MODIFICATION OF ERYTHROPOIESIS IN RATS
AS A RESULT OF MYLERAN ADMINISTRATION

by

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ABSTRACT

MODIFICATION OF ERYTHROPOIESIS IN RATS

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The effect of myleran on red blood cell production in the rat was measured by following erythrocyte concentrations, total numbers of erythrocytes, reticulocytes, total numbers of reticulocytes, blood volume and hematocrit for 22 days following injection. Data showed that 1) Myleran-treated rats demonstrated a weight loss at day 15. A slow recovery follows and normal values are reached at day 21. 2) Normal 200 gram rats exhibit increased erythropoietic activity and growth with increases in body weight. These changes are associated with increases in blood volume, total numbers of erythrocytes, and hematocrit. 3) Suppression of erythropoietic tissue by myleran apparently did not cause any observable effects on circulating blood cell parameters, with the exception of reticulocytes, until day 12. 4) The greatest observable effects occurred at days 16 to 22. Significant reductions occurred in hematocrit (p=0.05), and total numbers of erythrocytes (p=0.01) at day 16 and in erythrocyte concentrations (p=0.05), hematocrit (p=0.01), blood volume (p=0.01) and total number of erythrocytes (p=0.01) at day 22.

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CHAPTER I

INTRODUCTION

The past twenty years has been revolutionary in the understanding and description of processes regulating red blood cell production in mammals. During this period experimental techniques have been developed which detect the physiological properties of morphologically unrecognizable cells. Hence, knowledge about erythropoiesis is now more detailed. Figure 1. presents a current model of erythropoiesis.

This is a modification of a model for erythropoiesis proposed by McCulloch and Till.[47] The circles and squares represent cycling and resting states of the cell populations respectively. The majority of pluripotent stem cells (CFU'S) are in G_0 and can undergo a reversible transition with a small population of proliferating cells, capable of self-renewal.

Irreversible transitions of stem cells produce erythroid committed progenitor cells. These ESC's maintain high proliferative rates even in the absence of erythropoietin. The maturing, morphologically recognizable cells comprise the last compartment and represent the erythroid cell lineage. Erythropoietin accelerates both differentiation on ESC's and recognizable RBC precursors.

STEM CELLS

The in vivo spleen colony assay method developed by Till and McCulloch[69] has provided much information regarding hemopoietic stem cells. Briefly, suspensions of known numbers of nucleated bone marrow

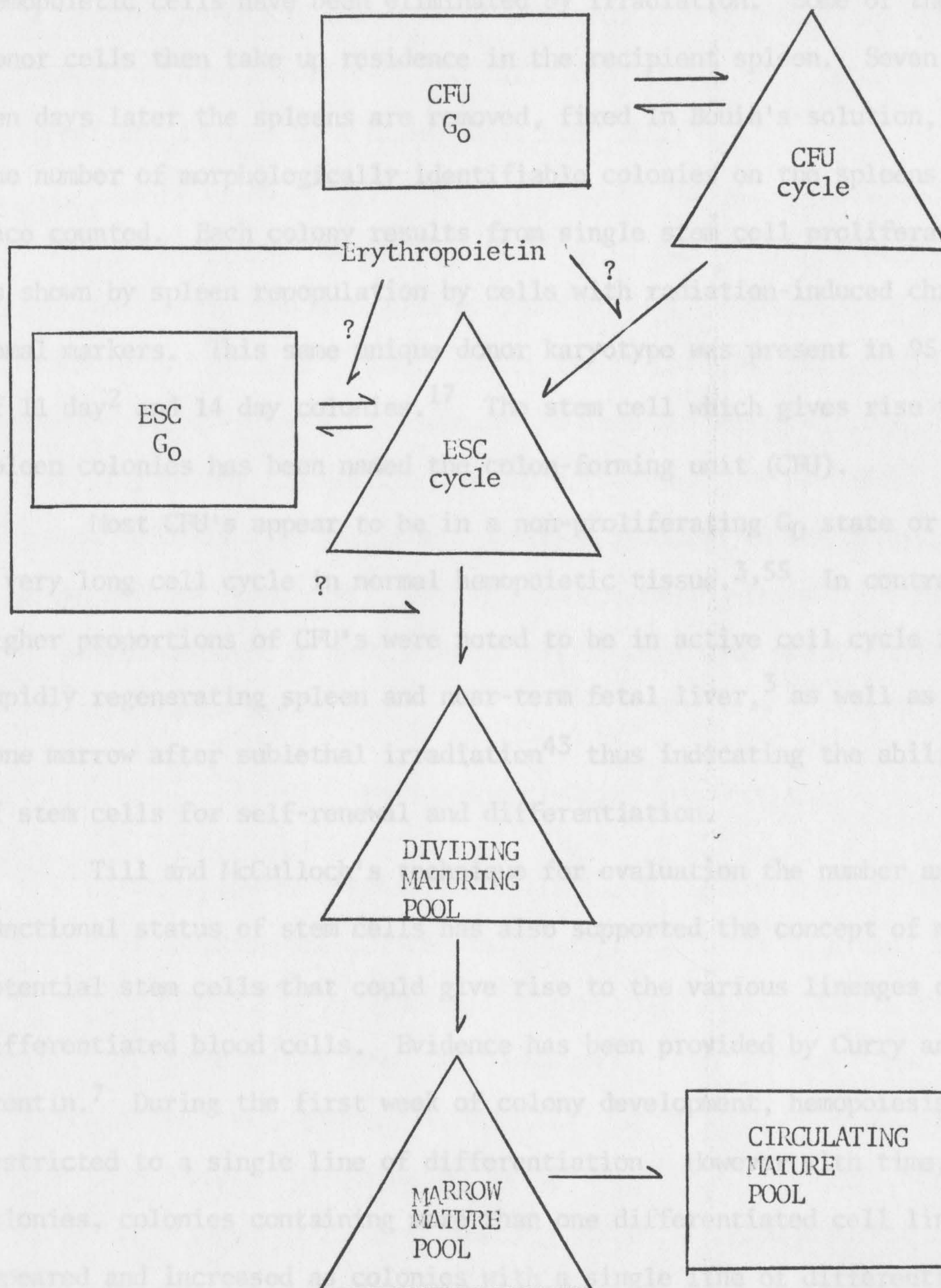

Figure 1. Model for Erythropoiesis.

cells are injected intravenously into recipients whose proliferating hemopoietic cells have been eliminated by irradiation. Some of the donor cells then take up residence in the recipient spleen. Seven to ten days later the spleens are removed, fixed in Bouin's solution, and the number of morphologically identifiable colonies on the spleens surface counted. Each colony results from single stem cell proliferation as shown by spleen repopulation by cells with radiation-induced chromosomal markers. This same unique donor karyotype was present in 95 to 99% of 11 day[2] and 14 day colonies.[17] The stem cell which gives rise to spleen colonies has been named the colon-forming unit (CFU).

