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THE EFFECT OF ACTINOMYCIN D ON MURINE HEMATOPOIETIC COLONY FORMING UNITS

City University of New York PH.D. 1980

The Effect of Actinomycin D on Murine Hematopoietic Colony Forming Units

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

1980

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

January 14, 1980
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ABSTRACT

The Effect of Actinomycin D

on Murine Hematopoietic

Colony Forming Units

by

Joan Glick Bieler

adviser: Eugene S. Handler

Actinomycin D inhibits bone marrow erythropoiesis without greatly affecting granulopoiesis. This phenomenon has been attributed to the drug's specific inhibition of processes stimulated by erythropoietin. In this study, Actinomycin D was injected i.p. into CF_1 mice and the effects of the drug were measured *in vitro* using colony culture techniques. Plasma clots were used to assay erythroid colonies and methyl cellulose cultures were used to assay granulocytic colonies. 1-20 μg doses of ActD / 30 g mouse decreased CFU-E's by 50-80% 16 hours after injection, whereas, 10 μg of ActD had little effect on CFU-C. The dose-response curve 16 hours after ActD plateaued at approximately 20% survival for CFU-E's and approximately 50% for CFU-C's. High specific activity ^{3}H-thymidine suicide caused 82% suicide for CFU-E's and only 48% suicide for CFU-C's. Hypertransfusion, phenylhydrazine and endotoxin caused alterations in the numbers of colony forming units and their susceptibility to ActD. In general, increased cell cycle activity caused colony forming units to become more sensitive to the drug. A dose as low as 0.2 μg of ActD caused a decrease in CFU-E's 6 hours after injection. Recovery after this dose was rapid. It is concluded that ActD will have a greater effect on populations with greater cell cycle activity. A very low dose (0.2 μg) must be used to exclusively study the counter effect of the drug on erythropoietin.

Acknowledgement

I am indebted to Professor Eugene Handler and Dean Evelyn Handler, who have served as my sponsors, for their financial, intellectual and emotional support. I would also like to thank Professor Costante Ceccarini for making himself available for question and discussions. Betty Semel is gratefully acknowledged for technical assistance and contributions above and beyond the call of duty. The years I have spent on this project have been enriched by friendships with my fellow graduate students and post-doctoral students at Hunter College. I am grateful to Jack Bieler for his expert typing and David and Paul Glick for access to a computer for the editing and phototypesetting of this thesis.

Dedication

To my parents, who have instilled in me a thirst for knowledge . . .

To my husband, who loves to teach and teaches others to love learning . . .

To my children, in whom we hope to nurture the ability to ask and answer questions.

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INTRODUCTION

The processes of cell proliferation remain both enormously interesting and surprisingly mysterious. Although many thorough and far-reaching investigations have been undertaken to elucidate the mechanisms involved in these processes, countless questions have yet to be answered. Lesions in proliferation are frequently associated with neoplasms. It is of utmost importance to understand the normal development of cell structure and function in order to comprehend what has gone wrong in the abnormal cell. Bone marrow, which never ceases dividing and differentiating throughout adult life, can serve as a useful model system for study. Disorders and malfunctions of bone marrow can be investigated to determine what happens under abnormal conditions. The study of regulatory control processes in bone marrow should help clarify similar processes in other systems.

1. Hematopoiesis as a Model of Differentiation

A recent scheme of differentiation involved in hematopoiesis (Cline and Golde, 1979) shows, as the earliest ancestor of blood cells, the pluripotent stem cell. This progenitor is endowed with the ability to differentiate into committed lymphoid, erythroid or myeloid stem cells. Further, known and unknown factors will influence the development of these stem cells into their functional progeny. The pluripotent stem cell spends most of its time resting in G_0 and is not generally in replicative cell cycle (Lajtha, *et al.*, 1962). The development of a pluripotent stem cell into a committed stem cell is thought to be a stochastic process (Till *et al.*, 1964). In effect, whether each individual stem cell reproduces another stem cell or differentiates into a committed stem cell is a random choice. Regulatory mechanisms, such as the microenvironment (Wolf and Trentin, 1968) and hormones (VanZant and Goldwasser, 1977), may influence the population as a whole and affect the outcome of differentiation.

Red blood cells are the product of erythropoiesis. The major factor affecting the erythropoietic process is erythropoietin (epo). This hormone, elaborated by the kidney, controls the proliferation and further differentiation of the epo-responsive cell (ERC). Epo may act to shorten the cell cycle time of red cell precursors and,thus, allow for rapid amplification under

conditions of stress (Lajtha and Schofield, 1974). The effects of epo on differentiation processes are varied and the initial direct effect of the hormone is still debated. Early research (Paul and Hunter, 1969) showed that prior to epo-induced hemoglobin synthesis in fetal mouse liver cells, several other events are necessary. DNA synthesis is "imperative" prior to hemoglobin synthesis and this DNA synthesis seems to depend on RNA and protein synthesis. Rifkind *et al.* (1976) agree that DNA synthesis is a requirement prior to hemoglobin synthesis and explained that accompanying changes in chromatin structure may lead to transcription of globin m-RNA. Epo does stimulate RNA and protein synthesis prior to DNA synthesis, however, these are not directly involved in hemoglobin synthesis. Rifkind *et al.* (1976) find that cultures of fetal liver cells synthesize RNA as early as 30 minutes after epo stimulation. Goldwasser (1975) has isolated a transient species of 150S RNA from 5 to 10 minutes after exposure to epo and has proposed that this is the primary result of hormone action. He has evidence to suggest that the ERC must be in G_2 in order to respond to epo in this manner. Epo-treated bone marrow cells produce a cytoplasmic protein which stimulates uridine incorporation by isolated nuclei from bone marrow, kidney and lung cells (Chang and Goldwasser, 1974). Other responses to epo include increases in varieties of RNA synthesis, increases in globin synthesis, expression of specific membrane antigens, glucosamine uptake and stimulation of the iron uptake system. These differentiation changes take place at the level of the pro- and basophilic erythroblast (Harrison, 1976).

The effect of epo on the committed erythroid stem cell population is less clear. Early work in this area equated the erythroid stem cell to the erythropoietin responsive cell (ERC). The ERC is functionally defined as the cell in a polycythemic mouse that responds to an injection of epo and becomes an ^{59}Fe-labeled reticulocyte 48 hours later. Reissmann and Ito (1966 and 1968) used actinomycin D (ActD) to specifically block the effect of epo on ERC's. They assayed not only the proliferating ERC compartment but also the differentiating erythroblasts and maturing reticulocytes. They found that ActD did not block the effect of epo on the latter two types of cells. They did find that in the presence of ActD, epo was unable to stimulate ERC's. Recovery from ActD was comparatively rapid (36-48 hours). They concluded that

ActD was not inhibiting epo-mediated stem cell proliferation, but was blocking epo-mediated differentiation of ERC's to immature erythroblasts. The epo-stimulated differentiation of erythroid normoblasts was not blocked due to the pre-existence of stable m-RNA, the translation of which is not blocked by ActD (Davidson *et al.*, 1963). In later studies by Reissmann *et al.* (1972), it is suggested that epo affects both differentiation and proliferation. The process stimulated by the hormone will depend on the "age" of the stem cell, the "younger" ones being more likely to proliferate, the "older" ones being more likely to differentiate.

Another theory of the action of epo on the erythroid stem cell relates the fraction of the surviving ERC population to the times between epo stimulation and hydroxyurea (HU)-induced death (Stohlman, 1970). It was found that as the time increased between epo and HU, the fraction of the population surviving decreased. Since the effect of HU is S phase kill, it was proposed that S was becoming a greater fraction of the total cell cycle time. This was occurring, according to Stohlman's hypothesis, due to epo-mediated shortening of G_1 in the ERC population. The recent development of colony assays has allowed for further study of the problem of the effect of epo on the committed erythroid stem cell and will be discussed later.

Murine erythroleukemia cells (MELC) serve as a model of abortive differentiation. *In vitro* culture of spleen or liver from Friend virus-infected mice result in cell lines which are erythroblastic (Friend *et al.*, 1966). These cells are arrested at the proerythroblast stage and are erythropoietin independent (Liao and Axelrad, 1975). Upon exposure of MELC to dimethyl sulfoxide (DMSO), a fraction of the population is induced to mature and express typically erythroid characteristics (Friend *et al.*, 1974). Upon differentiation, these cells lose, to some extent, their ability to induce Friend disease (Friend *et al.*, 1974). Many other inducers have been found but all seem to have membrane-active properties in common (Preisler *et al.*, 1976).

The events following DMSO induction of MELC are of great interest. Friend infected cells are arrested at the proerythroblast stage and have not yet begun the processes involved in hemoglobin synthesis. Using thymidine-synchronized Friend cells, Levy *et al.* (1975) found that DMSO must be present during or slightly after S phase and for the preceding 24 to 30

hours, in order for induction to take place. Preincubation may be necessary to accumulate adequate intracellular DMSO concentrations or to allow for synthesis of a molecule needed for induction. DMSO must be present during at least one S phase in order to get hemoglobin synthesis. DNA of MELC treated from 20-30 hours with DMSO has increased alkali lability and altered affinity for intercalating dyes (Rifkind *et al.*, 1976). This implies an altered structure which may be necessary for differentiation. The number of synchronized cells synthesizing hemoglobin increases with longer exposure to DMSO (Levy, *et al.*, 1975). This could mean that either cells are being recruited into active cell cycle from G_0 or that a definitive quantal cell cycle must occur for the cell to be able to differentiate (Holtzer, *et al.*, 1972). This system may be useful in clarifying questions concerning commitment to erythropoiesis and, more specifically, to hemoglobin synthesis and the process of differentiation in general.

2. Regulation and Inhibition Erythropoiesis

The hematopoietic system has the uncommon property of producing end products that are short-lived, thus, necessitating their replacement. The various types of blood cells are under strict positive and negative control. Changes in demand for any particular type of blood cell are carefully monitored by various sensitive regulatory mechanisms. A lesion in a single aspect of the control system generates pathological conditions such as anemias, leukemias and polycythemias. These conditions may be experimentally induced to allow study of different hematopoietic compartments and the control mechanisms that link them.

The principal target of bacterial endotoxin appears to be the granulocyte. After an initial granulocytopenia, granulocytosis follows (Cline, 1975). Endotoxin also exerts an effect on erythropoiesis. The increased utilization of the marrow for granulopoiesis is related to the diminution of marrow erythropoiesis. When administered prior to or along with epo to polycythemic mice, endotoxin abolished or reduced Fe^{59} incorporation. The site for erythropoiesis shifts to the spleen (Fruhman, 1967). Polycythemic mice have increased numbers of splenic erythroid colony-forming units (CFU-E) after endotoxin; however, splenic Fe^{59} incorporation does not increase, due to the lack of epo in the circulation (Reissmann, *et al.*, 1976). The

number of marrow CFU-E decreases when endotoxin is injected prior to or along with epo, although preincubation of marrow with endotoxin or serum of endotoxin has no effect on CFU-E (Udupa and Reissmann, 1977). It can be concluded that endotoxin causes erythropoiesis to be shunted to the spleen from marrow in mice. This could be attributed to a migration of stem cells to the spleen.

Erythropoiesis may be directly suppressed by increasing the red cell mass. This is accomplished by hypertransfusion or by exposing the animals to hypoxia and then returning them to normal atmospheric pressure. Exhypoxic rats have lower levels of erythrogenin and erythropoietin (Kaplan, *et al.*, 1977). Increasing the red cell mass with divided doses of packed red blood cells also decreases plasma epo titers. As a result the numbers of CFU-E decrease (Iscove, 1977 and Hara and Ogawa, 1977). Under these conditions the number of CFU-E in cycle also decreases slightly. Hara and Ogawa (1977) found that the less differentiated erythroid burst forming unit (BFU-E) increased in the femur but decreased in the spleen. It is possible that erythropoietic suppression causes a back-up into the pluripotent stem cell compartment, causing that population to increase (Preisler and Henderson, 1972). Iscove (1977) observes a slight increase in granulocytic colony-forming units. The fact that polycythemia-induced reduction of plasma epo levels causes a concomitant decrease only in CFU-E demonstrates that it may be the only colony-forming cell with a physiologic dependence on the hormone.

Stimulation of erythropoiesis is accomplished experimentally by bleeding or with injections of phenylhydrazine. In mice, phenylhydrazine causes damage to red blood cells which results in hemolysis and decreased hematocrit. As a result, CFU-E numbers begin to increase in the spleen alone (Hara and Ogawa, 1976). Other hematopoietic factors associated with phenylhydrazine-induced anemia involve extramedullary erythropoiesis. Erythroid cells and CFU-S increase in liver and spleen (Ploemacher, *et al.*, 1977). Extramedullary CFU-S are also found to be more actively in cycle. Similar hematologic effects are observed after anemia due to bleeding (Hara and Ogawa, 1977 and Iscove, 1977). Thus, suppression or stimulation of erythropoiesis may have a dynamic effect on other hematologic processes.

Many agents have been found to inhibit or promote hematopoietic cells. Erythropoiesis is stimulated by hormones, such as androgens (Byron, 1972_a, Moriyama and Fisher, 1975), adrenergic agonists (Byron, 1972_a), dexamethasone, growth hormone, prostaglandin E_2 and thyroid hormone (Golde and Cline, 1978). Although androgens similarly increase granulocytic CFU-C, dexamethasone and prostaglandin E_2 depress CFU-C numbers. A wide range of effects in hematopoietic cells is elicited by cancer chemotherapeutic agents (Marsh, 1976). With these agents at hand it is possible to construct schemes of alternate stimulation and suppression to achieve an affect desirable for experimentation.

3. The Mechanism of Action and Effects of Actinomycin D

Actinomycin D (ActD) is an agent which is useful in elucidating various properties of hematopoietic cells. The structure of the antibiotic is seen in fig. 1. The triple ring structure allows for intercalation between base pairs, specifically, next to G-C pairs. This causes some distortion in the DNA which results in the inability of DNA dependent RNA polymerase to transcribe the macromolecule (Muller, 1968).

The physicochemistry of the ActD-DNA complex in solution has been understood for some time. ActD's action *in vivo* and on cells *in vitro* is complicated by many additional variables. *In vivo* tissue uptake is proportional to the dose of ActD and is limited by tissue blood flow rate. Binding is rapid and reversible. Permeability of the cells does not appear to play an important role (Lutz *et al.*, 1977). In mice, ActD is active in external cellular fluid for up to 16-24 hours (Vietti and Valeriote, 1971). After drug treatment, neoplastic ascites cells *in vivo* generated apoptic bodies containing pieces of cell organelles. In solid tumors, these bodies were soon phagocytosed (Searle *et al.*, 1975). A consideration in the use of ActD as a chemotherapeutic agent is the increased affinity (20 times as great as normal cells) of dead cells for the drug (Mendecki *et al.*, 1975). Necrotic areas of tumor are more likely to bind the drug, thus decreasing the chances of its affecting active tumor tissue.

It is important to understand the basis of ActD's differential effect in mixed cell populations. In some systems it has been found that variations in the effect is due to permiability. A

drug resistant HeLa cell line was found to incorporate no ^{3}H-ActD as seen in autoradiographs, whereas, the sensitive line had a high degree of label over the nuclei (Goldstein *et al.*, 1966). In both mouse L cells and Chinese hamster lung cells resistant to ActD, altered membrane glycoproteins and glycosidases are found. Thus, in these resistant cell lines membrane permiability is also implicated (Bossman and Bruce, 1971). Williams and MacPherson (1975) demonstrated that, although virus transformed BHK21 cells took up less ActD than non-transformed cells, isolated nuclei of both types of cells had similar uptake rates. Transformed cells incubated with cyclic-AMP had a twofold increase in ActD incorporation, although, the cyclic nucleotide did not affect the normal cells' ActD uptake.

Other lines of experimentation have shown that the extent of ActD binding is dependent on the activity of the genome. Differentiated cell lines are not so seriously affected by the drug due to the presence of stable RNA's and proteins needed for mandatory metabolic activities. In the presence of ActD, synthesis of induced proteins will not take place but these are generally optional to the survival of the cell (Davidson *et al.*, 1963). HeLa cells, which have a greater DNA and protein content than mouse L cells bind ActD to a greater extent than mouse L cells (Brachet and Hulin, 1970). Drosophila neuroblast and myoblast cultures have decreased sensitivity to the drug with increased culture time and differentiation (Donady *et al.*, 1975). Myeloblasts and proerythroblasts have similar grain counts after ^{3}H-ActD exposure but as blasts mature they bind less ActD as well as incorporate less uridine (Pileri *et al.*, 1974_{b}). Human myeloid leukemia cells, which are proliferatively more sluggish than their normal myeoblast counterparts, also bind less ActD (Pileri *et al.*, 1974_{a}). Cytoxic effects of the drug are seen in rapidly regenerating livers of hepatectomized rats but not in normal rats (Schwartz *et al.*, 1965). These lines of evidence suggest that cells in cell cycle will be more susceptible to the effects of ActD. Other studies suggest that the portion of the cell cycle which will be most sensitive is S phase (Djordjevic *et al.*, 1968). Synchronized HeLa cells have the greatest amount of ^{3}H-ActD bound during the G_{1}/S interface. Isolated nuclei from these cells show the same preference, thus eliminating permiability considerations (Pederson and Robbins, 1972). The authors of the aforementioned study hypothesize that increased binding of the drug is due to looseness of

DNA packing and loss of some protective chromatin proteins at the onset of S phase. Bruce *et al.* (1966) classified the effect of ActD on normal and leukemic CFU-S as cycle active as opposed to phase active. This misclassification could have arisen due to the fact that a large proportion of CFU-S are out of cycle and may be enlisted as active CFU-S are affected by the drug. Cells that spend a large fraction of their cell cycle time in S phase would appear to be affected by the drug throughout their cell cycle.

