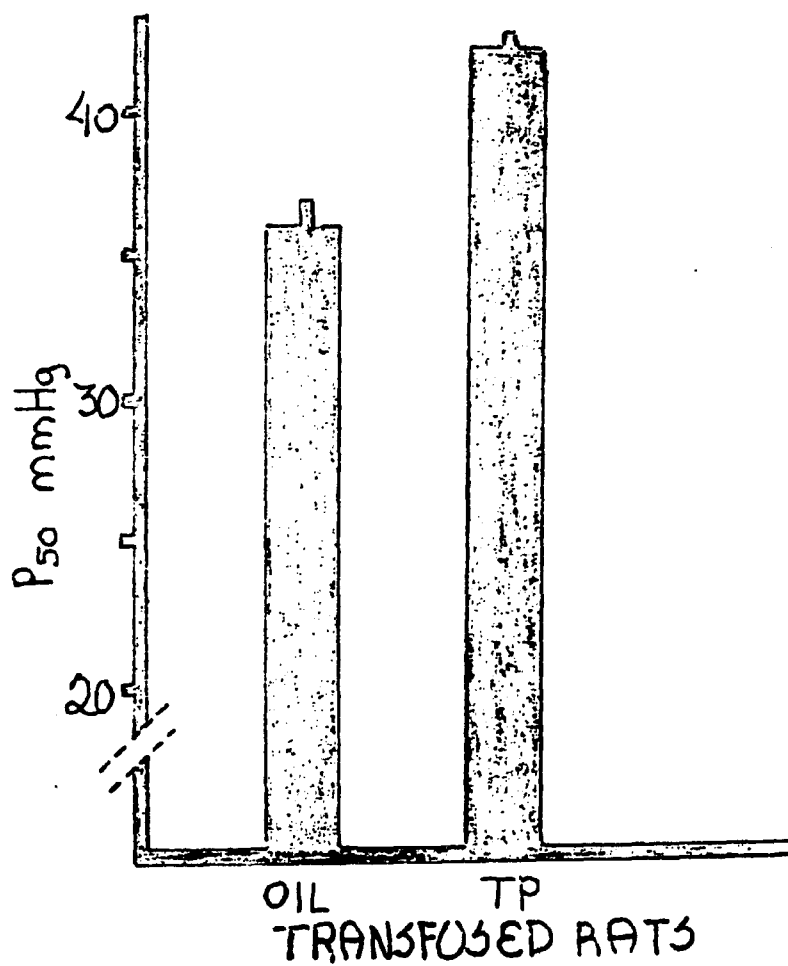
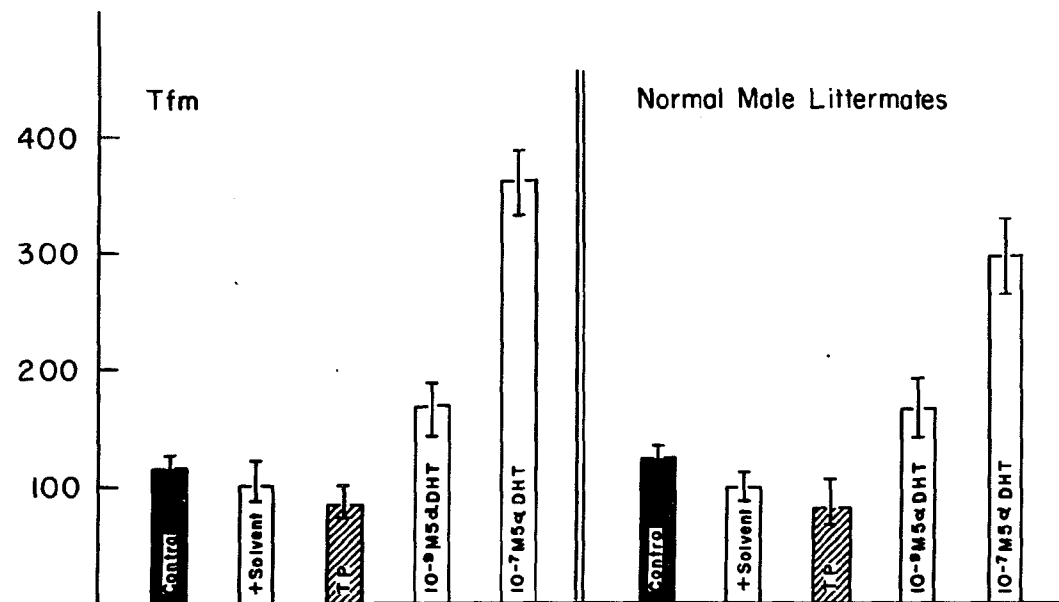


Fig. 7

Fig. 8. Effect of addition of 10^{-7} M testosterone or 10^{-7} M 5α -DHT to the plasma clot on erythroid colony formation of bone marrow from Tfm mice and their normal littermates.