Most CFU's appear to be in a non-proliferating G_0 state or in a very long cell cycle in normal hemopoietic tissue.[3,55] In contrast, higher proportions of CFU's were noted to be in active cell cycle in rapidly regenerating spleen and near-term fetal liver,[3] as well as in bone marrow after sublethal irradiation[43] thus indicating the ability of stem cells for self-renewal and differentiation.

Till and McCulloch's technique for evaluation the number and functional status of stem cells has also supported the concept of multipotential stem cells that could give rise to the various lineages of differentiated blood cells. Evidence has been provided by Curry and Trentin.[7] During the first week of colony development, hemopoiesis was restricted to a single line of differentiation. However with time, mixed colonies, colonies containing more than one differentiated cell line, appeared and increased as colonies with a single line of differentiated cell (homogenous colonies) enlarged and underwent second and third lines of differentiation. Transplantation of pure erythroid colonies (primary colonies) into a second group of irradiated mice still produced

all the various hemopoietic cell colonies (secondary colonies).[37,44] It appears as if the character of the secondary colonies was not dependent on the character of the cells from the primary colonies thus supporting the CFU as a pluripotent stem cell.

Work by Wu et al,[71] using radiation-induced chromosomal markers and recognizable differentiated fuctions of the erythrocytic or granulocytic series, provided good evidence for the existence of a pluripotent hemopoietic CFU. They found that of the 17 colonies examined, 90% or more of the dividing cells carried the same abnormal karyotype. In analyzing colony composition, he found that in 9 out of the 12 colonies metaphase cells with peroxidase-positive granules in the cytoplasm (characteristic of granulocytic cells) and metaphase cells which contained ^{55}Fe (characteristic of erythrocytic cells) were both present with the same chromosome marker.

ERYTHROPOIETIN-SENSITIVE CELLS

Erythropoietin-sensitive cells (ESC) (1) are more primitive than erythroblasts and (2) respond to erythropoietin by giving rise to erythroblasts.[40] The presence of erythropoietin-sensitive cells was first suggested by Bruce and McCulloch. They observed that an increased wave of erythropoiesis in four day hypoxic mice did not result in a decrease in the number of splenic CFU's. A major decline in CFU's followed 10-15 days later.[5] Stohlman[68] also observed a similar effect and concluded that erythropoietin did not act on the pluripotent stem cell[6,52,63,64] but rather on a descendant or progeny of the CFU.[5,63,64]

Subsequent experiments have confirmed the existence of the ESC.

It was found that injection of tritiated thymidine into polycythemic rats prior to erythropoietin administration produced a large number of labelled erythroblasts.[27] However when lethal amounts of the isotope was used (tritiated thymidine suicide), the erythropoietin response was drastically reduced to 30%.[56] Thus it appeared that a large number of erythropoietin-sensitive cells were in active cell cycle (in DNA-synthesis phase), in contrast to CFU's.

In development of the cells in the colonies and their maturation into erythrocytes occurred prior to the tenth day of examination, there would be a direct decrease in CFU's. However, Schooley[63] found that daily injection of erythropoietin into polycythemic hosts did not decrease the number of spleen colonies ten days after transplantation and therefore concluded that erythropoietin did not act directly on CFU's but upon some progeny of the CFU-the ESC. Similarly, Reissmann and Samorapoompichit[59] were unable to get a CFU decrease after stimulating with erythropoietin in mouse bone marrow using 5-fluorouracil to eradicate erythroid cells. They did however note a marked acceleration in erythroid regeneration and attributed it to the effect of the hormone on an induced inflow of precursors into the ESC population or a stimulation of ESC cell division.

DIFFENTIATING EFFECT OF ERYTHROPOIETIN

Erythropoietin was first shown to stimulate erythropoiesis in transfusion-induced polycythemic starved rats and hyperoxic rats which were injected with plasma from anemic animals.[36] Erythropoietin caused significant increases in ^{59}Fe incorporation into red blood cells.

Other studies[12,53] provided evidence that a single injection

of erythropoietin acts on the undifferentiated stem cells or an erythropoietin-committed cell in polycythemic mice. Serial observations on the spleen revealed an orderly sequential appearance of a proerythroblasts peak at 24 hours followed by a normoblasts peak at 72 hours and culminating at 72 hours with a reticulocytosis in the peripheral blood.

Corresponding to the morphological events previously cited, a very rapid increase in tritiated uridine incorporation into RNA occurred within 5 minuted after erythropoietin administration. This effect was also demonstrated in bone marrow cultures treated with erythropoietin preparations.[38] However when actinomycin D was added before or shortly after erythropoietin addition, no labelled RNA production was noted, possibly indicating that the hormone appears to effect DNA-dependent RNA synthesis.[19,38]

Nakao et al studied a series of enzymatic activities of heme synthesis in stem cells and noted that delta-aminolevulenic acid synthetase (ALAS), an enzyme which condenses glycine and succinyl CoA to form delta-aminolevulinic acid (ALA) increased within 8 hours in mouse spleen after erythropoietin addition. Actinomycin D when administered with the hormone abolished ALAS activity, further suggesting the action of erythropoietin to dependent of DNA-directed syntheisis.[50] ALAS has also been demonstrated in rat marrow mitochrondrial preparations.[34] It has been suggested that erythropoietin may act as a derepressor allowing genic expression of ESC differentiation into erythroid cells.[40]

Twelve hours after erythropoietin injection an increase in labelled DNA occurred and reached maximum values at 24 and 48 hours as observed in spleens of erythropoietic depressed hypertransfused mice.[30,61]

Powser and Brown[57] and Erslev[10] report increased DNA values with *in vitro* erythropoietin treatment of bone marrow cells.