The fact that ActD is an RNA inhibitor which acts at the beginning of the DNA synthetic phase of the cell cycle poses problems as to which specific macromolecular synthesis is primarily inhibited. Using epo stimulation of hemoglobin synthesis, Paul and Hunter (1969) demonstrated that ActD inhibits RNA synthesis prior to inhibiting hemoglobin m-RNA synthesis. Very low concentrations of the drug (0.02-0.08 μg/ml) selectively inhibit nucleolar r-RNA synthesis in mouse L cells (Rickinson, 1970). This inhibition takes place in early G_1 and prevents the cells from entering S. Low concentrations of ActD administered to cells in mid to late G_1 do not influence the cells' progress through S. Using exogenous m-RNA, increases in rate of translation in the presence of ActD have been noted, possibly due to changes on secondary structure of ribosomes (Leinwand *et al.*, 1977). Other studies have shown decreases in rates of initiation, however, in enucleated PHA stimulated lymphocytes no change in initiation rate is found (Cooper and Braverman, 1977). Cooper and Braverman suggest that changes in protein synthetic rate are not due to structural alterations of molecules, but, perhaps, to specific interference of t-RNA synthesis.

These controversies are important in considering the effects of ActD on normal and leukemic hematopoietic cells. Early studies by Reissmann and Ito (1966 and 1968) claimed that ActD specifically blocked stem cell differentiation to the erythroid line. Their evidence demonstrated no appreciable stem cell death. At the time of these studies only the pluripotent stem cell was known. Daily injections of 60 μg/kg had no effect on granulocytic precursors. A single 300 μg/kg dose of ActD decreases ^{59}Fe uptake into reticulocytes 3 days later but actually increases the number of granulocytes available for mobilization after endotoxin on day 3 (Constable and Blackett, 1973). This selective effect of ActD on the erythroid population was used

by Hershko *et al.* (1969) to synchronize cohorts of red cell precursors *in vivo*. When used as a method of erythropoietic suppression, ActD injections do not have as pronounced an effect on increasing the CFU-S population as exhypoxic- or hypertransfusion-induced plethora (Preisler and Henderson, 1972). Leukemic CFU of AKR mice are more sensitive to the drug than are CFU-S of normal mice (Valeriote *et al.*, 1973). At very low concentration (0.5-5 ng/ml), ActD induces differentiation of MELC, possibly by increasing DNA lability (Terada *et al.*, 1978) or by inhibiting the production of RNA necessary to maintain dedifferentiation. Daily injections of 75-82 μg/kg ActD into mice decrease CFU-E numbers while no effect on CFU-C and increases of CFU-S are observed. BFU-E may decrease in the spleen but do not decline in the marrow (Zuckerman *et al.*, 1978). Recent studies enable one to see that the effects of the drug are manifold. One cannot say that stem cells are not influenced. Questions center on which stem cells are affected, to what extent and why? Recent research has generated many techniques to assay various hematopoietic stem cells at many levels of differentiation.

4. Hematopoietic Colony Assays

The pioneer colony forming assay came about as a result of measurements of radiation sensitivity (Till and McCulloch, 1961). Mice are supralethally irradiated and are then injected with donor marrow suspensions. Recipient mice may then survive due to the replenishment of hemapoietic cells by donor marrow which proliferates and matures. Injected cells form macroscopic colonies on the surface of the spleen. The number of colonies is proportional to the number of cells injected. Many colonies are comprised of both erythroid and granulocytic cells. The clonal nature of the spleen colony was established using anemic mice of the W/W^v genotype. These mice are themselves stem cell deficient. The donor marrow used had unique radiation-induced genetic markers. Nearly all mitoses in the cells of a single colony were labeled with a unique marker and cells of a single colony were both erythroid and granulocytic (Wu *et al.*, 1967). Along with other evidence the spleen colony forming unit (CFU-S) was established as a pluripotent colony forming cell. Only 10% of CFU-S are killed by high specific activity ^{3}H-thymidine, whereas, in sublethally irradiated mice, 50% of CFU-S involved in mar-

row regeneration are susceptible (Lajtha, 1969). Thus, most CFU-S are either in G_0 or have very long cell cycle times. In-cycle CFU-S have a cell cycle time of 12 hours (Vassort *et al.*, 1971). The spleen colony assay allowed for the study of hematopoietic microenvironment. It could be established that mice of Sl/Sld genotype had CFU-S but did not possess the proper environment for their growth and differentiation (Lajtha, 1975).

The CFU-S assay had the particular disadvantage of having to be carried out using the mouse as medium. No other species is as effective for CFU-S growth. The search for an *in vitro* assay system resulted in the CFU-C (CFU in culture). Initially, semi-solid agar was used as culture media with a feeder layer of cells below in solid agar (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). Methylcellulose was also found to be suitable (Ichikawa *et al.*, 1966). Embryonic or neonatal mouse kidney cells, spleen cells, mouse L cells or leukemic cells could serve as feeder layer cells. These cells provided a factor without which colonies would not form. This colony-stimulating factor (CSF) can also be provided by serum or medium previously conditioned by CSF-producing cells. Cells comprising the colonies are either granulocytic or macrophage. The CFU-C is different from the CFU-S. It is not pluripotent. It has a higher sedimentation velocity than the CFU-S (Morton *et al.*, 1969). It also has a higher thymidine suicide rate of about 40-50% (Lajtha *et al.*, 1969).

A technique for erythroid colony culture was developed by Stephenson (1971) and improved by McLeod *et al.* (1974). This assay requires the support medium of a plasma clot and is dependent upon the hormone erythropoietin. Iscove *et al.* (1974) developed a technique based on methyl cellulose medium, however, their procedure requires higher concentrations of more highly purified epo and the addition of a thiol compound (Iscove and Sieber, 1975). Lack of dependence of CFU-E on erythropoietin is a characteristic of pathological conditions such as polycythemia (Prchal and Axelrad, 1974) and tumor colony formation by MELC (Axelrad *et al.*, 1976). The clonal nature of the CFU-E was confirmed using reverse time-lapse cinematography (Cormack, 1976). Erythroid colonies are smaller (8-60 cells) than CFU-C and are identified by their hemoglobin content or benzidine positivity. They are at a more advanced stage of differentiation than CFU-C (Gregory *et al.*, 1973) and have a higher thymidine suicide

rate of about 75% (Hara and Ogawa, 1977).

Further developments led to an assay for another erythroid stem cell. This precursor requires more epo and a longer culture period and forms larger (> 50 cells) colonies or groups of colonies (McLeod *et al.*, 1974). It was named the erythroid burst forming unit (BFU-E) because of this characteristic morphology. The BFU-E can also be cultured in methyl cellulose (Iscove and Sieber, 1975). The clonal nature of the BFU-E is supported by studies using mixed male and female hematopoietic cells and then staining for centromeric heterochromatin (Strome *et al.*, 1978). Recently there has been still further proliferation of colony precursor assays. BFU-E's have been divided into early and late types which also vary in size. In the mouse, earlier and smaller BFU-E peak on day 3 or 4 and in humans on day 8 or between days 10 and 12. The later and larger BFU-E are assayed on day 8 in mouse and between days 11 and 18 or 17 and 24 in humans (Gregory and Henkelman, 1977; Ogawa *et al.*, 1977; Gregory and Eaves, 1977). A statistical analysis of the contents of cell types in individual murine CFU-S resulted in a "family tree" demonstrating close relationships between 1) CFU-S and CFU-C, 2) CFU-S and 8-day BFU-E, 3) 8-day BFU-E and 3-day BFU-E, 4) 3-day BFU-E and CFU-E and 5) CFU-E and nucleated cell counts (Gregory and Henkelman, 1977). Thymidine suicide rates and sedimentation velocities increased as the colony forming units' relationship to CFU-S grew more distant (Gregory and Eaves, 1978).

The latest advance in culture techniques has been the development of *in vitro* mixed colony assays. Mixed colonies would presumably arise from pluripotent stem cells. Johnson and Metcalf (1977) used fetal mouse liver cells cultured with pokeweed mitogen stimulated spleen conditioned medium and epo. Micromanipulation was used to clone single cells from 2-6 cell clusters after 12-24 hours of culture. Resultant colonies contained up to 5 types of cells (erythroid, macrophage, neutrophilic, eosinophilic, and megakaryocytic). Hara and Ogawa (1978) get similar results using mouse bone marrow cells and spleen conditioned media. They found that the progenitor of the CFU-mix had a very slow sedimentation velocity (3.4 mm/hr) and almost no thymidine suicide rate. A factor from nonadherent cells which potentiates the effect of epo on the formation of erythroid bursts and colonies is thought to be a pluripotent

stem cell stimulator. It is derived from leucocyte conditioned medium (LCM) and increases epo-independent erythroid colony formation of cells from patients with polycythemia vera. It is also capable of supporting CFU-C growth (Aye, 1977). It is thought to work by stimulating CFU-S to differentiate.

5. Rationale

Early research by Reissmann and Ito (1966 and 1968) concluded that ActD blocked pluripotent stem cell differentiation into the erythroid line and that the drug did not cause significant stem cell death. They hypothesized that ActD was directly counteracting the effect of epo on stem cells and assumed that this was the reason why the granulocytic population was not affected. Later studies demonstrated that physiologic levels of epo do not act directly on the early stem cell population, rather on the more mature CFU-E (McLeod *et al.*, 1974). Thus, another explanation for the specific effect of ActD on the erythroid population was needed.

The development of *in vitro* assays for erythroid and granulocytic stem cells made it possible to quantitate directly the *in vivo* influence of ActD. At the time that this study was undertaken, the BFU-E assay had not yet been developed. However, existing colony assays would enable one to detect a differential effect of ActD on erythroid and granulocytic stem cells. Using these assays one could learn if these colony forming units were the drug's target. The major aim of this thesis was to discover if there is a differential effect of ActD on erythroid versus granulocytic colony forming units. A secondary aim was to clarify the reason for the differential effect. Drug sensitivity of various cell populations is an important factor in the treatment of neoplasms. The selection and scheduling of available chemotherapeutic agents must be done with the knowledge of the kinetics of these drugs. This thesis is an investigation of some aspects of the kinetics of ActD's effect on the murine hematopoietic stem cell population.

MATERIALS AND METHODS

1. Animals

All animals used were male CF_1 mice purchased, between the ages of 6 and 8 weeks, from Charles River Laboratories (Wilmington, Mass.). The mice were sacrificed when they were between 2 and 3 months of age. They were maintained on a diet of Purina Lab Chow and tap water *ad libitum*. All injections were administered intraperitoneally (ip). The CF_1 male mice used, generally weighed between 30 and 35 g. Substances to be injected were dissolved so that the appropriate dose for a 30 g animal was contained in 0.5 ml. Volumes injected into animals weighing more or less than 30 g were calculated accordingly.

2. Cell Suspensions

Cell suspensions were prepared from femoral bone marrow under aseptic conditions. The femur was removed intact and transferred to a 35 mm petri dish containing a small amount of supplemented Hanks Minimal Essential Media (HMEM, see below). Three femora, (one femur from each of three mice) were generally pooled for each experimental point. The femur heads were removed and the marrow remaining was completely flushed through with 1 ml of supplemented HMEM containing 2% heat-inactivated fetal calf serum into a sterile test tube using a 26 gauge needle and a 3 ml syringe. Clumps of cells were dispersed by pipetting up and down with a 5 ml sterile plastic disposable pipette (Falcon; BBL, Md.). Appropriate volumes of cells were then transferred to sterile tubes for later use in cell culture and a small aliquot was removed for determination of the number of nucleated cells using a hemocytometer. Cell suspensions were then centrifuged for 10 min at 1000 rpm in the cold and resuspended in sufficient fresh medium containing 2% FCS to bring the cells to the appropriate concentration for cell culture.

3. Cell Culture

3.1 Erythroid Colonies

Culture Ingredients: Erythroid colony and burst-forming units (CFU-E and BFU-E, respectively) were grown in plasma clot cultures according to a modification of the method of McLeod *et al.* (1974 and personal communication). Supplemented HMEM was prepared as follows: 10 ml MEM Hanks Base (10×), 1 ml non-essential amino acid solution (100×), 1 ml L-glutamine (200 mM), 1.25 ml 5% $NaHCO_3$ solution and sterile, distilled, deionized water up to a volume of 100 ml. Two ml of penicillin-streptomycin solution (5000 u/ml and 5000 mcg/ml respectively) were added to 100 ml of medium. This medium was used for cell suspensions used in erythroid cultures. The clot culture medium for both CFU-E and BFU-E was the same except for the erythropoietin concentration and the way in which citrated plasma was introduced into the culture medium.

Clot culture medium contained 1) one part by volume of beef embryo extract, 2) two parts heat-inactivated FCS, 3) one part 10% bovine serum albumin, 4) one part L-asparagine (0.2 mg/ml), 5) one part erythropoietin, 6) two parts NCTC-109 (Microbiological Associates, Bethesda, Md.), 7) one part cell suspension (5×10^6 cells/ml) and 8) one part citrated bovine plasma. To 100 ml of NCTC-109 2 ml of penicillin-streptomycin solution was always added. Lyophilized beef embryo extract was restored to its original volume with 10 ml supplemented HMEM. Small aliquots were stored at -20°C. A 1:6 dilution of the stock suspension was made with NCTC-109 for use in clot culture. FCS was heat-inactivated at 60°C for 30 min. Bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St. Louis, Mo. or Calbiochem, La Jolla, Ca.) was prepared according to a modified technique of Worton *et al.* (1969). It was dissolved in sterile, distilled, deionized water and detoxified with AG-501 × 8 (D) resin, a mixed bed resin (Bio-Rad Laboratories, Richmond, Ca.). It was brought to a concentration of 10% with Dulbecco phosphate buffered saline (PBS). To each 100 ml of 10% BSA, 2.5 ml of 7% $NaHCO_3$ and 0.5 ml of 0.4 g phenol red per 100 ml PBS was added. The resultant 10% BSA solution was sterilized by passage through a 0.2 μ Nalgene filter (Nalge, Rochester, N.Y.), stored at -20°C and used directly in the clot culture medium. L-asparagine was stored in a stock concentration of 2 mg/ml of supplemented HMEM at -20°C. It was sterilized by passage

through a 0.45 μ Millipore filter (Millipore Ltd., Bedford, Mass.). The stock solution was diluted 1:10 with NCTC-109 for use in the clot culture medium. Erythropoietin Step III (Connaught Medical Laboratories, Willowdale, Ontario, Canada) was derived from anemic sheep plasma. It varied in purity from 4-17 u/mg protein. For CFU-E cultures, the stock concentration was 10 u/ml supplemented HMEM. This was diluted 1:4 with NCTC-109 to a final concentration of 2.5 u/ml for use in CFU-E clots. Erythropoietin for BFU-E assays was stored and used at a concentration of 30 u/ml. Stock erythropoietin solutions were stored at -20°C. Citrated bovine plasma was stored in small aliquots at -20°C. Where β-mercaptoethanol was used, cultures contained a final concentration of 10^{-4} M. Except where specified, culture ingredients were purchased form Grand Island Biological Company (GIBCO), Grand Island, N.Y., 14072. Clot culture medium was kept on ice until plated.

CFU-E Incubation: Plasma clots assayed for CFU-E's were incubated in microtiter wells obtained from Cooke Engineering Co. (Alexandria, Va.). A block of 6 wells and a small 35 mm uncovered petri dish containing water for humidity were placed in a large petri dish. One part plasma was pipetted into a test tube containing the rest of the culture ingredients and the contents were gently mixed. Immediately, 0.1 ml of clot culture was delivered into each of the 6 microtiter wells. The culture medium took approximately 5 min to clot. Each clot thus contained 0.025 u of erythropoietin and 5×10^4 cells. The clot cultures were incubated in a water-jacketed incubator (National Appliance Co., Portland Or. and Forma, Oh.) at 37°C in an atmosphere of 5% CO_2 and high humidity for 2 days.

BFU-E Incubation: BFU-E plasma clots were plated in larger (2 ml, 15 mm) wells purchased from Linbro (Hamden, Ct.). Four wells and one small petri dish containing water were placed in the larger petri dish. To each well, 0.05 ml of plasma was pre-plated and spread around the well to prevent the clot from sticking upon removal. 0.45 ml of clot culture medium was then pipetted into each of the 4 wells. Prior to clotting, the contents of the wells were swirled gently to allow for mixing. Each BFU-E clot contained 2.5×10^5 cells and 1.5 u of erythropoietin. It was not necessary to feed BFU-E using this concentration of erythropoietin. Cultures were incubated at 37°C and 5% CO_2 in high humidity for 7 or 8 days.

Harvest and Fixation: At the end of incubation, clots were rimmed and removed from the wells and placed on slides. A piece of filter paper was placed over the clots to remove excess liquid. When the filter paper was saturated, clots were allowed to drain further by slanting the slides at approximately an 80° angle. Remaining moisture was blotted using a second layer of filter paper. Leaving the first piece of filter paper in place, several drops of 5% glutaraldehyde (Fisher Scientific Co., Fairlawn, N.J.) in 0.01M phosphate buffer (pH 7.0-7.2) were added and allowed to stand for 5-6 min. The filter paper was then carefully removed and the excess fixative was allowed to run off the slide. Slides were then placed in distilled water for 8 min, after which they were dried with cool forced air.