EFFECT OF 5α DHT ON ERYTHROID COLONY FORMATION OF BONE MARROW FROM Tfm AND NORMAL MALE LITTERMATES



All incubations were made in the presence of .2 units of erythropoietin

Fig. 8

Fig. 9. Effect of addition of graded doses of testosterone metabolites to the plasma clot on erythroid colony formation of bone marrow from normal, untreated mice.

EFFECT OF STEROIDS ON ERYTHROID COLONY FORMATION BY
NORMAL MOUSE BONE MARROW CELLS IN VITRO

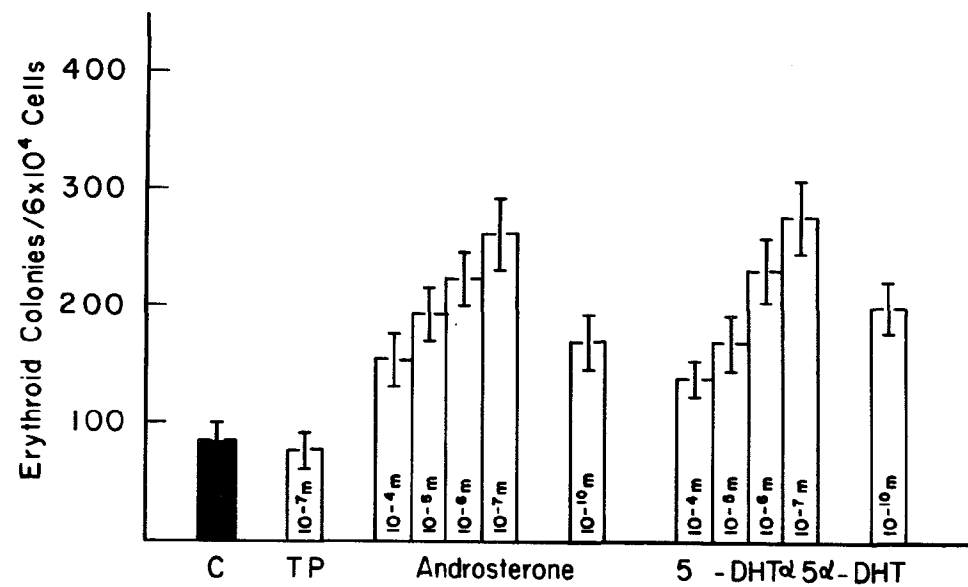


Fig.9

Fig. 10. Percentage of erythroid colonies per fraction of separated bone marrow determined by culturing each fraction from the Sta-put in the plasma clot.