By arresting erythroid cells in metaphase, Matoth *et al*[46] and Necheles *et al*[51] demonstrated that sheep erythropoietin or sera from anemic patients caused a significant increase in the mitotic index of erythroblasts in human bone marrow cultures. It was concluded that erythropoietin stimulated progenitor cells into mitotic division. The initial induction of erythropoietin-sensitive cells however appeared not by cell division. Schooley[62] showed that treatment of erythroid cells with colchicine, although causing inhibition of their proliferative activity, did not block induction of ESC by erythropoietin.

EFFECTS OF ERYTHROPOIETIN ON RECOGNIZABLE RBC PRECURSORS

Erythropoietin also plays a role in accelerating erythroblasts maturation and releasing marrow reticulocytes into the blood.[40] Fischer[13,15] reported various effects in starved rats and rabbits after erythropoietin administration. These animals showed a significant uptake of ^{59}Fe in plasma concentration decreased during this time. Since these newly formed red blood cells were released into circulation much earlier than expected if erythropoietin acted soley on erythropoietin-sensitive cell differentiation into proerythroblasts, he suggested that erythropoietin has more than one site of action in erythropoiesis, causing a direct effect on a reticulocyte releasing mechanism.

Hrinda and Goldwasser[32,33] found that this erythropoietin-

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induced stimulation of iron uptake is dependent on prior RNA and protein synthesis since actinomycin D, puromycin, and cyclohexamide all inhibited this uptake. Ferritin was identified as some of the protein fraction produced by erythropoietin stimulation. It has been suggested that erythropoietin induces the necessary hemoglobin machinery and the early increase in RNA could represent the required m-RNA synthesis.[54]

Many studies have reported significant increases in hemoglobin or heme synthesis in various erythroid cells (<u>in vitro</u> and <u>in vivo</u>) within 24-48 hours after treatment with erythropoietin.[18,33,39,51,54] The effect on reticulocytes, however, is not due to a direct action of the hormone.[18]

Erythropoietin has also been shown to directly stimulate reticulocyte release from bone marrow. Using an isolated perfusion technique on rat legs and femur, erythropoietin caused a considerable reticulocyte discharge into the perfusate 1-1½ hours after its addition. Further evidence that erythropoietin acts on already differentiated erythroblasts besides erythropoietin-sensitive cells, was observed in starved and hypertransfused animals-in which both conditions reduced the erythroid compartment. In both cases, reduced reticulocyte releasing effect was observed.[31,62]

ERYTHROPOIETIN

The first experiments demonstrating the site of erythropoietin production was done in 1957 by Jacobson <u>et al.</u>[35] It was observed that rats were unable to produce increased plasma levels of erythropoietin when subjected to various forms of hypoxia after a bilateral nephrectomy.[35,36] A number of other studies have confirmed the renal origin of erythropoietin (ESF or erythropoiesis stimulating factor).[16,49] However, it was found that perfused rabbit kidney released a very little ESF in the perfusate

unless it was incubated with plasma or serum.[26,41] The extraction of a renal factor from hypoxic rats and its intravenous injection or incubation with normal serum resulted in production of ESF.[21] It was therefore postulated that erythropoietin was produced by an erythropoietic factor released by the kidney and a substance present in plasma or serum.[45] The former has been named renal erythropoietic factor (REF) or erythrogenin and the latter referred to as a serum substrate or erythropoietinogen. The renal erythropoietic factor has been found in the light mitochondrial and micorsomal fractions of rat kidney.[48,65] It has also been identified in the renal tubules, cortex, medulla and glomeruli of rabbits, dogs, sheep, rats, pigs, and humans.[73]

Kinetic studies of the Ref-serum system have suggested that REF is an enzyme that converts the substrate erythropoietinogen into the active form of erythropoietin.[72]

Immunological evidence shows that the antibody capable of neutralizing ESF, produced in vitro by the interaction of REF and serum substrate, does not combine with either REF or serum substrate.[65]

Gordon and Zanjani[14,24,25] propose a scheme for the biogenesis of ESF (see Figure 2). In this scheme, renal tissue release erythrogenin when triggered by hypoxia, the fundamental erythrogenic stimulus. The interaction of erythrogenin with erythropoietinogen in the kidney and/or plasma results in the formation of ESF, thereby increasing red blood cell production[36] and marrow reticulocyte release[31] into the blood. There appears to exist a negative-feedback mechanism exerted by ESF on its own production by its ability to decrease the production and/or activity and/or release of the serum substrate.[23,74] Furthermore the increased numbers of circulating red cells that result from enhanced ESF production act in an inhibitory capacity on the synthesis and/or availability

of renal erythrogenin and the suppresion of ESF formation.

Physiochemical characterization of erythropoietin has been hampered by imperfect extraction techniques and its subsequent purification. Several studies, though, have demonstrated that ESF is resistant to boiling,[22] destroyed by trypsin[66] and consists of 65.5% protein, 13.0% hexoses, 8.9% hexosamine, and 7.5% sialic acid.[11] Amino acid composition reveals aspartic acid. threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, histidine, lysine, arginine, isoleucine, leucine, tyrosine, and phenylalanine to be present.[11] The carbohydrates identified are galactose, mannose, glucosamine, glucose and sialic acid.[20]

Molecular weight determination of the partially purified glycoprotein has led to variable results. Allen _et al_[1] proposes two molecular species for erythropoietin with molecular weights of 40,000 and 100,000. One researcher, using an inactiviation technique by ionizing radiation, reports a weight of 27,000 assuming an elongated shape or 66,000 assuming a spherical shape.[60] Another study reveals a molecular weight of 68,000 when using gamma ray inactivation.[29] Lukowsky and Painter[45] report a weight of approximately 60,000. And analysis of sheep plasma erythropoietin labelled with ^{125}I has a weight of 48,5000.[20]

The intent of this research involved depressing the erythropoietic system of albino rats with the radiomimetic chemical Myleran (Busulfan) and examining the effect on blood volume, red blood cell concentration, hematocrit, and circulating blood reticulocytes for 4,8,12,16 and 22 days after infection. The peripheral erythrocytic parameters measured during the drug-suppressive state of the erythroid tissue and its subsequent recovery are discussed in relation to the process of erythropoiesis.