Staining: Mounted dried clots were stained in a 1% solution of benzidine (Eastman, Rochester, N.Y.) in methanol, followed by 2.5% peroxide (Fisher Scientific Co., Fairlawn, N.J.) in 70% ethanol. They were then stained in Harris-Modified Hematoxylin stain (Fisher Scientific Co., Fairlawn, N.J.) solution and blued with 1% ammonia water. After the slides were dried with cool forced air, cover slips were affixed with Permount and allowed to dry.

Scoring of Colonies: All clots were scored using 125× magnification. When necessary, a grid (with 1 mm square boxes) drawn on a piece of cellulose acetate was used to facilitate accurate colony counts. A CFU-E was considered to be any group of 8 or more benzidine positive nucleated cells. BFU-E's were scored as any group of 50 or more associated nucleated cells, some of which were benzidine positive. Except where indicated, colony numbers refer to the mean number of colonies per clot.

3.2 Granulocytic Colonies

Culture Ingredients: Granulocytic colony-forming units (CFU-C) were cultured in methyl cellulose using a modification of the technique of Worton *et al.* (1969). Methyl cellulose was prepared by adding 4 g of the powder (Fisher Scientific Co., Fairlawn, N.J.) to 125 ml of hot autoclaved distilled water. The mixture was stirred and allowed to cool at which time 125 ml of McCoys 5a medium (2×; GIBCO, Grand Island, N.Y.) was added under sterile conditions. This mixture was allowed to stir at 4°C for 48 hr. It was then frozen and thawed and stored at

4°C. McCoys-methyl cellulose medium was used directly in culture and was made with 50 ml of dissolved methyl cellulose and 50 ml of supplemented McCoys 5a medium which contained 10 ml of fetal calf serum, 1.0 ml 7.5% $NaHCO_3$, 1.0 ml sodium pyruvate solution (100×), 0.4 ml MEM vitamin solution (100×), 0.8 ml MEM amino acids without glutamine (50×), 0.4 ml non-essential amino acid solution (100×), 0.4 ml L-glutamine (200mM), 0.04 ml L-serine (100×, 20 mM), 0.16 ml asparagine (10 mg/ml), and enough sterile distilled water to bring the volume to 50 ml. (All ingredients were purchased from GIBCO, Grand Island, N.Y. except asparagine.) Each 35 mm plastic petri dish (Falcon, Md.) contained 3 ml of McCoys-Methyl cellulose, 0.2 ml of an appropriate dissolution of colony stimulating activity (CSA, see below) and 0.1 ml of cell suspension (1×10^6 cells/ml) in McCoys 5a and 2% FCS. Four replicate plates were incubated for each assay point.

CSA was derived from the pooled serum of mice that had received 20 μg *S. typhosa* lipopolysaccharide W (Difco Laboratories, Detroit, Mi.) 3 hr prior to bleeding by cardiac puncture. The serum was sterilized by passage through a 44 μ Millipore filter and stored in small aliquots at -20°C. Before use in the culture system, various dilutions of CSA were assayed and an appropriate one selected for experimental use.

CFU-C Incubation: Granulocytic colony cultures were incubated for 7 days in an atmosphere of 7.5% CO_2 and high humidity at a temperature of 37°C.

In later experiments 2 ml of penicillin-streptomycin solution was added to each 100 ml of methyl-cellulose-McCoys medium. In this manner contamination was almost completely eliminated with no effect upon colony growth.

Scoring of Colonies: At the end of the incubation period, CFU-C were scored on an inverted microscope (Zeiss, N.Y.C.) at a magnification of 50×. A CFU-C was defined as any group of 50 or more granulocytic/macrophage cells. Except where indicated, colony numbers refer to the mean number of colonies per plate.

4. Tritiated Thymidine "Suicide"

"Suicide" experiments were carried out with high specific activity (60.36 Ci/m mole) tritiated thymidine (^{3}H-thymidine; New England Nuclear, Boston, Mass.). The concentration of thymidine was 4 μg/ml and the concentration of radioactivity was 1 mCi/ml. Pooled femoral marrow was washed and resuspended in supplemented HMEM with 2% FCS to a concentration of 5×10^6 nucleated cells/ml. Two ml of cell suspension were incubated with or without ^{3}H-thymidine for a period of 20 min in an atmosphere of 5% CO_2 at 37°C in a humidified incubator. It was determined that 50 μCi of ^{3}H-thymidine would be incubated with each 2 ml aliquot of cells (see Fig. 12). After incubation, cells were washed 3 times with 10 ml of ice cold NCTC-109 supplemented with 10% FCS and 100 μg/ml cold (non-radioactive) thymidine. Cells were then diluted to the appropriate concentration for use in the various culture systems.

5. Induction of Hemolytic Anemia

Mice were made anemic with 2 ip injections of phenylhydrazine hydrochloride (ph; Fisher Scientific Co., Fairlawn, N.J.). Phenylhydrazine was diluted in sterile saline to a concentration of 3.6 mg/ml. A phenylhydrazine treated mouse received 1.8 mg/ 30 g body weight in each injection. Injections were administered 48 and 24 hr prior to actinomycin D treatment. In Initial experiments hematocrits were monitored prior to phenylhydrazine injection and on days 1, 2, 3, 5 and 7 after the first injection. Hematocrits were gauged using tail blood and heparinized microhematocrit tubes (Clay Adams, Parsippany, N.J.). Anemia was defined as at least a 25% reduction in hematocrit.

6. Endotoxin-Induced Granulopoiesis

Twenty-four hours prior to actinomycin D treatment, mice received ip injections of 20 μg of endotoxin (Lipopolysaccharide W from *S. typhosa* 0901). The endotoxin was dissolved in sterile, non-pyrogenic saline to a concentration of 100 μg/ml.

7. Induced Polycythemia

Plethora was induced by hypertransfusion. Blood was obtained from donor mice by cardiac puncture using a 3 cc syringe and a 26 gauge half inch needle, rinsed in a solution of heparin. Heparin (Fisher Scientific Co., Fairlawn, N.J.) was dissolved in sterile saline to a concentration of 400 u/ 0.5 ml. Sixteen ml plastic test tubes in which blood was collected and pooled contained 0.5 ml of the heparin solution. The blood obtained was then centrifuged in the cold at 1000 rpm for 3-10 min after which the plasma was carefully removed. Red blood cells were subsequently washed 3 times in Dulbecco phosphate buffered saline. Recipient mice received 2 ip injections containing 0.9-1.0 ml of the resultant packed red blood cells, 30 and 6 hr prior to actinomycin D treatment. Tail blood hematocrits were taken prior to each transfusion and prior to actinomycin treatment. Plethora was defined as at least a 50% increase in hematocrit above controls.

8. Actinomycin D Treatment

Actinomycin D (ActD- A grade) was obtained in 200 μg amber injection vials from Calbiochem (LA Jolla, Ca.). It was brought to the appropriate concentration with sterile saline. After dissolving ActD, vials were covered with aluminum foil to protect them from light. It was stored at 4°C for up to 3 wk. Injections of ActD were administered ip.

9. Antiserum to Erythropoietin

Dr. Robert D. Lange (Univ. of Tenn., Knoxville) kindly sent rabbit antiserum to erythropoietin which, according to his laboratory, was capable of neutralizing 25 u of hormone / 1 ml of antiserum. Normal rabbit serum was obtained from the same source and used as a control. Antiserum was diluted and preincubated for 15 min at 37°C with those culture ingredients which could possibly contain small amounts of erythropoietin (beef embryo extract, FCS, bovine serum albumin and citrated bovine plasma). Controls were similarly treated with normal rabbit serum.

10. Sterile Conditions

All surgical equipment was dry heat sterilized at 220°C for 3 hr. Removal of femurs and manipulations of cells prior to centrifugation were conducted under a sterile hood reserved for animal procedures. All other sterile work was conducted under a separate vertical laminar air flow hood (Baker Co., Me.). Glassware was autoclaved at 125-135°C and 15-20 lbs pressure for 1 hr.

RESULTS

1. Some Characteristics of the Colony Culture Systems in Our Laboratory

1.1 Response to Erythropoietin

CFU-E : It was necessary to ascertain whether bone marrow cells from CF_1 male mice responded to erythropoietin in a manner similar to that reported for C_3H/Bi mice (McLeod *et al.*, 1974). Concentrations of erythropoietin ranging from 0.1-0.4 u/ml were used in CFU-E plasma clot cultures. Results indicate that the numbers of CFU-E plateau between 0.25-0.4 u/ml of erythropoietin (Fig. 2). 0.25 u/ml of erythropoietin was used throughout the course of experimentation. This corresponds to the dose used by McLeod *et al.*

BFU-E: In initial experiments it was determined that BFU-E's developed better if the entire amount of erythropoietin was added at the beginning of the culture period rather than feeding over the course of the week-long incubation. Concentrations of 1-5 u/ml of erythropoietin were tested and the numbers of BFU-E were assayed. In Fig. 3 it can be seen that the numbers of BFU-E peak at 3 u/ml of erythropoietin. The response to erythropoietin is linear for the lower doses. Higher doses of the hormone cause a slight decrease in bursts.

1.2 The Effect of β-Mercaptoethanol

Iscove and Sieber (1975) used β-mercaptoethanol (β-mercaptoethanol) in methyl cellulose cultures to increase the numbers of CFU-E and bursts. The effect of this thiol compound was studied in the plasma clot culture system. Results show that 10^{-4}M β-m increased the numbers of CFU-E in erythropoietin-containing-cultures (Table 1). A considerable increase was also seen in cultures not containing erythropoietin. It was also observed that the numbers of colonies with either β-mercaptoethanol alone or erythropoietin alone were nearly additive when compared to the number of colonies which appeared in the presence of both agents.

In order to determine the mechanism of action of β-mercaptoethanol, antiserum to erythropoietin was obtained. In one experiment in which a 1:6 dilution of antiserum was used, it was shown that no CFU-E appeared when erythropoietin was completely neutralized (Table 2).

Normal rabbit serum did not negate the effect of erythropoietin or β-mercaptoethanol. (β-mercaptoethanol was not used for the remainder of the experiments because it was seen to have no specific action of its own.)

1.3 Colony Stimulating Activity (CSA) and the Numbers of CFU-C

Various batches of sera-containing CSA had quantitatively different effects *in vitro*. Fig. 4 shows the resultant numbers of CFU-C for 3 different batches of CSA-containing sera. Although the strength of CSA varied, it always caused a linear increase in the colony numbers. Dilutions of CSA were chosen to give between 40 and 70 colonies so as to improve accuracy and facilitate colony counts.

2. Time Course of Actinomycin D Response

2.1 Effects on the CFU-E Population

1-20 μg: A dose of ActD was sought that would allow for complete recovery of colony numbers within 48 hr. Fig. 5 shows the time course of events following the injection of different doses of ActD. As little as 1 μg/30 g mouse caused a significant decrease in CFU-E number within 6 hr after injection. By 16 hr post-injection, colony numbers reached a nadir for all doses between 1-20 μg. These doses of ActD did not allow for complete recovery by the 48th hr after injection. The pattern of colony response is similar for doses of 10 and 20 μg. It can be seen that saline injection had no significant effect on the numbers of CFU-E.

The Effect of 0.2 μg ActD: When the dose of ActD was decreased to 0.2 μg/30 g mouse, little effect was expected. However, as shown in Fig. 5, there is a significant decrease ($p<0.001$) in the number of CFU-E 6 hr following injection. Unlike the pattern for higher doses, by 16 hr, CFU-E's have recovered to their normal levels after the 0.2 μg dose. Although a slight decrease in colony number is seen at later times, by 48 hr there is no significant difference between colony numbers of injected and uninjected mice ($p>0.8$).

These data were confirmed in another experiment where CFU-E's were assayed at shorter intervals after the time of injection of 0.2 μg of ActD (Fig. 6). In this study, colony decrease

was greatest 8-12 hr after ActD. Recovery to normal levels of CFU-E was reached between 16-24 hr. Again as before, colony number returned to normal by 48 hr, after a significant decrease between 28 and 40 hr. If the dose of ActD was decreased further to 0.1 μg/30 g mouse, no significant decrease could be measured, except at 4 hr ($p<0.025$, Table 3).

In order to ascertain whether or not 0.2 μg of ActD results in the cumulative depletion of the CFU-E compartment, this dose was administered 4 times to a group of mice every 12 hr. The data reported in Table 4 demonstrate that the effects of this regimen do not differ markedly from the effects of a single injection.

2.2 Effect on the BFU-E Population

The erythroid burst population was assayed at various times after administration of 0.2 μg ActD / 30 g mouse. The pattern of recovery of BFU-E's is somewhat similar to that of CFU-E's (Fig. 7). The initial decline has its nadir 6 hr after injection. Complete recovery, however, is not reached until the 24th hr. Although by 48 hr, recovery is not significantly different than control values ($p>0.4$), there is a major decline in the numbers of BFU-E 30 hr post-injection ($p<0.01$).

2.3 Effect on the CFU-C Population

When the time course of events after 0.2 μg ActD / 30 g mouse was followed for CFU-C's, no significant decrease could be detected (Fig. 8). Furthermore, significant ($p<0.05$) increases in CFU-C were observed at 2 and 16 hr. When the dose was raised to 10 μg, a small decrease in CFU-C number was observed 6 hr after injection. Recovery was achieved by the 16th hr. This pattern of response is somewhat similar to that of CFU-E's after 0.2 μg ActD. A slight increase over control values was evident at 48 hr ($p<0.02$).

3. Dose-Response Curves

3.1 CFU-E's

Figure 9 shows the relationship between the dose of ActD administered and the response of CFU-E's 16 hr following injection. The graph shows a slight increase in colony numbers after

0.2 μg, the dose at which recovery is achieved by 16 hr. The number of surviving CFU-E's at higher doses decreases dramatically. It was seen above (Fig. 5) that the fraction of surviving CFU-E's is similar at 10 and 20 μg ActD. Thus, it seems that the percent of the CFU-E population which can withstand the effects of high doses of ActD is between 10 and 20%.

3.2 BFU-E's

The numbers of erythroid bursts 16 hr after various doses of ActD were also determined (Fig. 10). As with CFU-E's, there is a slight increase due to 0.2 μg ActD. At higher doses the numbers of surviving BFU-E's sharply decreases. The fraction of BFU-E's surviving 20.0 μg ActD, the largest dose used, is less than 10%.

3.3 CFU-C's

The dose-response curve for CFU-C's was compiled from assays done 16 hr after doses of up to 80 μg ActD / 30 g mouse (Fig. 11). Doses higher than this (100 or 120 μg) caused death in most animals ($L.D._{50}$= 1200 μg/kg or 36 μg/ 30 g in mice). Unlike the erythroid population, CFU-C's did not show a sharp decline following increasing doses of ActD. A slight decrease in CFU-C number was observed after 20 μg ActD, however, increasing the dose to 80 μg did not have a further dramatic effect. In fact, even at 80 μg, approximately 50% of control CFU-C's survived ActD treatment.

4. ^{3}H-Thymidine Suicide Experiments

4.1 Optimum Radioactivity Needed for Suicide

It was necessary to ascertain how many μCi of ^{3}H-thymidine were needed to achieve the maximal suicide effect. From 0-100 μCi of thymidine were added to 2 ml of suspension containing 5×10^6 cells/ml. In this study CFU-E's only were assayed. Fig. 12 shows that the percent suicide rate plateaus at 40 μCi. This experiment shows that approximately 85% of the CFU-E population is susceptible to the action of ^{3}H-thymidine. In subsequent studies of the various colony populations, 50 μCi of ^{3}H-thymidine were incubated with the cell suspensions.

4.2 Effect of ^{3}H-Thymidine on Colonies

The response of the various hematopoietic precursors to ^{3}H-thymidine is shown in Table 5. The same samples of pooled marrow were used for each of the three assays. CFU-C's seem to have the greatest "resistance" to ^{3}H-thymidine with only 48.4% suicide. The erythroid colonies are much more susceptible; 81.5% of CFU-E's and 97% of BFU-E's are killed by the ^{3}H-thymidine.

When the percent survival of hematopoietic precursors after treatment with ^{3}H-thymidine is compared to the percent survival 16 hr after 20 μg ActD (Fig. 13) it can be seen that these two parameters are similar. The slope of the line for the 3 colony types is nearly 1 ($m=1.14$) and the correlation is high ($r=0.971$). Thus, there is reason to believe that the mode of action or target of the two agents is similar.

5. Alteration of Cycling Hematopoietic Precursor Populations by External Means

5.1 Effects of Agents Used

The rationale behind these experiments was to perturb the various colony-forming populations and see if these disturbances influenced the ActD response.

Phenylhydrazine is known to produce hemolytic anemia. Two ip injections, 24 hr apart caused the hematocrit to decrease (Table 6). The day after the first injection, the hematocrit dropped 23%. The second injection caused a further decrease in hematocrit. At the time the mice were sacrificed, 16 hr after they had received ActD, their hematocrits were nearly 30% below controls. When the colony-forming ability of phenylhydrazine treated animals that had not received ActD was compared to uninjected controls, it was shown that the former produced 172% of control CFU-E's (Fig. 14) and 58% of control CFU-C's (Fig. 15).

Endotoxin stimulates granulocytopoiesis in the marrow through various mechanisms. This was evidenced by a 164% increase in the number of CFU-C of endotoxin treated mice over the number of CFU-C in uninjected controls (Fig. 15). Inversely, CFU-E's of endotoxin treated animals were 54% of control values (Fig. 14).

The method of *hypertransfusion* was used to increase hematocrit, thereby reducing marrow erythropoiesis. One injection of packed red blood cells induced a rise in average hematocrit from 48.4 to 64.5 (see Table 7). At the time of sacrifice, about 22 hr after a second transfusion and 16 hr after ActD, the mean hematocrit had risen to 78.9, a 63% increase over controls. Hypertransfusion caused an 82% reduction in the number of CFU-E's (Fig. 14) accompanied by an increase to 130% of control CFU-C values (Fig. 15).