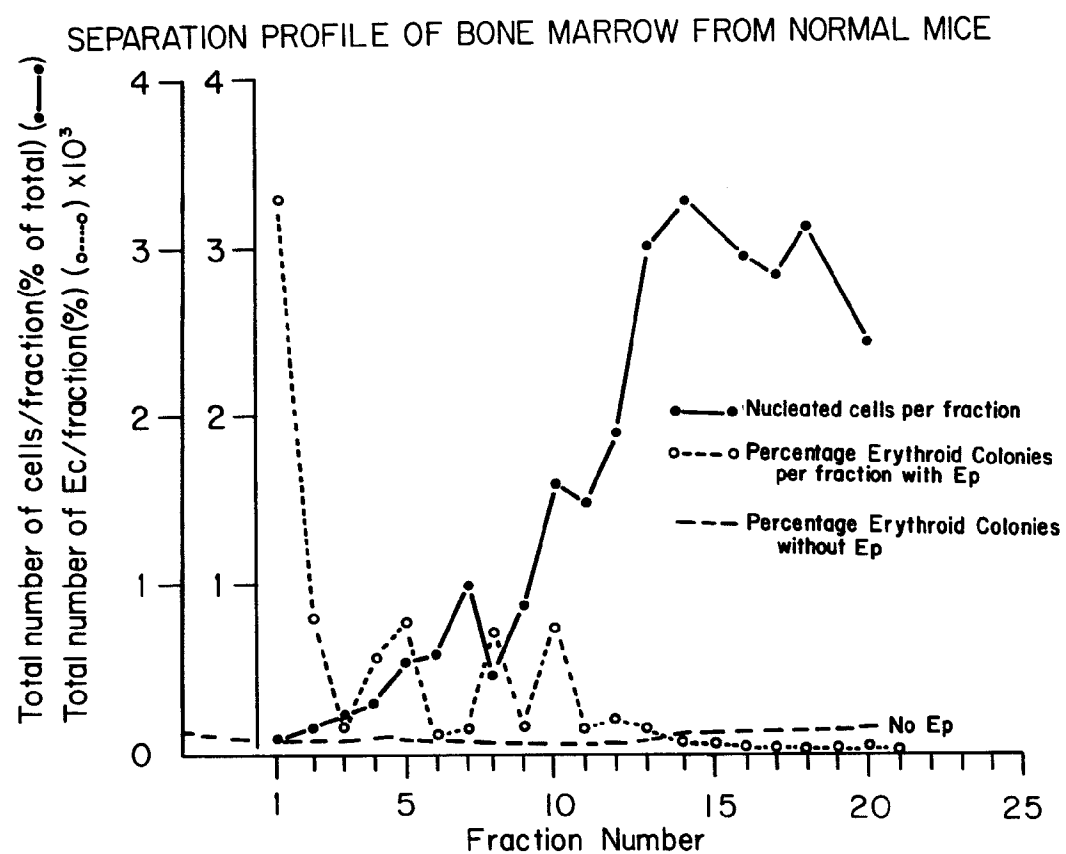


Fig. 10

Fig. 11. Effect of addition of 10^{-7} M 5α -DHT to cultures of fractions from normal mouse bone marrow.

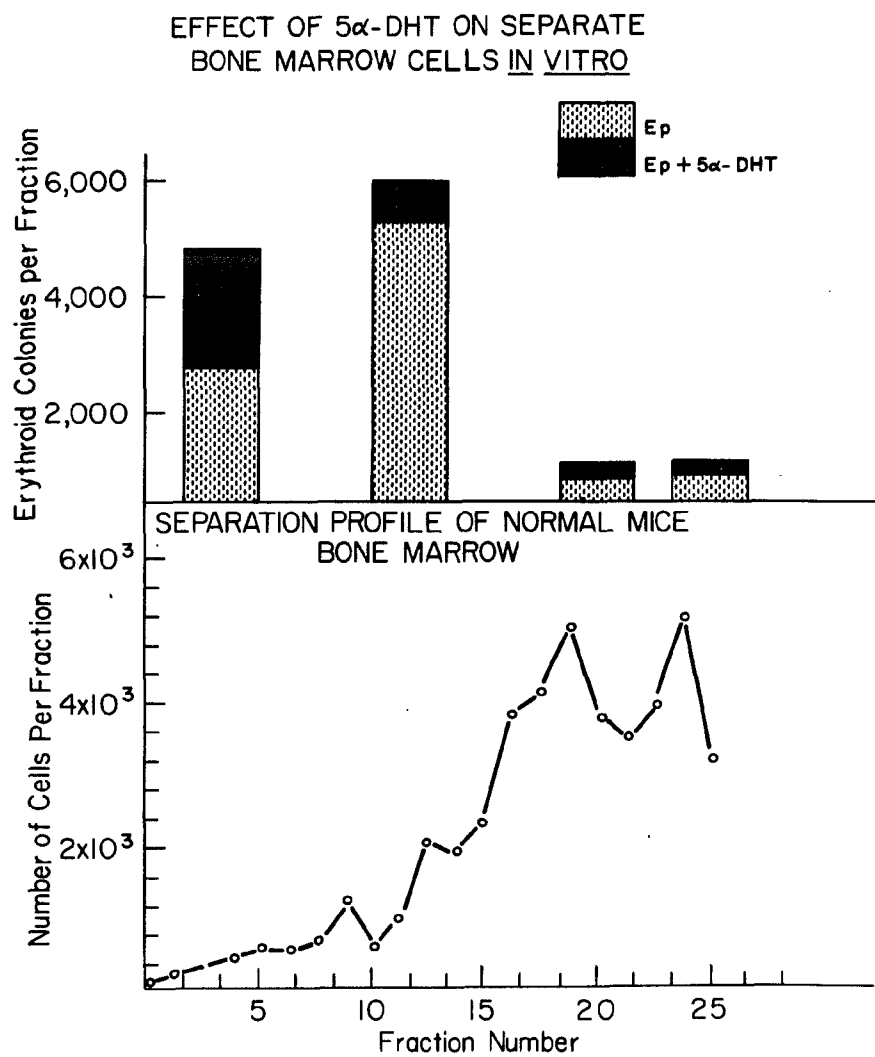


Fig. 11

Fig. 12. Effect of testosterone metabolites with different configurations on the colony forming ability of bone marrow from normal, untreated mice cultured in the plasma clot.