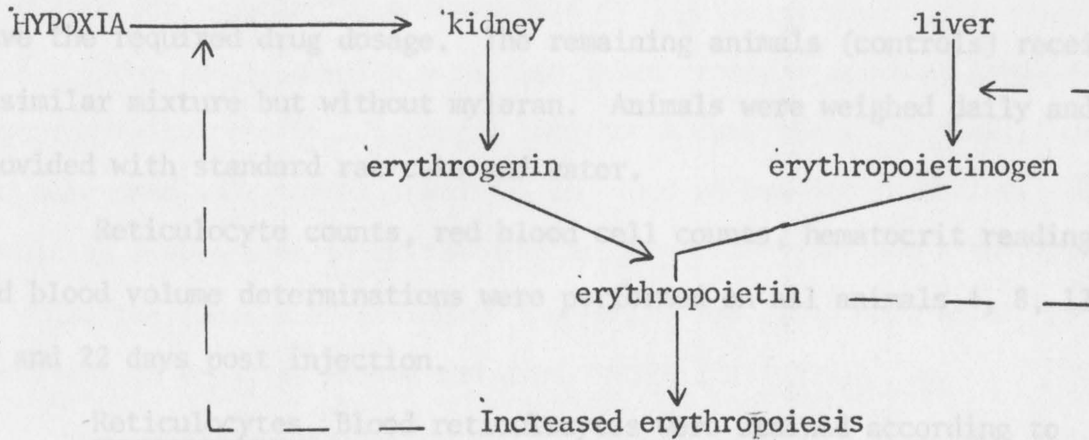


Figure 2. Scheme for biogenesis of ESF.

CHAPTER II

MATERIALS AND METHODS

Seventy 200±5 gram male albino rats (Wisconsin Holtzman strain) were used. Half received a single dose of Myleran (15 mg. per Kg.) via stomach tube. Myleran pills (Burroughs Welcome) were ground with a mortor and pestle and suspended by tragacanth in water (20 mg. tragacanth/ 1 ml. water) so the 1 ml. of the mixture introduced directly into the stomach gave the required drug dosage. The remaining animals (controls) received a similar mixture but without myleran. Animals were weighed daily and provided with standard rat cake and water.

Reticulocyte counts, red blood cell counts, hematocrit readings and blood volume determinations were performed on all animals 4, 8, 12, 16 and 22 days post injection.

Reticulocytes Blood reticulocytes were stained according to Bhuyran.[4] One drop of blood obtained by cardiac puncture was placed in a small glass tube in which 2 to 3 drops of a solution containing 120 mg% ammonium oxalate and 80 mg% potassium oxalate have been dried. Three drops of serum were added to a similar tube in which 3 drops of 1% new methylene blue in absolute alcohol have been dried and then the dye is dissolved in the serum. The latter contents were added directly to the tube containing the blood, agitated well and wet mounts prepared after 5 minutes. Reticulocytes were expressed as percentage of 1000 cells counted.

Red Blood Cell Counts Blood taken from the tail vein was diluted with formalin-saline-albumin solution[58] and red blood cells counted by means of a hemocytometer.

Blood Volume One half ml. of blood was taken by intracardiac puncture from an etherized rat and was transferred to a small culture tube containing 0.2 ml. acid citrate-dextrose solution (ACD). Twenty Ci of $Na_2{}^{51}CrO_4$ was then added to the blood and gently mixed. After 30 minutes of incubation at 37^0C, 0.5 ml. of the radioactive red blood cell suspension was reinjected back into the heart of the same anesthetized animal. Ten minutes later, 2 duplicate blood samples, each of 50 l., were taken from the tail vein and dispensed into individual heparinized hematocrit tubes, centrifuged and hematocrit determined. These blood samples were then used for radioactive measurements.

Four 50 l. samples were also taken from the original labelled erythrocyte suspension, dispensed into hematocrit tubes and centrifuged. Two were used as external standards in blood volume determination. The remaining 2 were used in determining tagging efficiency which involved freezing the tubes and cutting at the packed red blood cell interphase, resulting in separate plasma and erythrocyte sections.

All radioactivity counts of ^{51}Cr were made in a well type γ-scintillation counter using a 2 inch thallium activated sodium iodide crystal. Counts were made on duplicate samples for 2 minutes.

Method of calculating total circulating blood volume was based on the isotope dilution principle. These blood volume determinations were calculated from the equation:

$$\text{Blood volume (ml.)} = \frac{\text{Std x 0.5 ml.}}{A_{10}}$$

Where Std = activity (cpm) of the external standard,

0.5 ml. = volume of ^{51}Cr-labelled blood cell suspension

A_{10} = activity (cpm) of the blood sample obtained 10 minutes after labelled blood reinjection.

All counts were corrected for background readings.

Plasma volume was calculated from the total circulating blood volume measurements x (100 - hematocrit readings) and red cell volume was determined from blood volume x hematocrit readings/100.

Total red blood cell and reticulocyte values were obtained by multiplying erythrocyte and reticulocyte concentrations times blood volume values, respectively. Expressions of erythrocyte and reticulocyte numbers per gram of animal weight were derived by dividing the cell numbers by the animal weight as given in Appendix A.

Statistics The Student's "t" test was the principal test used in comparing the drug treated and control sample means as described in Steel and Torrie.[67] It was assummed that both populations were normally distributed and had a common variance. The weighted average of the sample variances, s^2, was used in calculating $s_{\bar{d}}$ because n, the number of observations was not equal in experimental and control groups. Thus the "t" test, $t = \frac{\bar{d}}{s_{\bar{d}}}$, where $\bar{d} = \bar{x}_1 - \bar{x}_2$ and $s_{\bar{d}} = s_{\bar{x}_1 - \bar{x}_2}$ = standard deviation appropriate to a difference between two random means from a normal population, was rearranged accordingly to

$$t = \frac{\bar{x}_1 - \bar{x}_2 \sqrt{\frac{n_1 n_2}{n_1 + n_2}}}{\sqrt{\frac{s_1^2 (n_1 - 1) + s_2^2 (n_2 - 1)}{(n_1 + n_2 - 1}}}$$

CHAPTER III

DATA

Erythrocytes (# cells/mm. blood)

Day	Experimental x (S.E.)	Control x (S.E.)	Calculated "t" value
4	665.8(27.3)	654.0(30.9)	0.285
8	646.5(29.6)	631.0(28.1)	0.785
12	707.2(22.2)	735.2(35.5)	1.137
16	707.2(22.2)	769.6(22.1)	0.853
22	669.8(22.8)	792.0(30.3)	2.282*

Total Circulating Erythrocytes ($x10^9$ cells)

Day	Experimental x (S.E.)	Control x (S.E.)	Calculated "t" value
4	77.3(3.8)	70.0(5.0)	1.161
8	93.1(6.9)	88.8(3.1)	0.645
12	98.5(8.6)	113.8(6.0)	0.279
16	86.9(9.8)	114.4(9.4)	4.060**
22	95.2(8.0)	132.1(10.9)	43.860**

Total Circulating Erythrocytes/gram of body weight ($x10^7$ cells/gm.)