5.2 Specific Effects on Colony Forming Units

The 16 hr ActD dose-response curves for CFU-E's after various perturbations are seen in Fig. 14. Anemic animals that received saline after phenylhydrazine had an increased level of CFU-E'S compared to animals that had not received phenylhydrazine. The ActD dose-response curve for anemic mice is only slightly different than the dose-response curve for normal mice. The greatest difference in sensitivity between the two groups is the response to 0.2 μg of ActD. Normal mice show a slight increase in CFU-E's 16 hr after this low dose of ActD. CFU-E's of anemic mice decrease significantly ($p<0.001$) following this same low dose. With increasing doses of ActD, CFU-E's of normal and anemic mice respond similarly. An 89% decrease of anemic mouse CFU-E's is observed in response to 20 μg of ActD, whereas normal mouse CFU-E's are reduced by 87%.

Endotoxin has the side-effect of decreasing the number of CFU-E's. This reduced subpopulation demonstrated a great increase in colony number in response to the lowest dose of ActD administered (Fig. 12). The slope of the line from 0.2 to 5 μg ($m=-11.25$) for endotoxin treated animals was slightly less negative than the slope between the same doses for normal animals ($m=-17.5$). The number of surviving CFU-E at 20 μg for endotoxin treated animals was below the sensitivity of this assay.

Hypertransfusion polycythemia so reduced the CFU-E population that further treatment with any dose of ActD caused their number to be too small to measure.

The comparison of CFU-C dose-response curves to ActD after various perturbances is seen in Fig. 15. Mice that had been treated with endotoxin prior to saline have elevated CFU-C's as

compared to mice that have not been pre-treated. 20 μg ActD caused a greater decrease in endotoxin treated mice than in normal mice. The slope of the decrease is -4.41, four times more negative than the slope for the same stretch of the normal dose-response curve (m=-1.06). The dose-response curve, after endotoxin, levels off between 20 and 80 μg at numbers of CFU-C not significantly different from the normal numbers ($p>0.4$ at 20 μg).

Anemic animals, whose response to Ph has been an increase in CFU-E's, have a corresponding decrease in CFU-C's 16 hr after saline injection. The moderately depleted CFU-C population increases slightly 16 hr after 20 μg ActD and decreases in response to 80 μg.

Hypertransfused animals, on the other hand, have elevated numbers of CFU-C complementing the corresponding decrease in CFU-E. This increased population responds similarly, as compared to the normal mouse CFU-C's, to a 20 μg dose of ActD ($m_{hypertransfused}$= -1.68; $m_{control}$= -1.06). However, 80 μg of ActD causes the numbers of CFU-C in polycythemic mice to drop below the sensitivity of the assay.

The response of BFU-E's to ActD after various treatments was also measured. At the time of these studies, all assays generated depleted numbers of BFU-E's. Though many factors were altered, burst numbers remained consistently low. Thus, the sensitivity of these studies is somewhat impaired. Some generalizations can be made. 5 μg of ActD following any of the pretreatments used caused very little change in the number of assayable BFU-E's (Fig. 16). In all cases the numbers of bursts were slightly elevated 16 hr after 5 μg. However, 16 hr after 20 μg the numbers of bursts were so reduced that they were below the sensitivity of the assay.

DISCUSSION

1. Background

The hematopoietic population is made up of at least four subpopulations: 1) pluripotent stem cells which may duplicate themselves or differentiate into committed stem cells of erythroid, granulocytic or other type; 2) committed stem cells which are "unipotent" but retain the capacity to regenerate as well as differentiate; 3) differentiating and dividing blasts which, though they still undergo division, cannot reproduce themselves and are destined to differentiate; and 4) maturing cells which no longer retain the ability to divide. Methods are currently available to study all of the compartments mentioned (Till and McCullough, 1961; Pluznik and Sachs, 1965; Bradley and Metcalf, 1966; and McCleod *et al.*, 1974). The red cell population of bone marrow readily lends itself to study because in addition to its assayable and identifiable components, the stimulator (erythropoietin) of the population is known, available and purifiable to near homogeneity (Miyake *et al.*, 1977).

The effect of epo on identifiable erythroblasts has been extensively studied. The earliest observable effect of epo on cultured rat marrow cells is the formation of 150 S RNA, detectable after only 15 minutes (Gross and Goldwasser, 1969). Protein and DNA synthesis follow and other species of RNA are subsequently synthesized. By 6 hours, hemoglobin synthesis is detected. There is some controversy as to whether DNA synthesis is required for eventual hemoglobin synthesis, however, this controversy could be due to the fact that hematopoietic cells are a heterogeneous population (Goldwasser, 1975). The effects of epo on one segment of the population could require DNA synthesis while the other subpopulation need not rely on DNA synthesis. Thus, disparate observations could be due to culture conditions which better support one or the other subpopulation. The specific effects of epo on proliferating and repopulating cells are not as thoroughly documented as epo's action on late differentiating and maturing cells. Therefore, studies concerning the effect of epo or inhibitors of epo must be careful to consider the effects on individual subpopulations.

The early work of Reissmann and Ito (1966 and 1968) used methodology available at the time to pinpoint the site of ActD inhibition. It was assumed that ActD was directly counteracting the stimulatory effect of erythropoietin. At the time, they classified all stem cells into one compartment. They concluded that ActD at low doses (1-2 μg/mouse) was not killing stem cells. Assuming that "stem cells" means pluripotent CFU-S, this conclusion is probably correct. Zuckerman *et al.* (1978) demonstrate that 2.0-2.5 μg ActD/mouse does not cause a decline in CFU-S in either bone marrow or spleen. However, committed stem cells were not directly assayable at the time of Reissmann's work. At present, techniques exist for assessing granulocytic CFU (CFU-C; Pluznik and Sachs, 1965 and Bradley and Metcalf, 1966) and an entire range of erythroid CFU including CFU-E and BFU-E (McCleod *et al.*, 1974; Iscove *et al.*, 1974 and Iscove and Sieber, 1975) and BFU-E at different stages of maturity (Gregory and Henkelman, 1977; Gregory and Eaves, 1977; and Ogawa *et al.*, 1977). Developments in some very recent work (Johnson and Metcalf, 1977 and Hara and Ogawa, 1978) may lead to *in vitro* assays for pluripotent stem cells. All of these advances make it possible to further clarify the level at which ActD is inhibiting and thus, perhaps, the target of epo action on the early erythroid population.

The fact that many different erythroid colony forming units have been discovered, at different levels of maturation is compatible with Holtzer's (1972) understanding of differentiation. In order to ensure that a mature cell carries out only one specific function, its progenitors must differentiate gradually. The necessity for DNA synthesis prior to a differentiation step would allow for quantum, step-wise jumps from one level of maturation to the next. A cell cycle during which such a jump is made is termed a quantal cell cycle (Holtzer *el al.*, 1972). In this way, cells would gradually become specialized avoiding the risk of becoming "schizoid". Such an age structure amongst the erythroid population has been hypothesized (Reissmann *et al.*, 1972). Derepression of genes which program for "luxury" molecules, i.e., those molecules which are specialties of a given cell type, is more sensitive to inhibitors than the synthesis of "essential" molecules, i.e., those necessary for cell survival (Davidson *et al.*, 1963). Davidson *et al.* studied the effect of ActD on ARE, connective tissue cell line, and

found the decay of acid mucopolysaccharide synthesis after the drug had a half-time of 8 hr. The synthesis of succinic dehydrogenase, however, was unaffected 23 hr after the cessation of RNA synthesis due to ActD. The sensitivity of acid mucopolysaccharide synthesis is probably due to the lack of large pools of specific m-RNA's or specific protein molecules for "luxury" molecules. Thus, an inhibitor such as ActD, may allow a cell to survive on the one hand, but block its ability to differentiate, on the other.

A model of hematopoietic differentiation proposes a completely different hypothesis (Goldwasser, 1975). It has been suggested that as pluripotent stem cells go through the cell cycle they develop and lose, successively, different receptors for different "poietins". Thus, at one phase of the cycle a stem cell would be sensitive only to erythropoietin and at another only to granulopoietin. If this were true, then granulopoietins would compete with erythropoietin for stem cells. There is recent evidence against such competition. Studies show that mixed colonies arise from presumably one cell in the presence of epo and conditioned media (Johnson and Metcalf, 1977; Hara and Ogawa, 1978). A factor from leucocyte conditioned media which potentiates both erythroid and granulocytic colony growth has also been detected (Aye, 1977).

The results presented above are explained in light of this information. These observations will be related to the current general understanding of differentiation.

2. Time Course Studies

On the basis of the kinetic studies of Lajtha *et al.* (1962) it was determined that if recovery of colony number did not occur within 48 hours of ActD injection then colony forming unit-death could not be ruled out. In order to achieve recovery of CFU-E numbers, the dose of ActD had to be reduced to 0.2 μg/mouse. At this dose, peak inhibition was reached at 6 hr after which CFU-E number began to rise. It is likely that at such low concentrations of the drug, CFU-E are merely being temporarily blocked in S. Support for this possibility comes from experiments carried out with synchronized mouse L cells and low concentrations (0.4 μg/ml) of ActD (Rickinson, 1970). Cells exposed to the drug in the first 2 hrs of G_1 delayed their entry into S by 6 hrs. After this time cells progressed through S relatively

normally. Delayed entry into S was not demonstrated by cells treated with ActD past the fourth hour of G_1. This fact could explain the secondary decrease of CFU-E and BFU-E at 30 hrs. Those CFU-E and BFU-E which are affected by the drug past a certain point in G_1 and retain drug molecules may not be blocked until they make another revolution about the cell cycle.

Repeated injection of 0.2 μg of ActD every 12 hours had no cumulative effect on CFU-E. This result is to be expected if there is no toxicity of the drug. It may, therefore, be concluded that 0.2 μg of ActD is not causing CFU-E death and is probably either temporarily blocking or delaying CFU-E progress through the cell cycle.

This same low dose of ActD (0.2 μg) had a slight stimulatory effect on CFU-C. At low concentrations (0.5-5 ng/ml) ActD may cause the induction of murine erythroleukemia cells (MELC: Terada *et al.*, 1978). It is hypothesized that induction could be due to slight increases of lability in DNA (as measured by alkaline sucrose gradient analysis) caused by ActD. Increased lability of DNA might also explain the increases of CFU-C at very low doses.

The time course of CFU-C response to 10 μg ActD is similar to the response of CFU-E to 0.2 μg. It would seem that as long as the inhibitory effect of the drug is overcome by 6 hr or as long as it does not last until 16 hr, then recovery of colony number by 48 hr is possible and colony forming unit death may be ruled out. Reasons for the differential sensitivity between CFU-C and CFU-E to the drug are discussed later.

All doses administered, above 0.2 μg, did not allow for CFU-E recovery by 48 hours. Even a dose of 1 μg ActD, which caused a decrease similar to the one caused by 0.2 μg at 6 hrs, resulted in a further decline of CFU-E's at 16 hrs (Fig. 5). It may, therefore, be concluded that if colony forming units have not begun to recover by the 16th hour after injection of ActD, they will not fully recover by 48 hours. Since recovery would be expected by 48 hours if no cell death were to occur (Lajtha, 1962), it must be concluded that doses of 1 μg and greater do have some lethal effect on CFU-E. This effect may be exerted by delaying entry into S for longer than a certain critical period. In order to exclusively study the inhibitory effect of ActD,

the effect which directly counteracts epo stimulation of CFU-E, one should choose low doses (less than or equal to 0.2 μg) of ActD.

3. Dose Response Studies

Bruce *et al.* (1966) classified several chemotherapeutic agents based on the shapes of dose-response curves of normal and lymphoma CFU-S. Drugs which allowed the numbers of CFU to reach a plateau were classified as phase active, i.e., they acted only in one phase of the cell cycle. Those agents which caused a logarithmic decrease in colony number throughout the dose range were classified as cycle active, i.e., any cell in active cell cycle would be affected. The fact that some drugs were administered in divided doses and some in single injections was not considered. ActD, which was administered six times every 4 hours, was classified as cycle active based on the fact that it caused a logarithmic decrease in CFU-S. However, because a time course study was not done, it is not known whether the divided doses used were having the effect of a single injection at low doses and a cumulative effect at high doses.

In this study, the dose response curves were done at 16 hours after a single ActD injection because this was the time at which there was maximal decrease in CFU-E for all doses above 0.2 μg/mouse. For CFU-E and BFU-E the dose-response curve shows a slight increase above control levels at 0.2 μg (Figs. 9 & 10). At 16 hours, erythroid stem cells have already recovered from this low dose. Very small amounts of the drug remaining in the circulation may have a stimulatory effect on colony forming units similar to the induction effect on MELC (Terada *et al.*, 1978). Doses above 0.2 μg cause a logarithmic decline in CFU-E and BFU-E to about 10% of control levels. The effect of the drug on CFU-C was much less severe, plateauing at 20 μg (Fig. 11). The maximal decrease in CFU-C at 80 μg was about 50%. If ActD is cycle active then it might be concluded that no more than 50% of CFU-C are in cycle and possibly greater than 90% of CFU-E and BFU-E are in cycle. If ActD is phase active then these percentages apply to the number of colony forming units which enter into a particular phase of the cell cycle during the 16 hr course of the experiment. Differences in sensitivity to the drug might also be due to intrinsic differences of the colony forming units' susceptibility. Thus, the

survival of a granulocytic CFU may be less dependent on RNA synthesis than the survival of a CFU-E. In order to be able to relate ActD action to cell cycle activity, thymidine suicide studies were carried out.

4. Thymidine Suicide Studies

High specific activity tritiated thymidine suicide studies were carried out in order to ascertain the relationship between S-phase and ActD effect. Fig. 13 shows that the effect of ActD and the effect of ^{3}H-thymidine are nearly identical. Thymidine can cause cell death only during S-phase when it is incorporated into the DNA. The fact that the ActD effect is similar to the ^{3}H-thymidine effect might be expected on the basis of studies showing that the drug binds more strongly to G_1/S phase HeLa cells or HeLa cell nuclei (Djordjevic *et al.*, 1968 and Peterson and Robbins, 1972). The position of the individual points, which lie above or below the linear regression line, may be indicative of differences between colony forming units. That is, CFU-E are more sensitive to ActD than to ^{3}H-thymidine. This additional sensitivity would be accounted for if CFU-E were also susceptible to drug action in another part of the cell cycle. Working with synchronized MELC, Rifkind *et al.* (1976) found that inducer (DMSO) had to be present during S and part of G_2 in order to stimulate erythroid differentiation. In addition it was found that cells had to be preincubated with inducer for 24 hours prior to S/G_2. Since MELC are probably derived from CFU-E (Harrison, 1976) it is possible that CFU-E cell cycle phases other than S may be sensitive to the inhibitory effect of ActD. It may be Hypothesized that CFU-C are sensitive to ActD during only a portion of S phase thus making them more susceptible to thymidine than to ActD. Another possibility that would account for the data is that S phase is a greater proportion of total cell cycle time for CFU-E than for CFU-C. Until methods become available to purify and synchronize these hematopoietic subpopulations, it is not possible to conclusively test out this hypothesis.

Assuming that S phase is the portion of the cell cycle most susceptible to ActD, then the dose-response curves may be more clearly explained. During the 16 hour course of the dose-response study, no greater than 50% of CFU-C enter into S. Since the entire cell cycle is pro-

bably between 8 and 12 hours (Vassort *et al.*, 1971 and Vilpo and Rtomaa, 1973), all cycling cells are likely to enter into S. It may be concluded that 50% of CFU-C are in G_0. This estimate is consistent with the one given by Lajtha *et al.* (1969). The plateau in the dose-response curve would suggest that no CFU-C in G_0 is being called into cell cycle within the 16 hour duration of the experiment. CFU-E have a cell cycle time of about 11 hours (Cormack, 1976). The fact that the CFU-E dose-response curve at 16 hours does not plateau, may mean that CFU-E are being recruited either from G_0 or from a previous compartment.

Recruitment from the BFU-E compartment to replenish CFU-E losses might explain the logarithmic decrease of the dose-response curve for BFU-E. The high sensitivity of BFU-E to ActD was not expected, nor was the high suicide rate. Hara and Ogawa (1977) and Iscove (1977) reported a thymidine suicide rate of between 30 and 36% for BFU-E cultured in methyl cellulose. The heightened sensitivity of BFU-E to both ^{3}H-thymidine and to ActD could reflect differences in the method of culture or the animals used and their living conditions. A factor produced by leucocytes has been found to potentiate BFU-E growth (Aye, 1977). Animals in one laboratory may be manufacturing this factor in higher concentrations. Different lots of fetal calf serum may also vary in the concentration of unknown factors. The results are internally consistent, in that BFU-E are as sensitive to ^{3}H-thymidine as they are to ActD. As with CFU-C, BFU-E are slightly more sensitive to ^{3}H-thymidine than to ActD. This may signify that BFU-E are susceptible to the effect of ActD during a portion of S phase.