Effect of TP, ETIO, 5 α -DHT on Erythroid Colony
Formation of Bone Marrow from Normal Mice in vitro

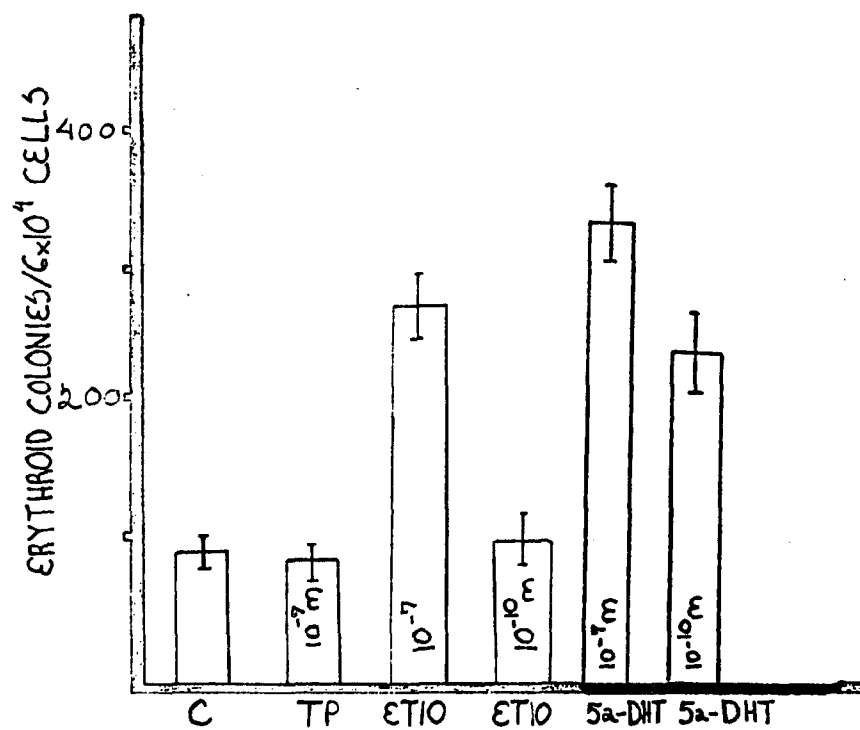


Fig. 12

Fig. 13. Comparison of velocity sedimentation patterns of erythroid precursors from normal and Tfm mice as determined by unit gravity sedimentation and culture in the plasma clot.

SEPARATION PROFILE OF BONE MARROW
FROM Tfm AND NORMAL LITTERMATE MICE

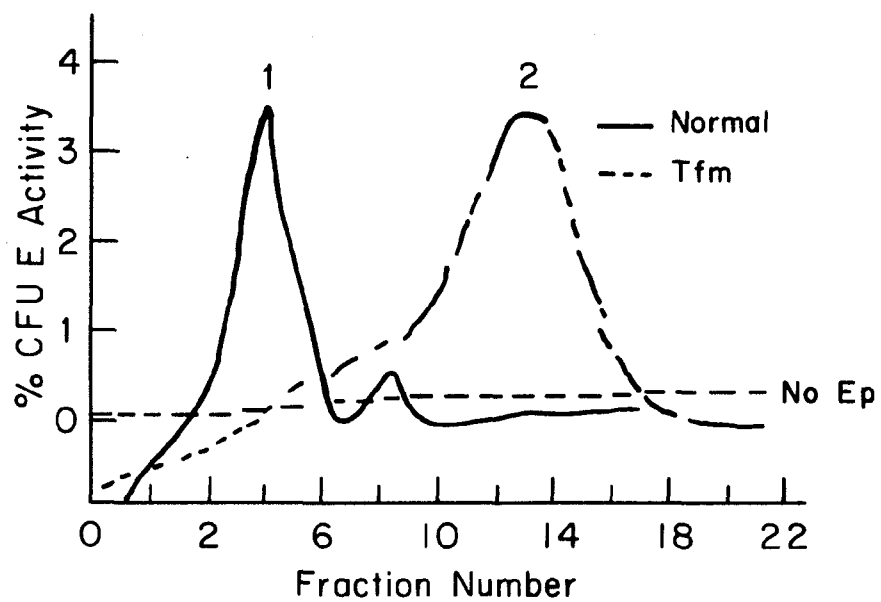


Fig. 13

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