Day	Experimental x (S.E.)	Control x (S.E.)	Calculated "t" value
4	34.6(2.1)	32.5(1.7)	0.768
8	38.4(2.9)	35.5(2.3)	0.802
12	38.0(3.2)	41.1(2.0)	0.839
16	30.1(2.5)	39.8(2.7)	2.659*
22	31.9(2.6)	43.6(3.3)	4.422**

Total Circulating Reticulocytes ($x10^9$ cells)

Day	Experimental x (S.E.)	Control x (S.E.)	Calculated "t" value
4	1.18(0.068)	2.50(0.026)	4.108**
8	8.38(2.300)	2.74(0.236)	2.766*
12	3.32(0.561)	4.04(0.560)	0.908
16	2.88(0.532)	3.96(0.456)	1.547
22	4.33(0.472)	4.25(0.561)	0.166

* - Significant at p=0.05
** - Significant at p=0.01

Total Circulating Reticulocytes/gram of body weight ($x10^7$ cells/gm.)

Day	Experimental $\bar{x}$ (S.E.)	Control $\bar{x}$ (S.E.)	Calculated "t" value
4	0.527(0.021)	1.159(0.102)	6.796**
8	3.452(0.937)	1.125(0.107)	2.791*
12	1.274(0.326)	1.457(0.192)	1.050
16	1.165(0.326)	1.377(0.140)	0.599
22	1.447(0.148)	1.402(0.175)	0.307
Blood Volume (ml.)			
4	11.68(0.81)	10.75(0.87)	0.785
8	14.50(1.39)	14.17(0.64)	0.243
12	13.84(0.81)	15.47(0.25)	1.756
16	12.37(1.54)	14.81(1.04)	1.309
22	14.71(0.48)	16.53(0.87)	3.064**
Hematocrit			
4	34.8(1.80)	37.0(2.12)	0.809
8	34.6(2.37)	38.3(1.50)	
12	36.2(1.49)	38.9(1.36)	
16	34.4(1.85)	41.9(1.57)	3.089*
22	34.0(1.34)	43.2(1.01)	7.889**

* - Significant at p=0.05
** - Significant at p=0.01

CHAPTER IV

RESULTS

The effect of Myleran

The changes in body weight of control and drug-treat animals are given in APPENDIX A.

Circulating Erythrocyte and Reticulocyte Cell Counts

Erythrocytes The changes occurring in red blood cell concentrations following myleran injection are presented in graph 3. Average values for erythrocyte concentrations do not deviate from control values until day 12, after which they plateau and drop off after day 16. No significant difference is evidence until day 22 when control values are considerably larger than experimental values.

The means (and S.E.) of the absolute numbers of erythrocytes are presented graphically in graph 4. The total red cell count following drug treatment maintains a level similar to control values until day 12. Thereafter highly significant differences (p=0.01) at both 16 and 22 days were observed. Absolute numbers at day 22 were slightly higher than day 16, but still less than day 12. Control values, as well as those of red blood cell concentration, reveal a progressive increase concommitant with the increase in body weight of control animals.

Essentially very similar results were obtained when these values were expressed as absolute numbers of circulating red blood cells per gram of body weight except that the apparent recovery of day 22 is less noticeable. See graph 5.

Graphs 11 and 12 show relative changes in total numbers of

circulating erythrocytes and erythrocyte concentration respectively. Total numbers of erythrocytes continually decline throughout the 22 days of the experiment. Erythrocyte concentration, with the exception at day 8, also declines throughout the experiment. In both cases, lowest values were obtained at day 22.

Reticulocytes Graph 6 illustrates that minimal numbers of these cells are noted at 4 days after myleran treatment. Subsequent to this, a temporary reticulocytosis ensues, approximately tripling the normal proportion of reticulocytes in the blood at day 8. Normal values are then retained throughout the duration of the experiment.

Analysis by Student "t" test reveals that the concentration of circulating reticulocytes of experimental animals are significantly different (p=0.05) at day 8 and highly significantly different (p-0.01) at day 4 when compared to values of control animals.

In graph 5, this is also the case when expressed in terms of blood reticulocytes based on a per gram weight basis.

Blood Volume Differences in blood volume between experimental and control groups are essentially unchanged until about day 12, when a suggestion of departure initially occurs as seen in graph 9. Four days later, the difference becomes more apparent as a lower volume is attained, although a statistical analysis fails to show significance. And finally at day 22, although blood volume of experimental subjects start to increase, there occurs a statistically lower volume (p=0.05) in experimental animals.

Graph 10 depicts the changes in plasma, blood, and red cell volumes after treatment with myleran. Values plotted relate relative changes in these parameters since they are expressed as per cent of control values obtained at the corresponding days. Essentially all values closely parallel each

other until day 16. At this time, plasma volume begins to increase and reaches above control values at day 22. Increases in blood volume and red cell volume do not occur until sometime after day 16. Thus, the apparent increase in blood volume at day 22 appears to result primarily from great increases in plasma and only secondarily from increases in cell volume.

Hematocrit

Hematocrit readings of control and myleran-treated animals are represented in graph 8. A steady increase in red cell volume is observed throughout the experiment in controls. These findings are consistent with an increase in weight, an increase in total numbers of erythrocytes and on a per weight basis. Average values for experimental rats undergo notable changes. Between 4 thru 8 days, essentially little change is evident. However, following a transient increase at day 12, there occurs a gradual decline until day 22 where values are at the lowest and are significantly different (p=0.05).

CHAPTER V

DISCUSSION

Femoral bone marrow examination following a single dose of myleran (15 mg./kg. of body weight) reveals a rapid decrease in the numbers of erythroblasts to nearly 40% of normal values 48 hours after drug administration.[9] Thereafter, erythroblast regeneration occurs until day 6, afterwich a profound depression of erythroblasts occurs with minimal counts of approximately 1% of normal values at day 11 to 13. A slow, but steady recovery follows until day 25 when normal erythroblasts levels are reached.