5. Perturbation Studies

The various hematopoietic perturbances induced in this study evoked increases or decreases in different stem cell populations. The resultant populations are either more or less sensitive to the effects of ActD. If we assume, based on the data discussed above, that the ActD effect is exerted primarily on cells in S phase, then several kinetic factors might explain increases and decreases in numbers of colony forming units and sensitivities to the drug. *1) The fraction of the population in G_0* — A greater fraction of cells in G_0 would not affect the total number of assayable stem cells. Conversely, a smaller fraction of G_0 cells would be indicated by increased

drug sensitivity. *2) Duration of cycling time* — Increased total cell cycle time would effectively reduce the total cell population. Since the duration of S phase is relatively constant (Mitchison, 1971), the fractions of cells in S would also be reduced, thus, decreasing the sensitivity to the drug. Decreased cell cycle time would have the opposite effect. *3) Recruitment: a) From the previous compartment* — In this way a specific population may be increased. However, the ActD sensitivity is dependent upon the additional factors of the cycling time and the G_0 subpopulation of the expanded compartment. *From* G_0 — This would increase sensitivity to ActD without a concommitant increase in total compartment population. The cessation of recruitment from either previous compartment or the G_0 subpopulation would result in the opposite effects. *4) Suppression of differentiation into the next compartment* — The total number of assayable stem cells would increase but the sensitivity of this larger population to ActD would depend on the cell cycle activity of the population. Stimulation of differentiation would result in the opposite effect. These possibilities allow for interpretation of the results presented in Figs. 14-16.

The total CFU-E population after phenylhydrazine treatment is increased. Sensitivity to ActD is greater at lower doses than controls and similar to controls at higher doses. This may be due to both shorter cycling time for the CFU-E and greater recruitment from the previous compartment following phenylhydrazine treatment. There are somewhat fewer BFU-E due to phenylhydrazine, however, this is not thought to be significant. It is not likely that the increased numbers of CFU-E are due to suppression of differentiation since erythropoietin titers, which are known to be increased following phenylhydrazine treatment, cause increased differentiation into normoblasts. Stohlman (1970) had previously shown that a shortened cell cycle was an effect of epo.

There is a reduced population of CFU-E due to endotoxin. It has been hypothesized that this reduced population is due to the shunting of BFU-E to the spleen (Reissmann *et al.*, 1976; Udupa and Reissmann, 1977). The CFU-E remaining in the marrow have a decreased sensitivity to ActD (Fig. 14) which may be due to an increased cell cycle time. The decreased activity of CFU-E is indicative of an unfavorable erythropoietic microenvironment in the

marrow as it is taken over for granulopoietic activity (Fruhman, 1967).

Due to the extremely low numbers of CFU-E present in the marrow after hypertransfusion polycythemia, their sensitivity to ActD could not be determined. If 60% or more of the CFU-E population were in G_0 due to hypertransfusion, their resistance to ActD could have been detected. Hara and Ogawa (1977) found no change due to hypertransfusion in the proliferative status of CFU-E. Thus, a 60% decrease in CFU-E's, as was seen in controls after 5 μg ActD, would not be assayable with such a small initial population. The depleted CFU-E population may be the result of a lack of differentiation into the compartment. Levels of epo in polycythemic mice are not detectable. The lack of epo in the circulation of polycythemic mice may dam the flow into the CFU-E compartment, thus, causing an increase in BFU-E numbers (Iscove, 1977).

About 50% of the CFU-C population is in G_0. Thus, only the other 50% is vulnerable to ActD. Direct stimulation of granulopoiesis using endotoxin results in increased CFU-C's (Fig. 15). These additional CFU-C's are more sensitive to ActD as can be seen by the increased slope over controls from 0 to 20 μg. However, the point at which the dose-response curve levels off is the same for endotoxin and control mice. Thus, the absolute numbers of G_0 CFU-C remain the same. The increased numbers of total CFU-C are very likely due to a decreased cell cycle time. Shortened cycling time may result from increased levels of a granulopoietin and may be responsible for both the increased number of CFU-C and the heightened sensitivity to doses of up to 20 μg of ActD.

The effects of phenylhydrazine and hypertransfusion on CFU-C's are not direct ones. CFU-C of phenylhydrazine treated mice are fewer in number and less sensitive to ActD. At 80 μg, approximately 50% of the total initial CFU-C's remain. Thus the proportion of G_0 CFU-C is not altered. A longer cycling time might account for both the decreased total population and the decreased sensitivity to ActD. The equilibrium between G_0 and cycling cells is left unchanged.

CFU-C of hypertransfused mice are increased in number and sensitivity to ActD. The num-

bers of CFU-C, after 80 μg of the drug, are not assayable. Thus, it would seem that there are few CFU-C remaining in G_0. This may account for the increased sensitivity. Increased numbers of CFU-C may be due to any of the possibilities listed above. The observed increase in granulopoietic activity in the marrow may result from decreased erythropoiesis and a more favorable granulopoietic hematopoietic microenvironment.

It is unfortunate that the numbers of BFU-E at the time of the perturbance studies were so low (Fig 16). It was impossible to account for the decreased plating efficiencies. All hematopoietic stimulators and suppressors used resulted in similar effects on BFU-E, i.e., a slight but insignificant increase at 5 μg and an undetectable number at 20 μg. Others (Hara and Ogawa, 1977; Iscove, 1977; and Gregory and Eaves, 1978) have found no change or a slight increase in the numbers of BFU-E after hypertransfusion. Phenylhydrazine-induced anemia caused a decrease in femoral BFU-E (Hara and Ogawa, 1976). These sources of erythropoietic suppression and stimulation do not cause increased suicide (hara and Ogawa, 1976 and 1977) and, therefore, probably do not change the number of BFU-E in G_0. It has been theorized that the decrease in femoral BFU-E in anemic mice is due to migration of BFU-E through the blood stream to the spleen (Hara and Ogawa, 1976 and 1977; Iscove, 1977). Decreased BFU-E due to erythropoietic stimulation and slight increases in BFU-E due to erythropoietic suppression may also be attributed to a direct effect of epo on BFU-E. Altered levels of epo may have no effect on BFU-E proliferation, however, they may have an effect on BFU-E differentiation into CFU-E. Thus, high levels of epo due to anemia may deplete the BFU-E compartment. Factors responsible for BFU-E proliferation are presently under investigation (Gregory and Eaves, 1978; Aye, 1977).

6. Summary, Speculations, and Implications

ActD may reversibly inhibit or may exert a lethal effect on cells in active cell cycle. If one wishes to exclusively study the specific counter-effect of ActD on epo stimulated proliferation and differentiation of CFU-E, very low doses ($\sim$0.2 μg/mouse) must be employed. Doses greater than or equal to 1 μg ActD/mouse will result in some CFU-E death. ActD does indeed

block epo-stimulated proliferation and differentiation of CFU-E in cell cycle. Inhibition most likely occurs during S-phase of the cell cycle. Although epo is probably not the normal physiologic stimulator of BFU-E proliferation, it may promote differentiation of BFU-E and CFU-E. The erythroid population is more sensitive to the inhibitory effect of ActD than is the granulocytic population. Increased sensitivity to the drug is a result of greater cell cycle activity on the part of the red cell population. The vulnerability of committed granulocytic stem cells to the drug may be increased by stimulating their cell cycle activity.

It is interesting to note that CFU-E are exquisitely sensitive to ActD. Rickinson (1970) found that concentrations of ActD between 0.02 and 0.08 μg/ml specifically inhibited r-RNA synthesis in mouse-L cells. This is the case despite the fact that ^{3}H-ActD at these concentrations will bind to DNA not only in the nucleolar region but throughout the genome. If in a 30 g mouse, 0.2 μg of ActD distributed itself over 2.5 to 10 ml of body fluid, the resulting concentration of the drug would fall in the range of specific r-RNA synthesis inhibition. Thus, it may be speculated, that the primary site of epo stimulation on CFU-E may be the nucleolus. Further investigation is needed in this area.

The use of MELC as a model of erythroid differentiation may also be taken advantage of. MELC are a pure population and may be synchronized. They can be induced to differentiate using, amongst other agents, DMSO. The effects of varying concentrations of ActD on DMSO-induced differentiation may also be a fruitful area of investigation.

Figure 1

The structure of Actinomycin D (Muller, 1969).

Figure 1

THE STRUCTURE OF ACTINOMYCIN D

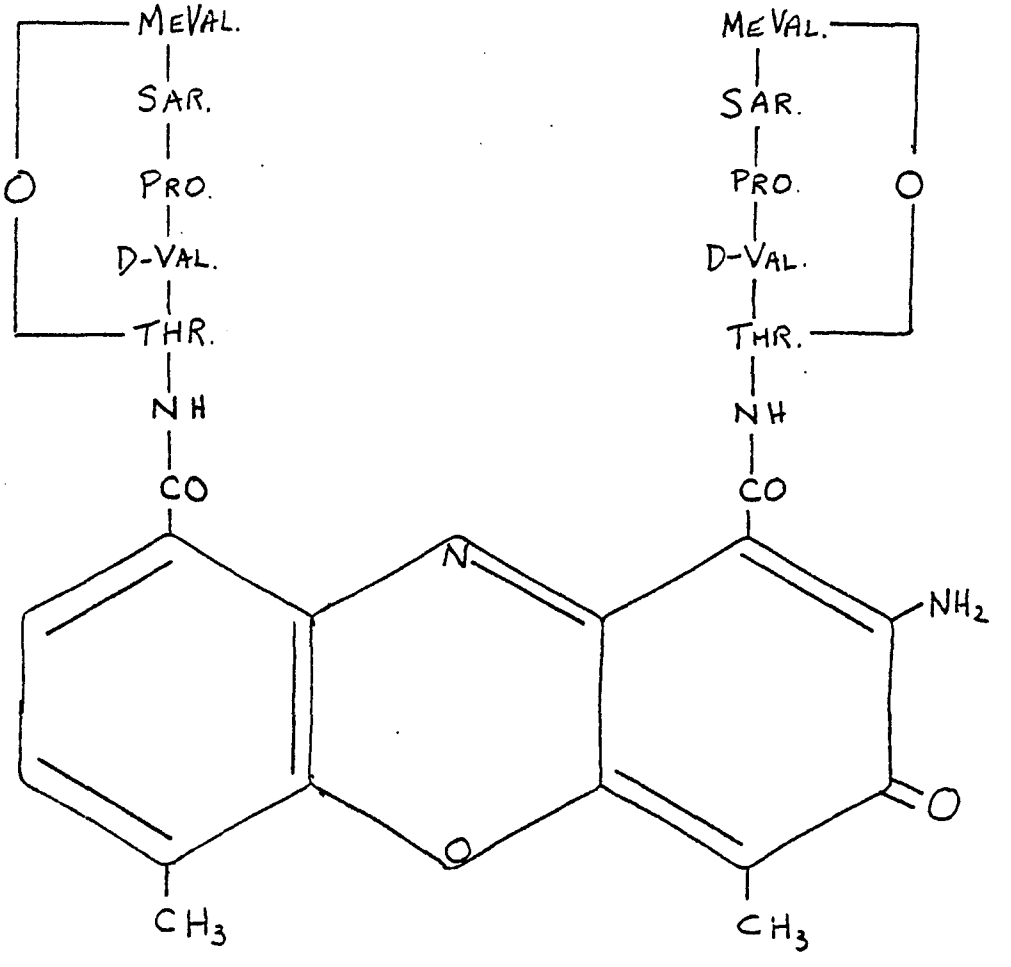

Figure 2

The effect of increasing concentrations of epo on the numbers of CFU-E. Each point represents the mean number of colonies in six clots and the s.e.m. Cultures contained the pooled marrow of three mice femora.

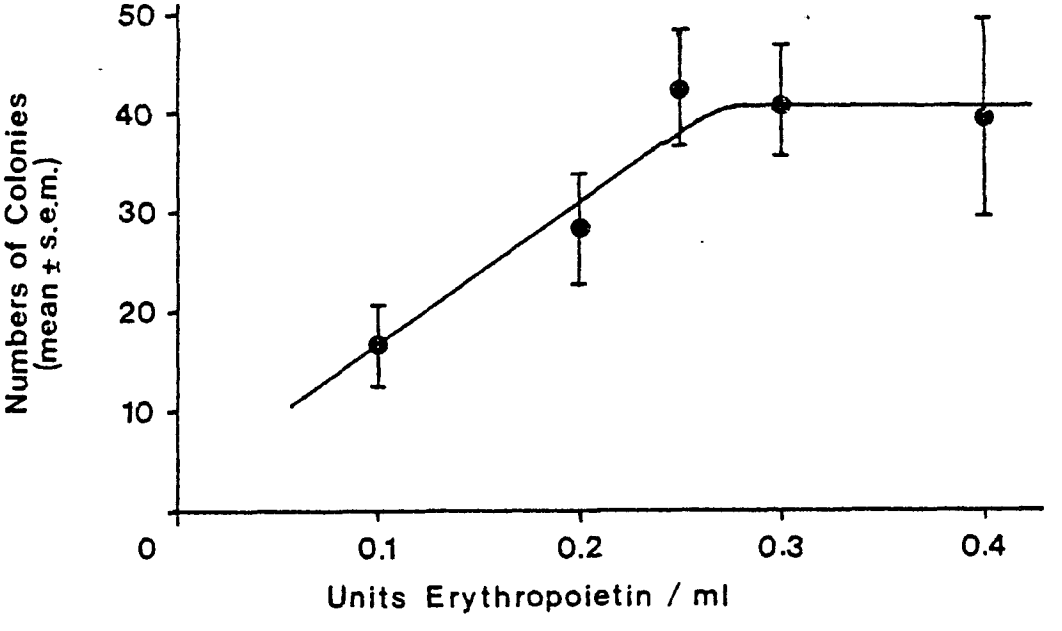
Numbers of Colonies
(mean ± s.e.m.)
50
40
30
20
10
0
0.1
0.2
0.3
0.4
Units Erythropoietin / ml

Figure 3

The effect of increasing concentrations of epo on the number of BFU-E. Each point represents the mean number of bursts in four clots and the s.e.m. Cultures contained the pooled marrow of three mice femora.

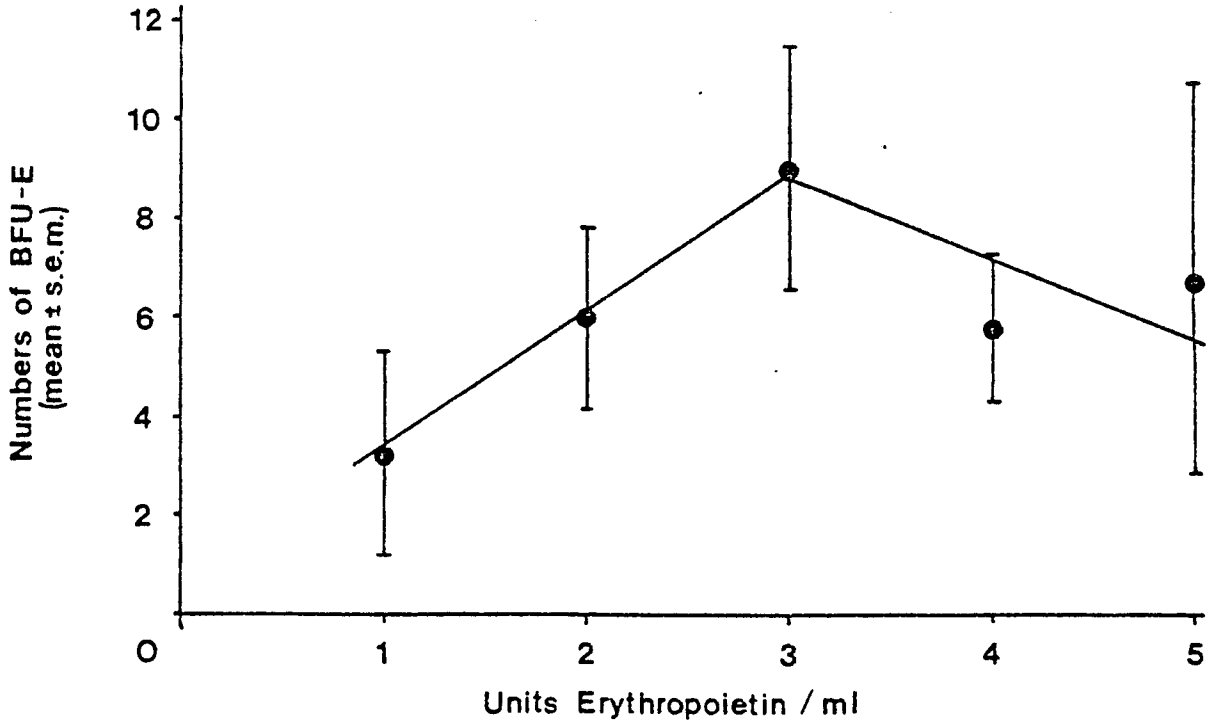
Numbers of BFU-E
(mean ± s.e.m.)
12
10
8
6
4
2
0
1
2
3
4
5
Units Erythropoietin / ml

Figure 4

The effects of dilutions of three different batches of CSA on the numbers of CFU-C.

A: Each point represents the mean number of CFU-C in 5 plates and the s.e.m. Each plate contained the pooled marrow of three mice femora.

B: This point represents the mean number of colonies in 4 plates and the s.e.m. Each plate contained the pooled marrow of three mice femora.

C: Each point represents the mean number of CFU-C in 4 plates and the s.e.m. Each plate contained the pooled marrow of 2 mice femora.