In this experiment the early effect of myleran on RBC precursors in the marrow was not apparent in the number of circulating erythrocytes. Due to the long life-span of erythrocytes, the temporary reduction in erythroblasts and the rate of delivery of mature erythroid cells into the circulation should not alter peripheral blood counts. In fact, graphs 4, 5, 9, and 12 indicate a rise in certain erythrocytic depression in the blood during this time.

Graph 3 however shows a slight decrease in both control and experimental erythrocyte concentrations at day 8. Control values decrease faster and are slightly lower than those of drug-treated animals. Interpretation of these results is ambiguous. However, most control values progressively increase with time throughout the experiment, indicating the increased erythrocyte concentration, as well as an increase in the total number of circulating red blood cells, total circulating RBC's per gram of animal weight, and percentage of RBC's in the blood imply an increased erythropoietic output with increasing animal weight (see Appendix A). This increase

in RBC's is generally reflected in an increased blood volume in control animals (graph 9).

Estimations of circulating reticulocyte numbers (graph 6), however do provide evidence of an effect due to myleran on erythropoietic tissue. Approximately 96 hours after myleran treatment, a highly significant reduction ($p=0.01$) in total circulating reticulocytes, followed by a significant reticulocytosis in the blood at day 8 ($p=0.05$) closely parallels marrow activity. This sequence of events is associated with the brief reduction of marrow erythroblasts at 48 hours and the subsequent abortive regeneration during days 2 to 8 of erythroblasts which have undergone maturation and release into the circulation. This agrees with other studies[8,9] when allowing for a total marrow transit time for erythropoiesis in the rat of 48 to 60 hours as reported by Harriss.[28] Thus the changes in the marrow precede those in the blood by 48 to 60 hours. Graph 7, expressing total circulating reticulocytes per gram of animal body weight also reflects the extent of damage to marrow erythroblast.

A more profound depression follows erythroblast regeneration with extensive depletion of erythroblasts reported at days 11 to 13.[9] Peripheral blood measurements reveal this event somewhat with some uncertainity. Graph 3 shows that the highest red cell concentration for drug-treated animals is obtained at day 12, with a very slight, almost unnoticeable decrease occurring at day 16, and a decrease at day 22. However this pattern may not reveal the true picture, since changes between the 8 to 12 day interval were not measured and the erythrocyte concentrations recorded at day 8 are ambiguous as previously discussed. Seven days after myleran, a rapid reduction of marrow erythroblast ensues and continues until day 12[70] or day 13.[9] Considering such results, then the erythrocyte concentration values

at day 8 could show an increase slightly below or equal to day 12 values resulting from the transient increase of erythroblasts in the marrow at day 4 to 6 and their subsequent release 2 to 2.5 days later into the blood. Thus the value at day 12 might represent a plateau in RBC concentration. This would be reasonable with the following observation. The decrease in blood volume, plasma volume, and red cell mass at day 12 (graph 10) could be attributed to severly depressed marrow precursors occurring about day 8, that is diminishing erythropoiesis as reflected by a leveling off of RBC concentration. The return of reticulocyte numbers to normal levels starting after day 8 and continuing for the remainder of the experiment are in agreement with other investigations.[9]

The depressant effect of myleran on peripheral blood measurements is most obvious at day 16. Significant decreases in the total number of circulating erythrocytes per gram of animal weight (graph 5) and in hematocrit reading (graph 8) reflect the degree of damage. The most drastic effect is on the total number of circulating erythrocytes. Such severe effects due to the failure of myleran-damaged stem cells to maintain erythroblast cells loss by differentation and to replace red cell loss is to a lesser extent shown in the marked reduction in blood volume, red cell mass, and erythrocyte concentration. Analysis by Toepfer[70] of total erythroid cells in the bone marrow reveal definite decreases in this cell population occurring within 4 days after myleran administration and reaching minimal values (approximately 60% of control values) at 12 days before a recovery period occurs. A similar pattern is reported by Elson[9] during this time. It therefore appears that observable effects on circulating mature erythrocytes occur about 16 days after injection. Up to this time, the little of not inflow of RBC's from bone marrow to the blood was possibly offset by the relatively long life span of the circulating erythrocytes. A decrease in animal body weight

is also present at this time (Appendix A) in myleran-treated animals, which is in agreement with other studies.[8,70]

At day 22, the blood response pattern shown in all the graphs can be associated with the reappearance of erythroblasts in myleran depleted bone marrow as described by Elson[58] and Toepfer.[70] Increases in marrow erythroblasts, although beginning at day 14, do not show substantial numbers until days 18 to 20 when they are approximately 50% of normal. In a second study,[70] normal values of erythroid cells in bone marrow occur two days earlier (16 to 18 days after injection). This study reveals a leveling off or a slight increase in all parameters measured except RBC concentration and hematocrit. Apparently these increases correspond to the maturation and release of the marrow erythroblasts into the blood. The decrease in erythrocyte concentration and hematocrit cannot be explained except that the slight increase in blood volume, perhaps mainly due to increased plasma volume rather than increased erythropoiesis, can account for the decreased values reported at day 22.

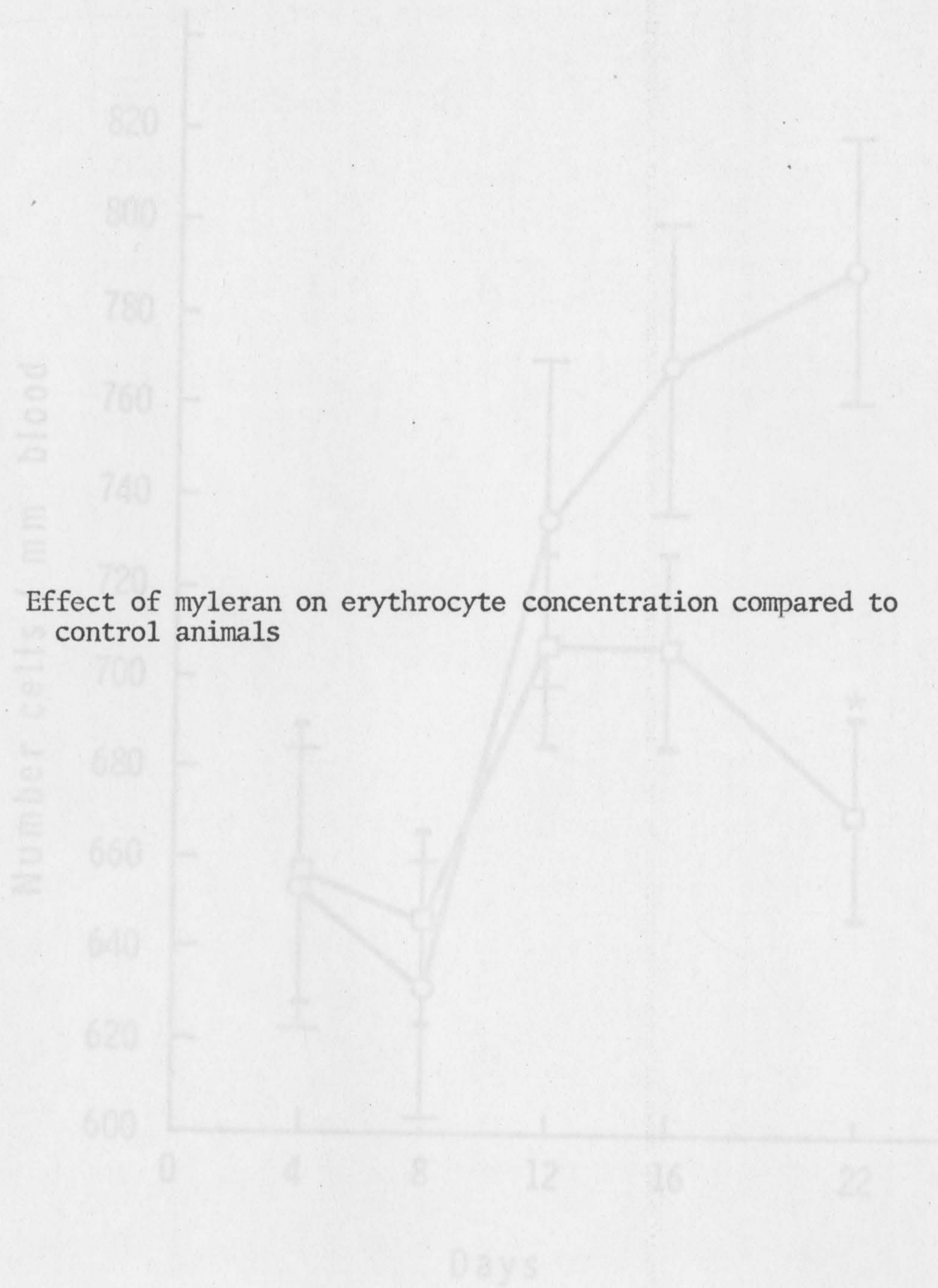
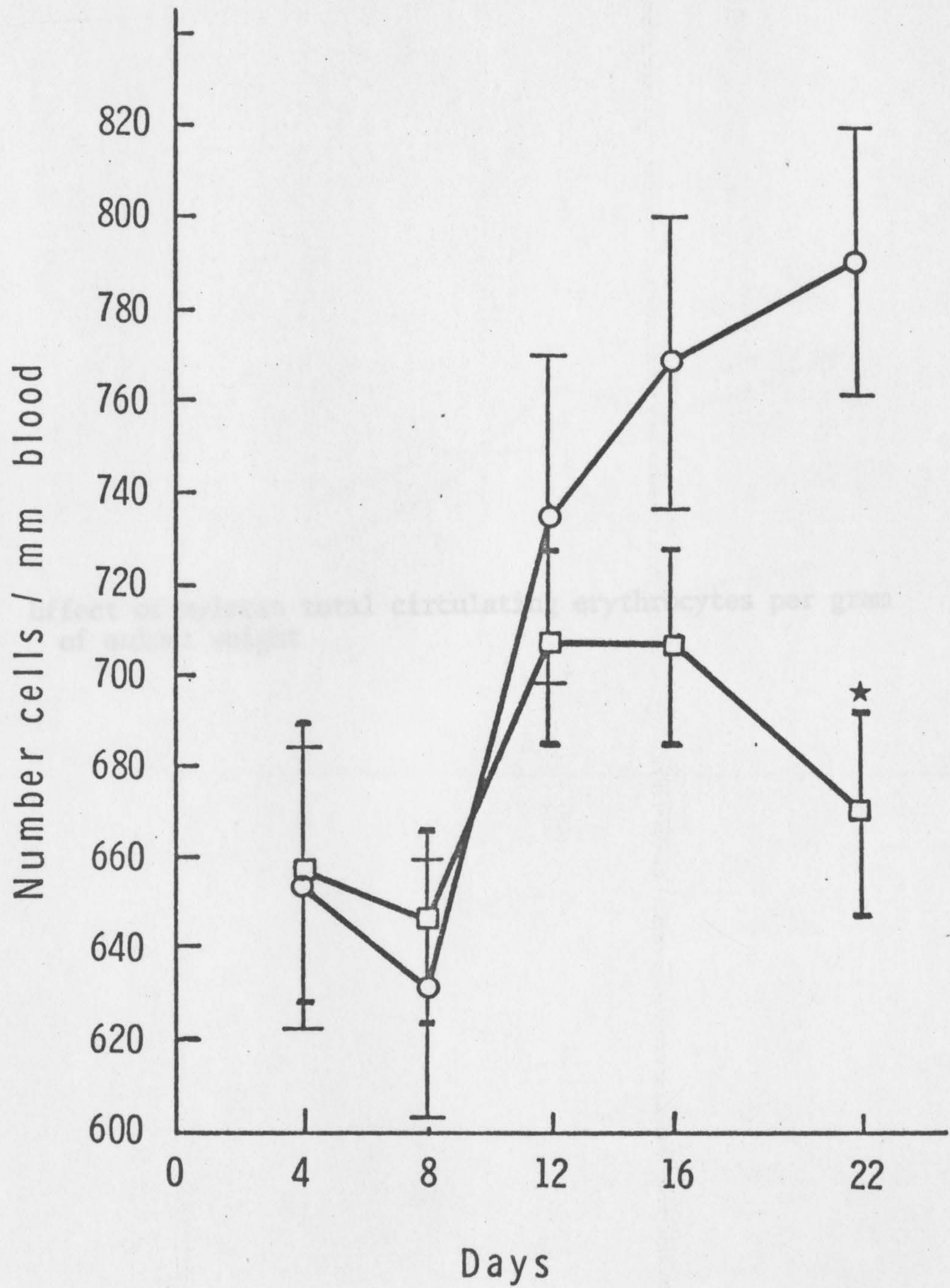


Figure 3. Effect of myleran on erythrocyte concentration compared to control animals