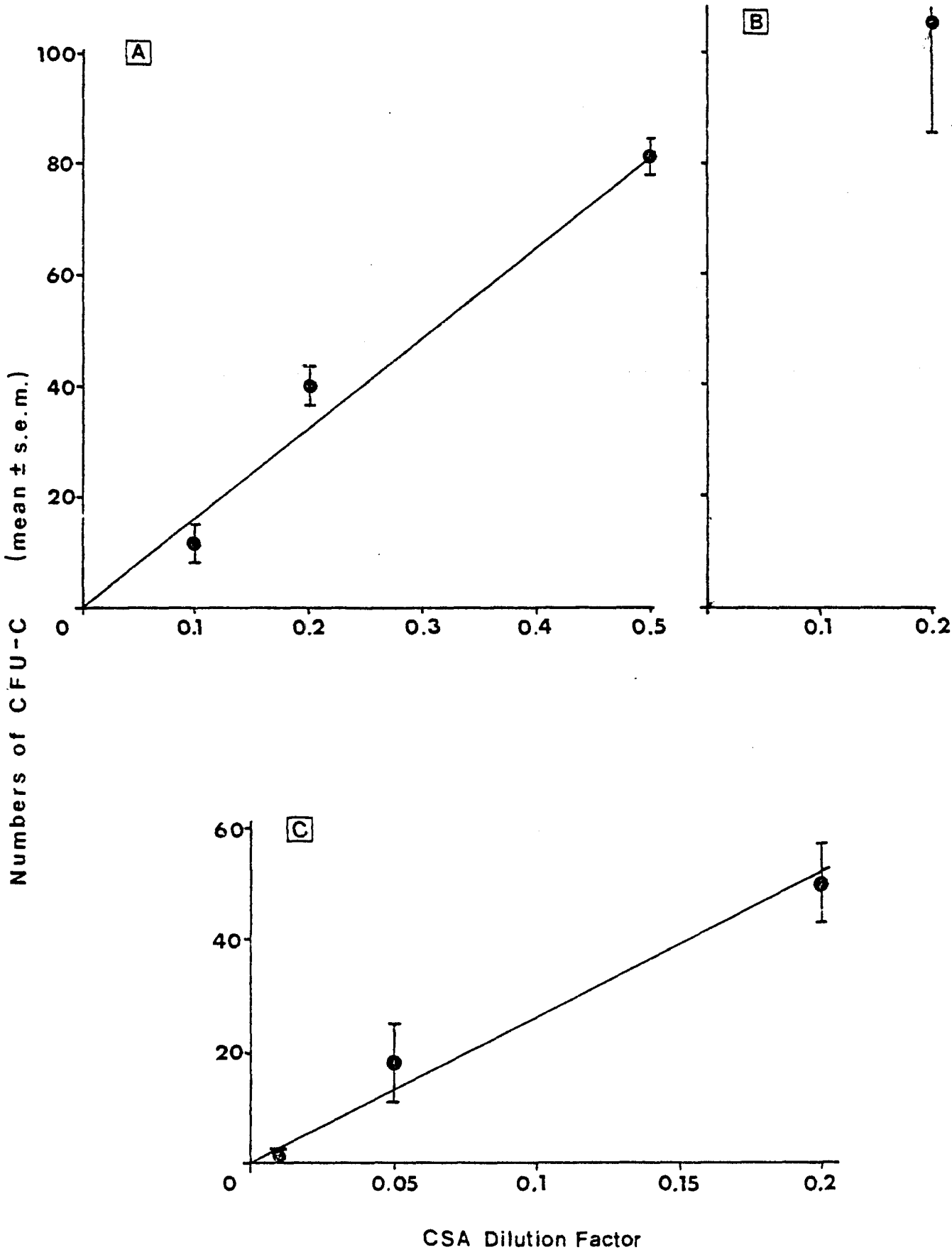
A
B
C
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.1
0.2
60
40
20
0
0.05
0.1
0.15
0.2
Numbers of CFU-C (mean ± s.e.m.)
CSA Dilution Factor

Figure 5

The percent of control CFU-E remaining after saline and various doses of ActD as a function of time. Each line represents at least three experiments. In each experiment three mice femora were pooled for each sample point and six clots were plated for each bone marrow sample. Marrow from uninjected controls was similarly plated for each experiment. The means ± s.e.m. for controls ranged from 60.5 ± 4.8 to 75.7 ± 4.5.

CFU-E Response to Various Doses of Actinomycin D

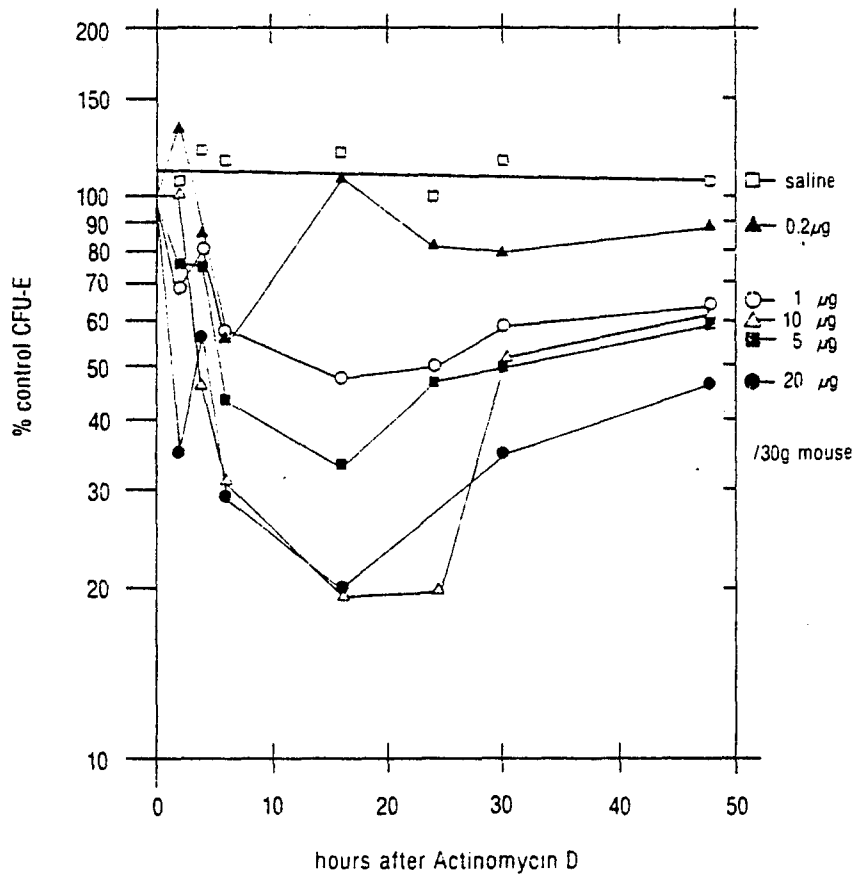

Figure 6

The number of CFU-E following 0.2 μg ActD / 30 g mouse as a function of time. Each point represents the mean number of colonies in six clots and the s.e.m. Femoral marrow from three mice was pooled for each sample.

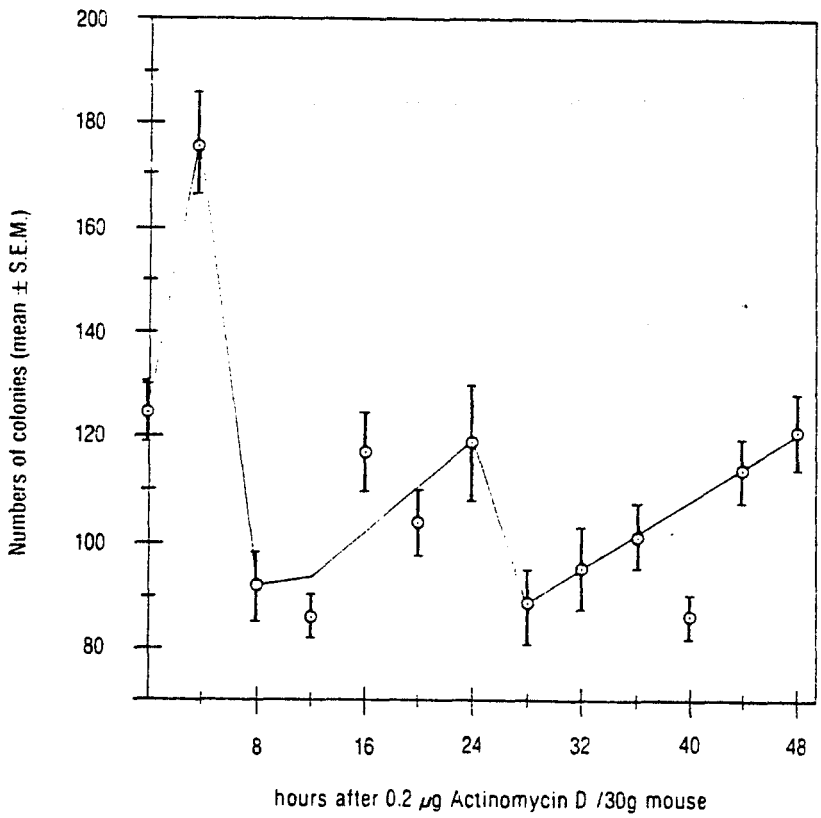
200
180
160
140
120
100
80
Numbers of colonies (mean ± S.E.M.)
8
16
24
32
40
48
hours after 0.2 μg Actinomycin D /30g mouse

Figure 7

The percent of control BFU-E's following 0.2μg ActD / 30 g mouse as a function of time. Each point represents the results of 2 experiments, in each, of which four clots were plated from pooled femoral marrow of three mice. The mean $\pm$ s.e.m. for uninjected controls was 29.8 $\pm$ 3.9.

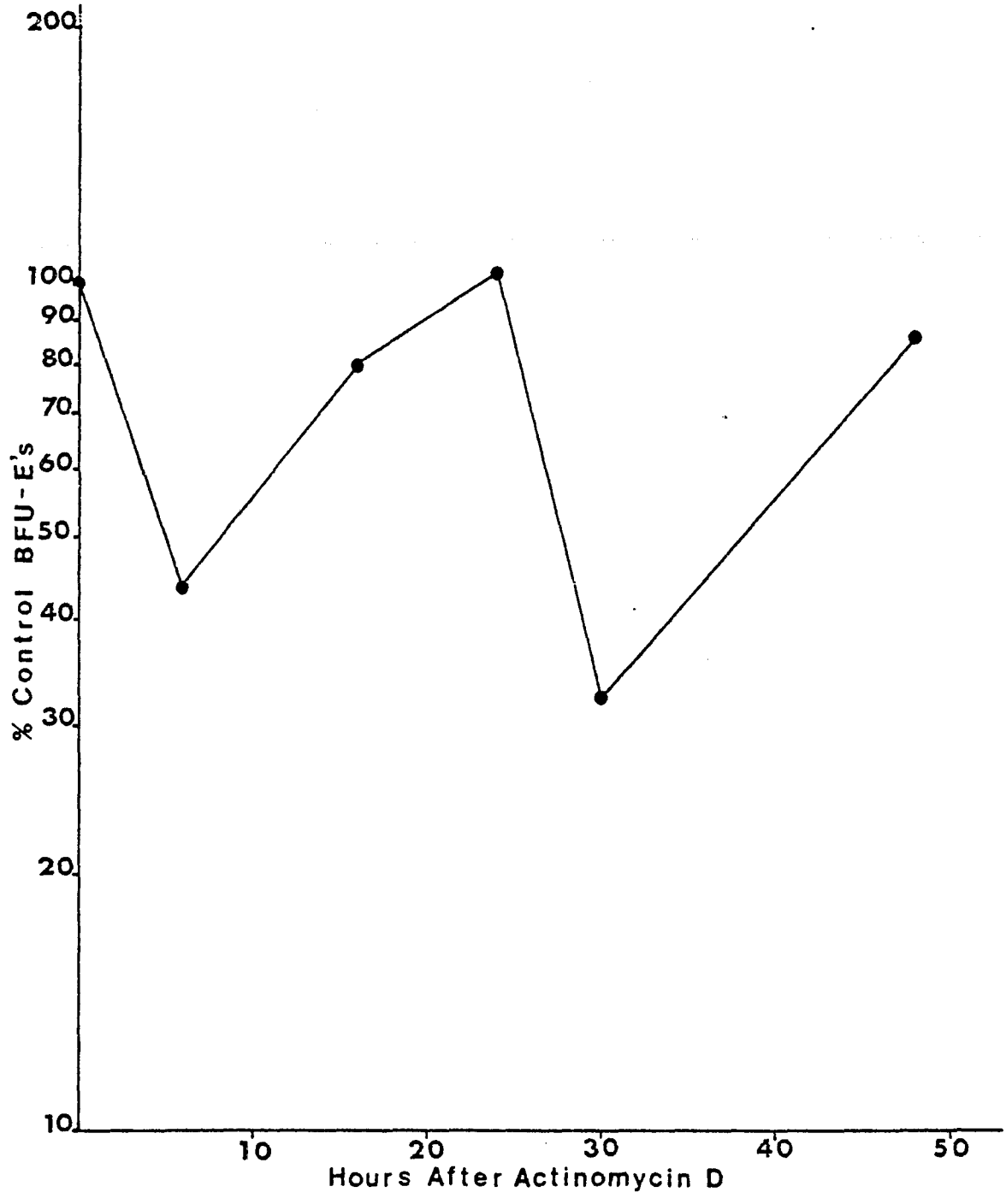
200
100
90
80
70
60
50
40
30
20
10
% Control BFU-E's
10
20
30
40
50
Hours After Actinomycin D

Figure 8

The percent of control CFU-C's following 0.2 μg (•) ActD and 10 μg (o) ActD / 30 g mouse as a function of time. Each point represents the results of an experiment in which pooled femoral marrow from three mice was plated in quadruplicate. The mean ± s.e.m. for uninjected controls was 67.0 ± 4.0.

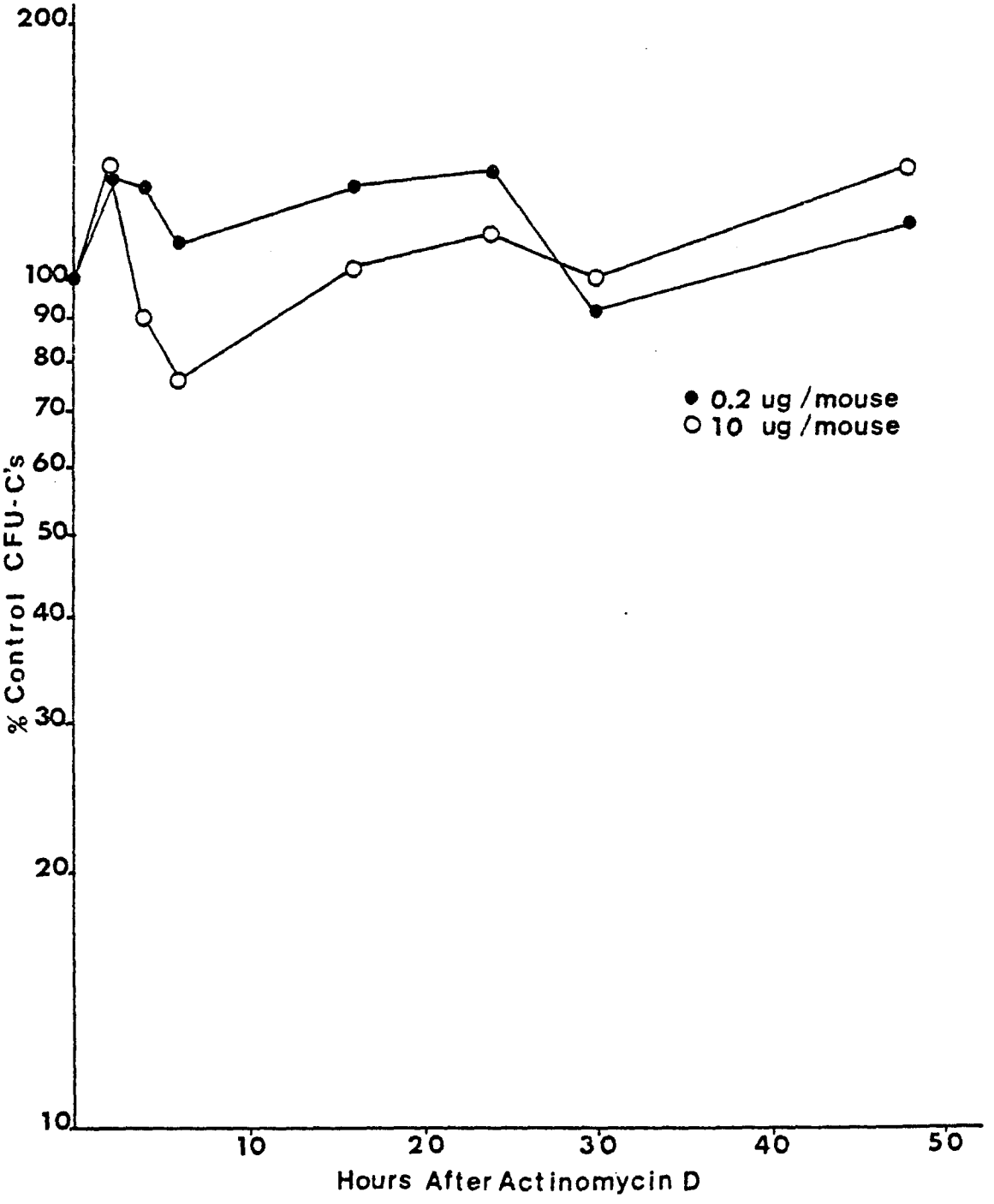
200
100
90
80
70
60
50
40
30
20
10
% Control CFU-C's
● 0.2 ug /mouse
○ 10 ug /mouse
10
20
30
40
50
Hours After Actinomycin D

Figure 9

The numbers of CFU-E 16 hours after various doses of ActD. Each point represents the results of 2 experiments, in each, of which 6 clots were counted. Femoral marrow from three mice was pooled for each sample in both experiments.

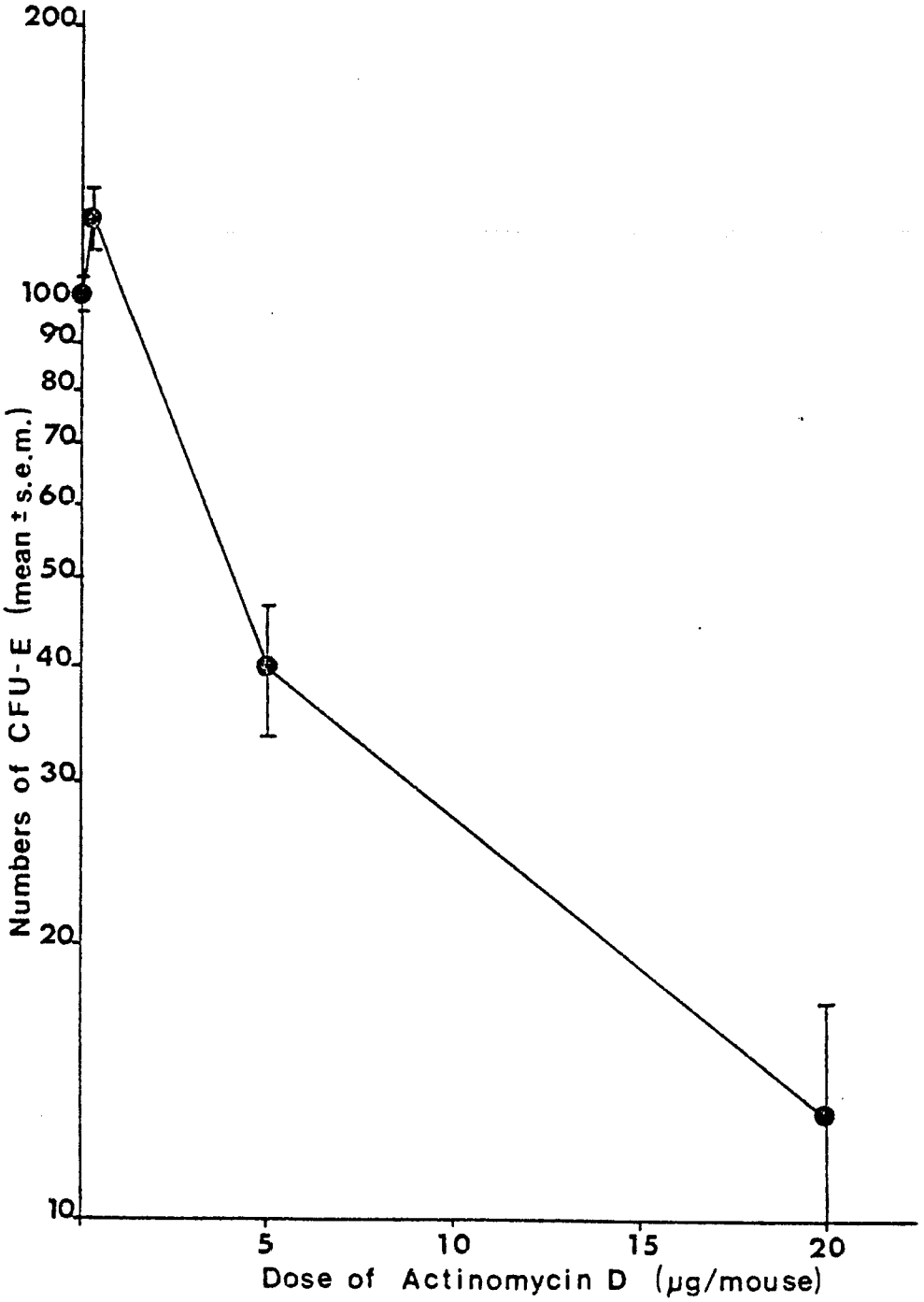
200
100
90
80
70
60
50
40
30
20
10
Numbers of CFU-E (mean ± s.e.m.)
5
10
15
20
Dose of Actinomycin D (μg/mouse)

Figure 10

The number of BFU-E 16 hours after various doses of ActD. Each point represents the results of 2 experiments, in each, of which 4 clots were counted. Femoral marrow from three mice was pooled for each sample in both experiments.

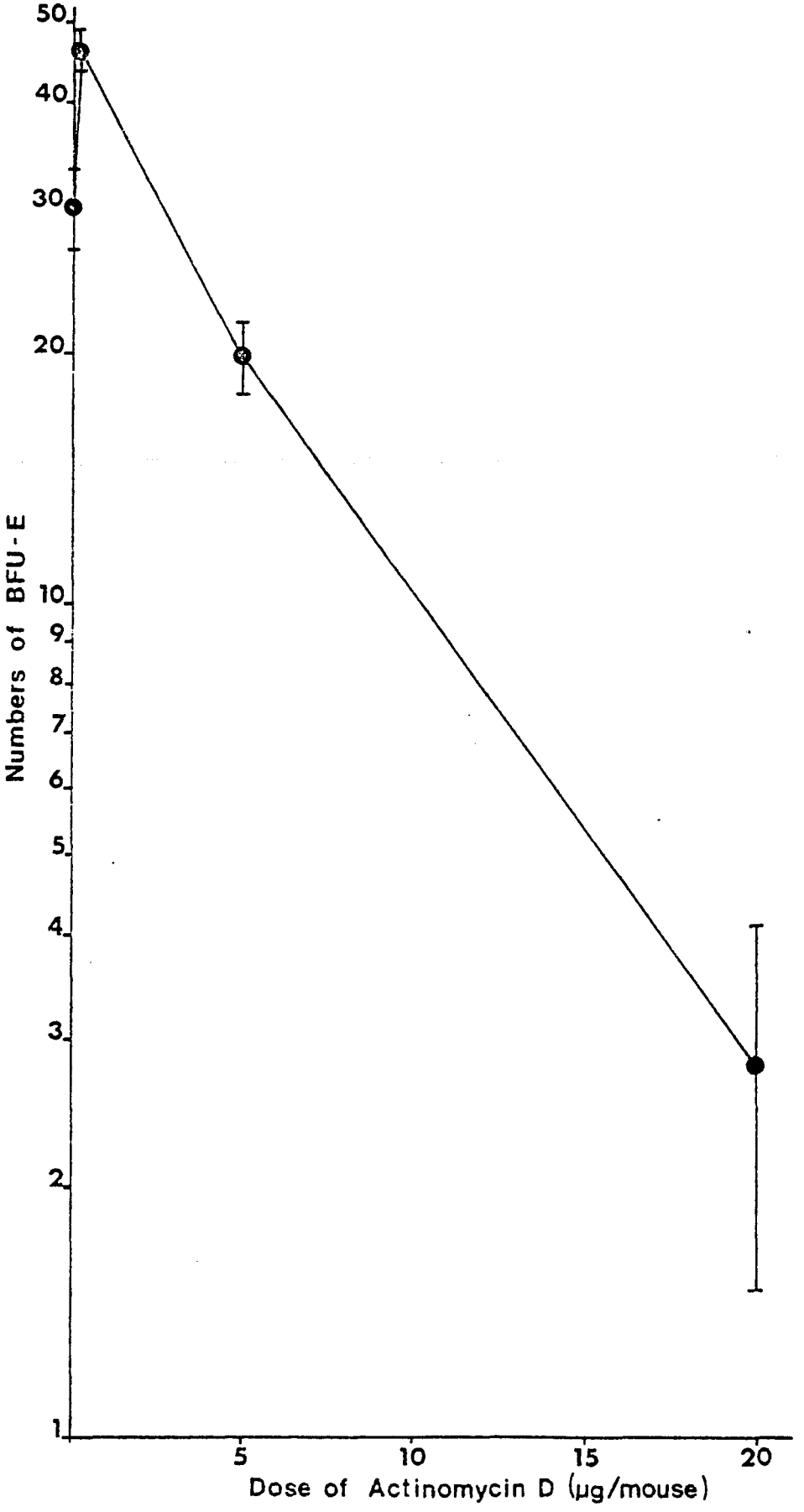
50
40
30
20
10
9
8
7
6
5
4
3
2
1
Numbers of BFU-E
5
10
15
20
Dose of Actinomycin D (μg/mouse)

Figure 11

The number of CFU-C 16 hours after various doses of ActD. Each point represents the results of 2 experiments, in each, of which pooled femoral marrow from three mice was plated in quadruplicate.

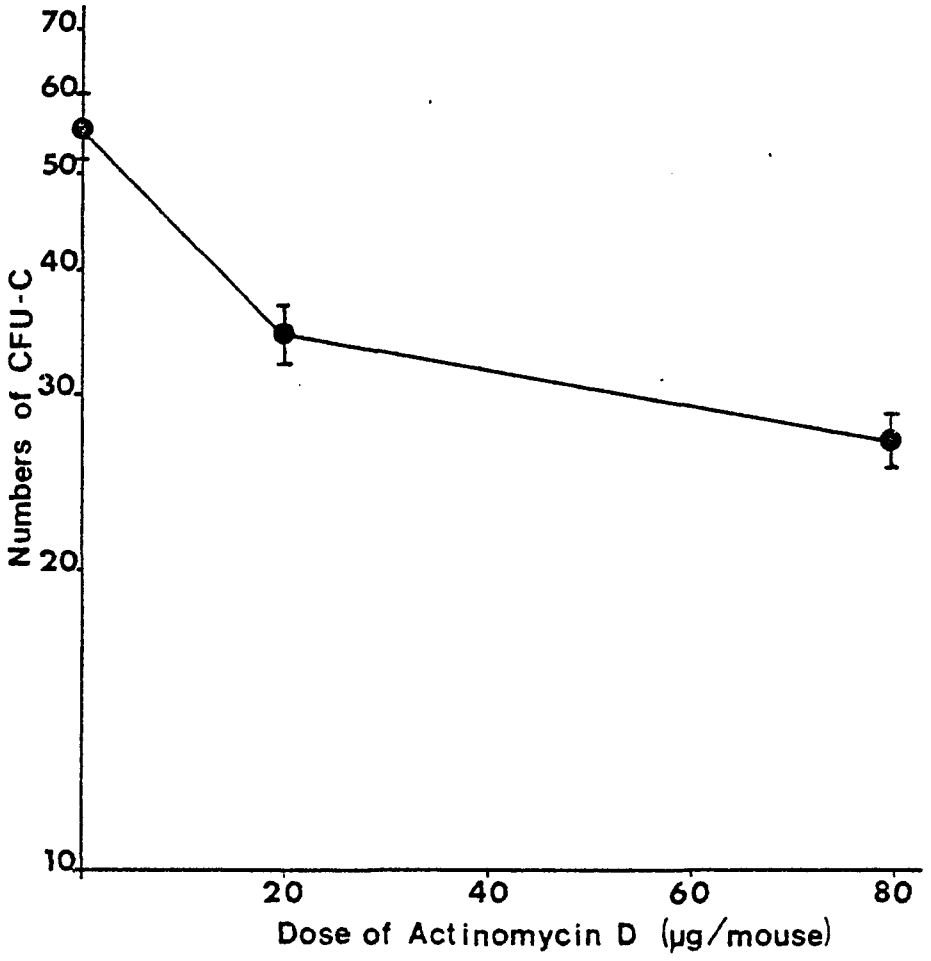
70
60
50
40
30
20
10
Numbers of CFU-C
20
40
60
80
Dose of Actinomycin D (μg/mouse)

Figure 12

The percent of CFU-E suicide due to increasing amounts of high specific activity ^{3}H-thymidine. 5×10^{6} marrow cells / ml pooled from three mice were incubated for 20 minutes at 37°C in 2 ml quantities with specified amounts of ^{3}H-thymidine. Treated cells were then plated in sextuplicate. Cultures containing cells which were preincubated without ^{3}H-thymidine had 29.3 $\pm$ 3.5 CFU-E (mean $\pm$ s.e.m.).

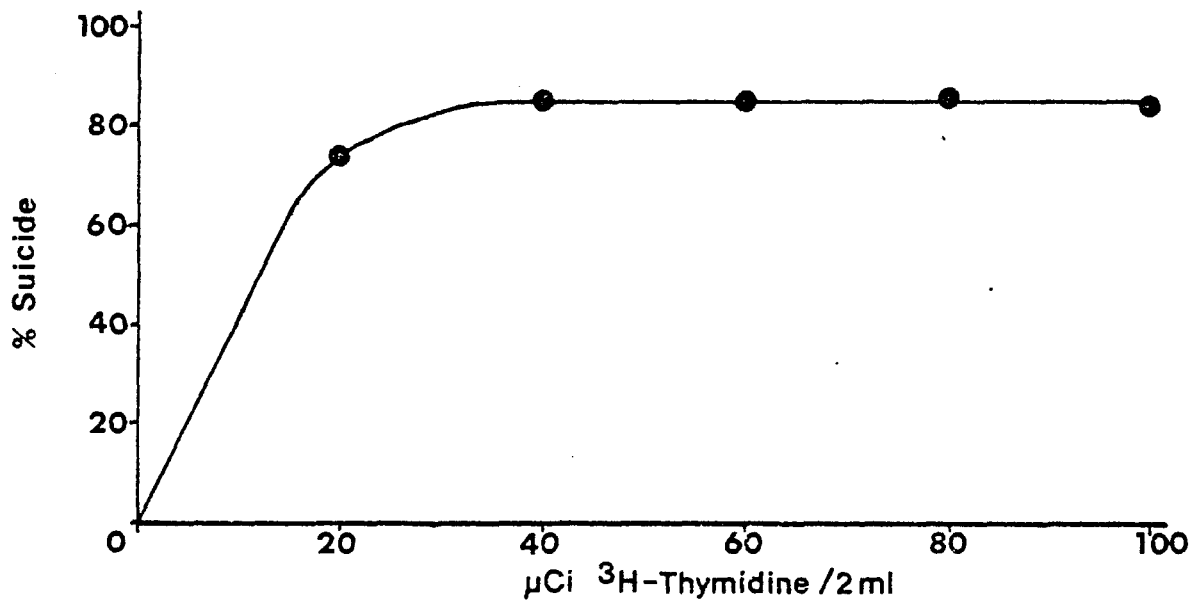
100
80
60
40
20
0
% Suicide
20
40
60
80
100
µCi ³H-Thymidine /2 ml

Figure 13

The percent survival of BFU-E CFU-E (○), and CFU-C (▽) 16 hours after 20 μg ActD / 30 g mouse as a function of percent survival after high specific activity ^{3}H-thymidine. Values are taken from Table 5 and Figure 5. r=0.971, m=1.14

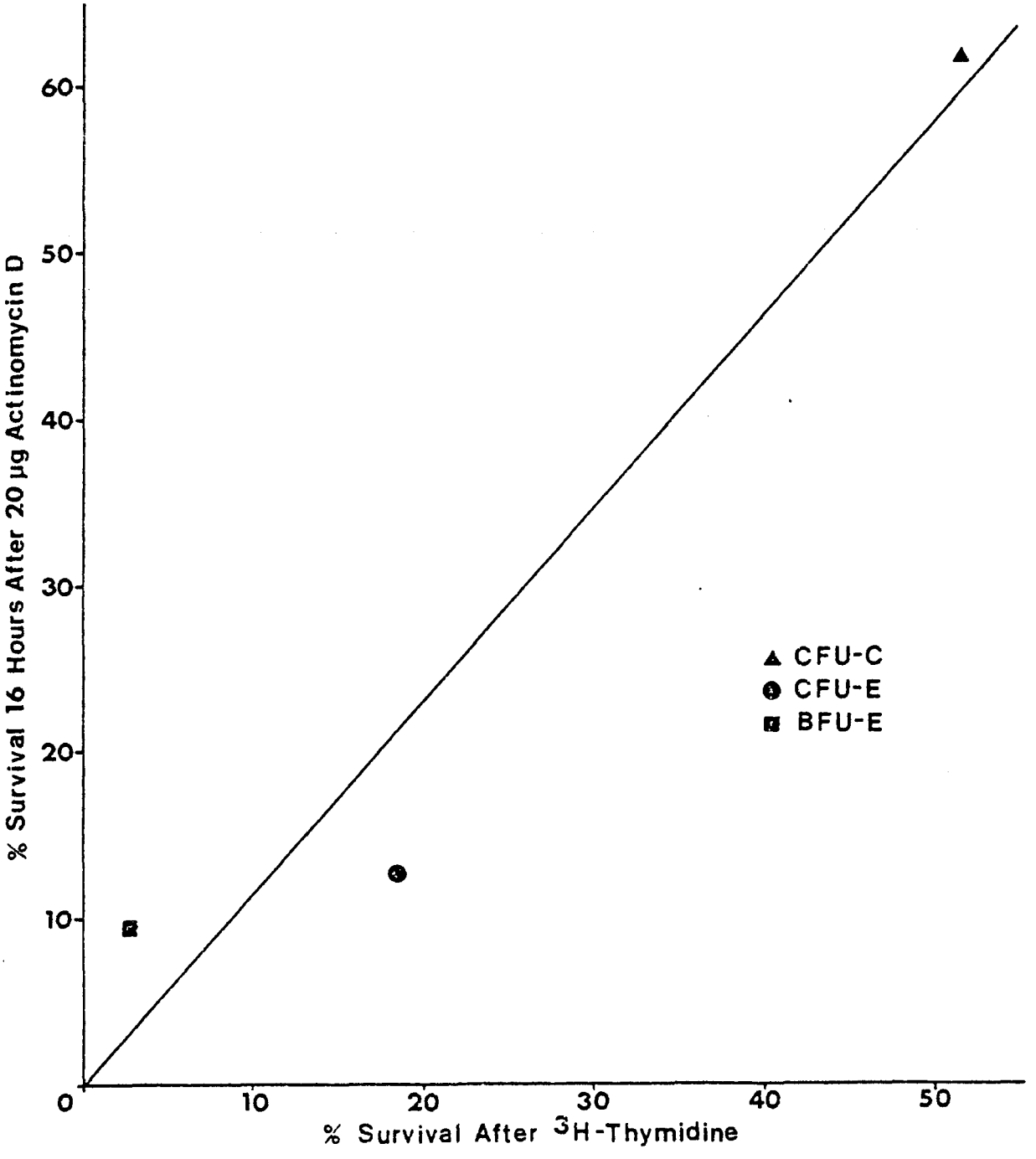
% Survival 16 Hours After 20 µg Actinomycin D
60
50
40
30
20
10
0
10
20
30
40
50
% Survival After ^{3}H-Thymidine
CFU-C
CFU-E
BFU-E

Figure 14

The numbers of CFU-E 16 hours after various doses of ActD in pretreated mice. Phenylhydrazine treatment consisted of 2 injections (1.8 mg / 30 g mouse) 48 and 24 hours prior to ActD. Another group of mice received 20 μg of endotoxin 24 hours prior to ActD. Hypertransfused mice received 0.9 to 1.0 ml of packed washed red blood cells 30 and 6 hours prior to ActD. Control mice received no pretreatment prior to ActD. Zero μg ActD points represent mice injected with 0.5 ml saline after specified pretreatment. Each point is the result of 2 experiments in which the femoral marrow of three mice was pooled and plated in sextuplicate.

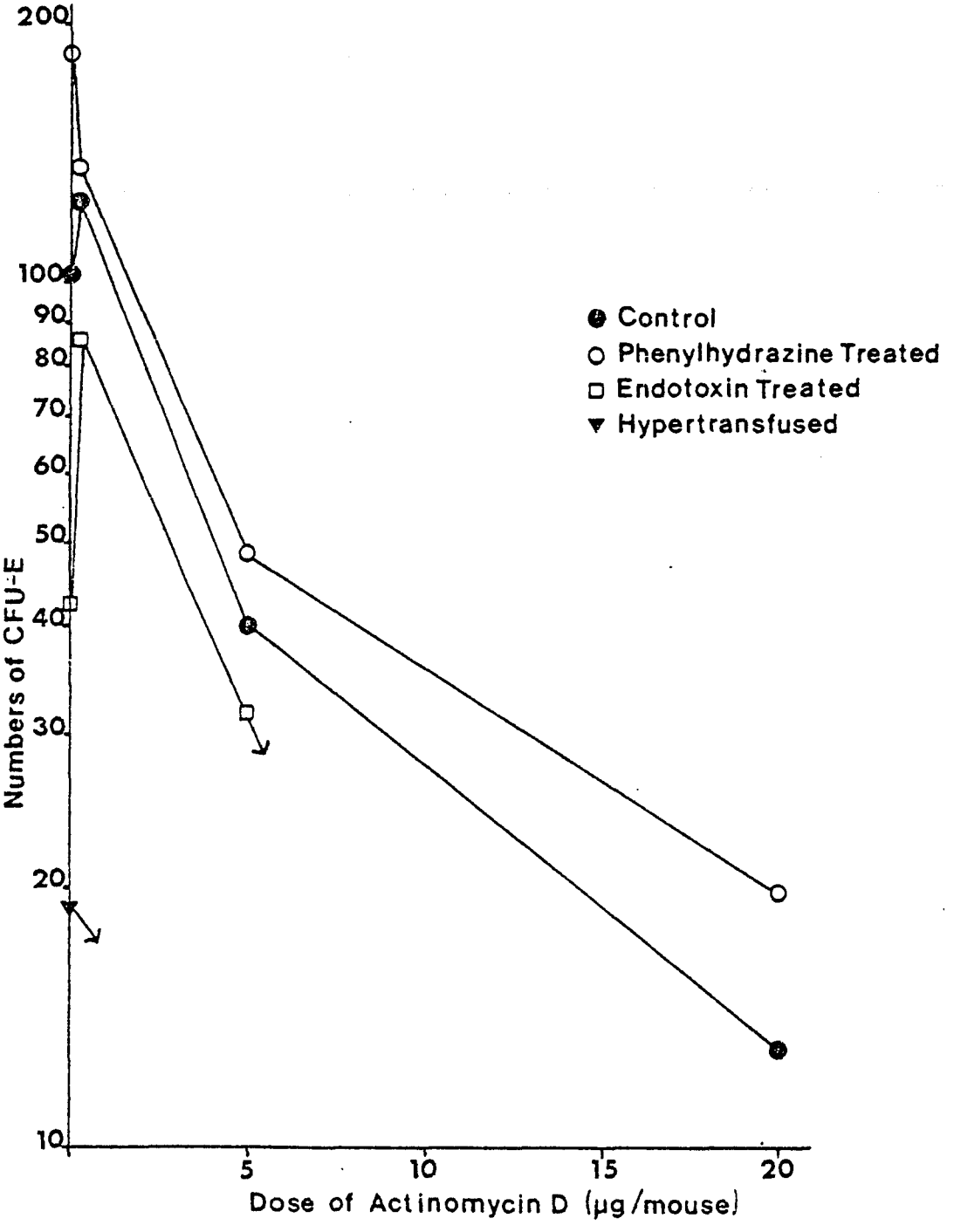
200
100
90
80
70
60
50
40
30
20
10
Numbers of CFU-E
5
10
15
20
Dose of Actinomycin D (μg/mouse)
Control
Phenylhydrazine Treated
Endotoxin Treated
Hypertransfused

Figure 15

The numbers of CFU-C 16 hours after various doses of ActD in pretreated mice. Pretreatments were as described in Figure 14. Each point is the result of 2 experiments in which the femoral marrow of three mice was pooled and plated in quadruplicate.

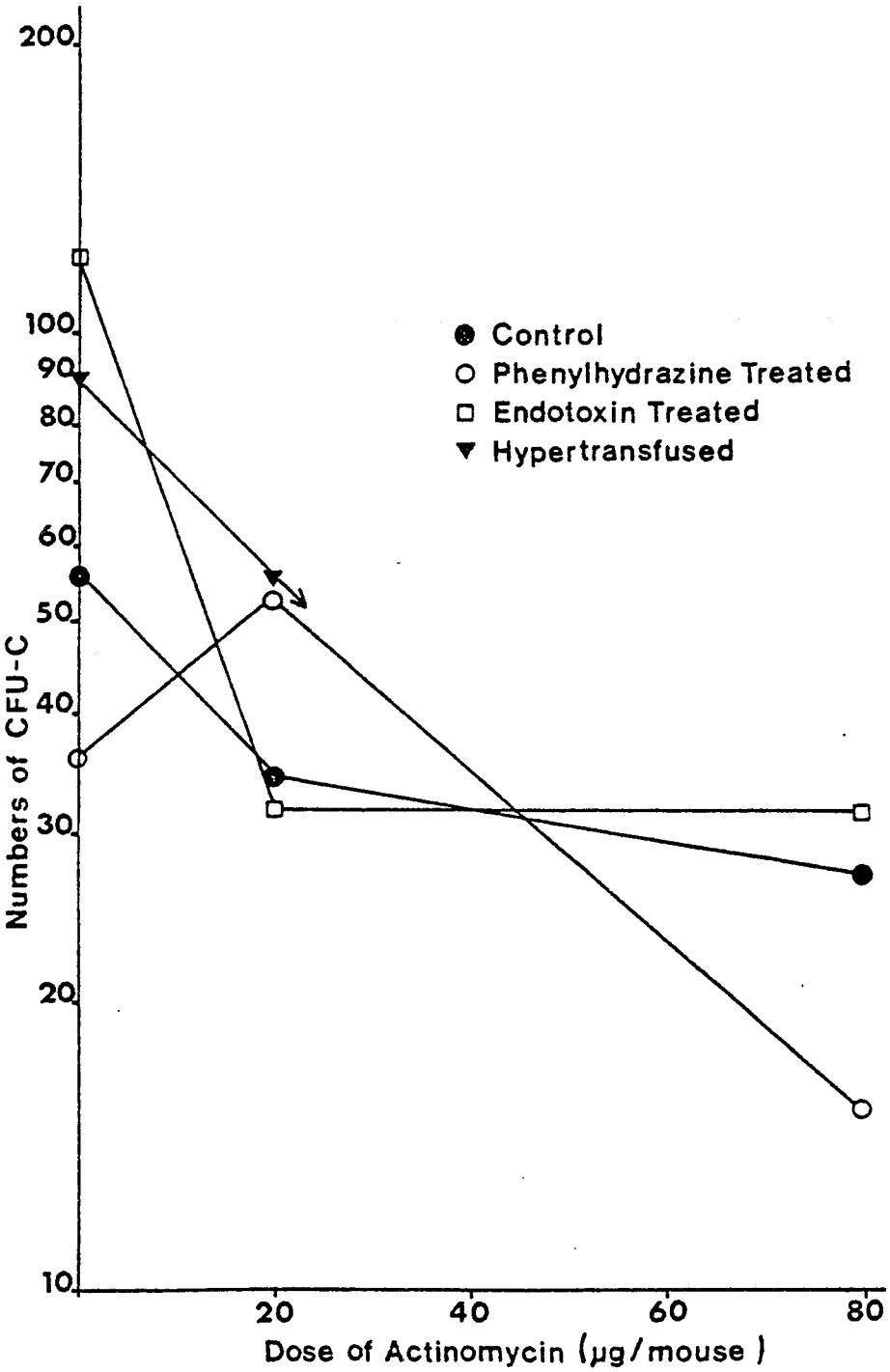
200
100
90
80
70
60
50
40
30
20
10
Numbers of CFU-C
Control
Phenylhydrazine Treated
Endotoxin Treated
Hypertransfused
20
40
60
80
Dose of Actinomycin (µg/mouse)

Figure 16

The numbers of BFU-E 16 hours after various doses of ActD in pretreated mice. Pretreatments were as described in Figure 14. Each point is the result of 2 experiments in which the femoral marrow of three mice was pooled and plated in quadruplicate.

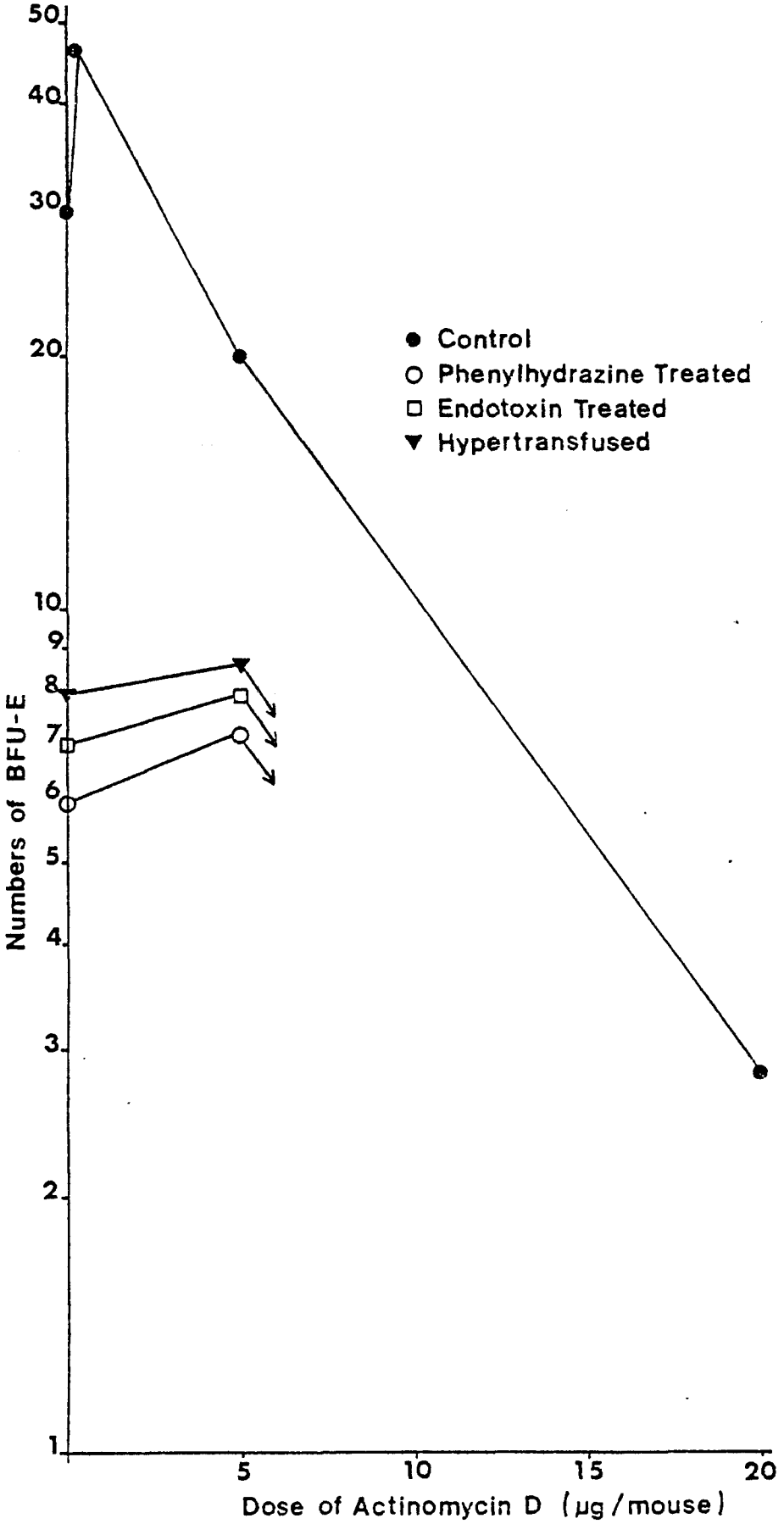
50
40
30
20
10
9
8
7
6
5
4
3
2
1
Numbers of BFU-E
Control
Phenylhydrazine Treated
Endotoxin Treated
Hypertransfused
5
10
15
20
Dose of Actinomycin D (μg/mouse)

TABLE 1

The Effect of β-Mercaptoethanol on CFU-E				
Erythropoietin	β-mercaptoethanol	Mean	±	s.e.m.'
-	-	8.0	±	1.7
+	-	81.8	±	2.7
-	+	75.4	±	3.3
+	+	175.7	±	7.0

' The mean number of CFU-E in 6 clots prepared with the pooled marrow of three mice femora.

TABLE 2

The Effect of Antiserum* to Erythropoietin on β-Mercaptoethanol and CFU-E						
Epo	β-m	Anti-serum	Control Serum	Mean**	±	S.E.M.
-	-	-	+	4.3	±	0.8
+	-	-	+	63.5	±	4.0
-	+	-	+	19.0	±	2.0
+	+	-	+	81.8	±	4.9
-	-	+	-	0		-
+	-	+	-	0.2	±	0.2
-	+	+	-	0.2	±	0.2
+	+	+	-	1.5	±	0.6

* Antiserum was diluted 1:6.

** The mean number of CFU-E in 6 clots using the pooled marrow of four mice.

TABLE 3

The Effect of 0.1 μg Actinomycin D / Mouse			
Hours After Actinomycin D	Numbers of Colonies Mean*	±	s.e.m.
2	82.7	±	5.1
4	63.5	±	3.0
6	70.8	±	4.7
17	86.0	±	4.2
23	81.2	±	3.2
48	73.7	±	2.7
Control	89.8	±	7.4

* The mean of number of CFU-E in 6 clots prepared from the pooled femoral marrow of 3 mice at each time point.

TABLE 4

The Effect of 4 injections of 0.2 μg ActD Every 12 Hours Compared to Single Injection		
	% Control After	
~Time After Last Injection (Hrs.)	Single Injection 0.2 μg ActD*	4 Injections of 0.2 μg ActD**
6	55.5	69.7
24	82.1	86.4
48	88.5	87.3

* Values taken from Fig. 5.

** Percent control values are based on 2 experiments in each of which 6 clots were plated from the pooled marrow of 3 mice femora. The mean ± s.e.m. for uninjected controls in the "single injection" experiment was 71.4 ± 3.2 and was 77.6 ± 3.0 for the multiple injection experiments.

TABLE 5

The Effect of ^{3}H-Thymidine on Hematopoietic CFU			
	Mean Numbers of Colonies		
	CFU-C′	CFU-E″	BFU-E‴
- ^{3}H-Thymidine	52.7	36.2	26.8
+ ^{3}H-Thymidine	27.2	6.7	0.8

′ 10^5 cells plated in quintuplicate (2 experiments).

″ 5×10^5 cells plated in sextuplicate (3 experiments).

‴ 2.5×10^5 cells plated in quadruplicate (2 experiments).

TABLE 6

The Effect of 2 Daily ip Phenylhydrazine* Injections on Hematocrit			
	Hematocrit (Mean ± S.D.)		
Control	50.8	±	3.3
Day After First Injection			
1	39.0	±	1.6
2	35.2	±	5.0
3	35.7	±	4.5

* 1.8 mg/30 g mouse was injected on day 0 and day 1 into 6 mice. Hematocrits were taken on day 0 before injection and on days 1, 2, 3, 5 and 8. The values given here are for days 1, 2 and 3 only.

TABLE 7

The Effect of Hypertransfusion on Hematocrit*			
	Hematocrit (Mean ± S.D.)		
Control	48.4	±	3.9
Day After 1st Transfusion	64.5	±	7.3
Day After 2nd Transfusion	78.9	±	4.2

* Means represent hematocrits of 27 mice measured prior to and after two transfusions 24 hours apart.

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