Number cells / mm blood
820
800
780
760
740
720
700
680
660
640
620
600
0
4
8
12
16
22
Days
★

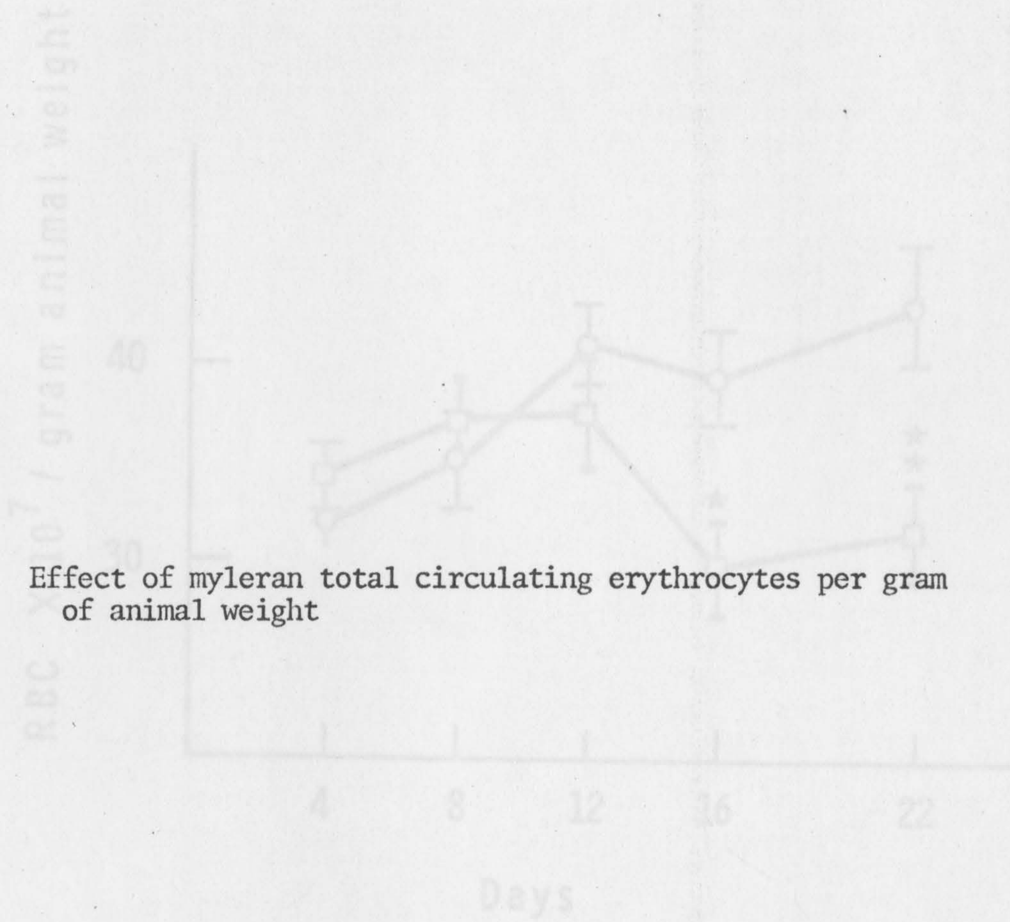
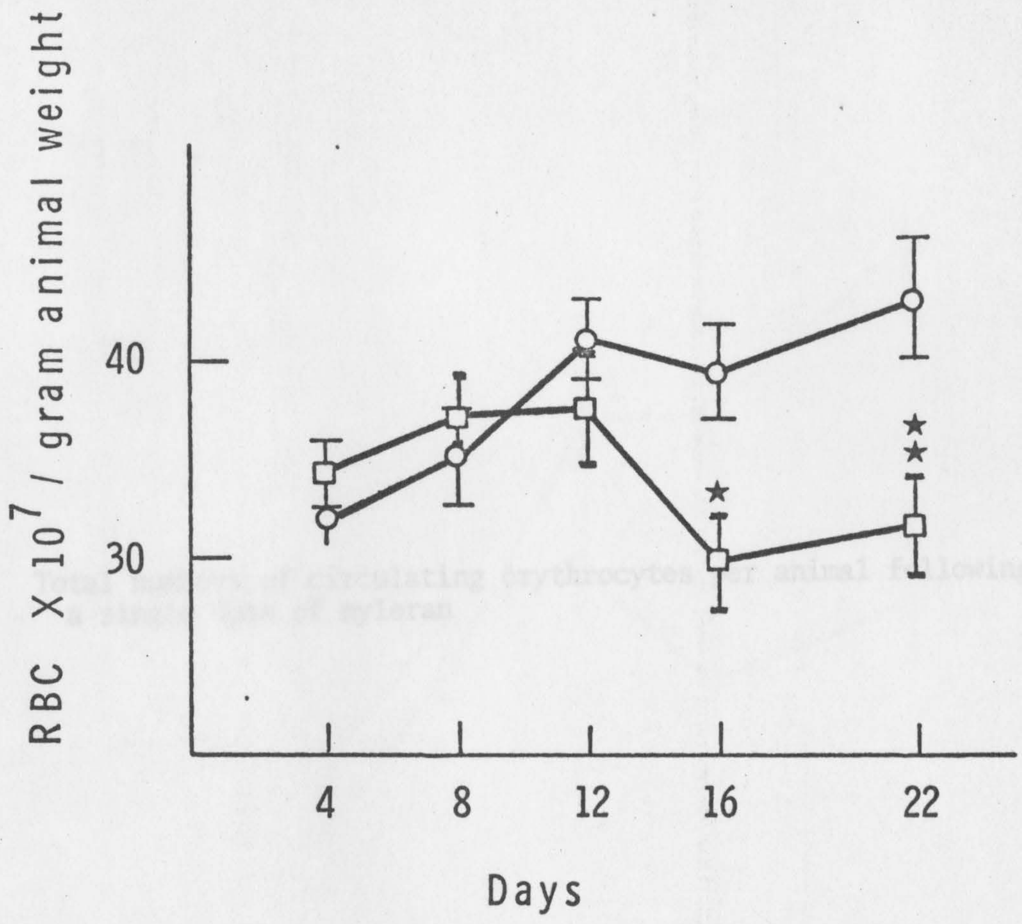


Figure 4. Effect of myleran total circulating erythrocytes per gram of animal weight


RBC X 10^7 / gram animal weight
40
30
4
8
12
16
22
Days

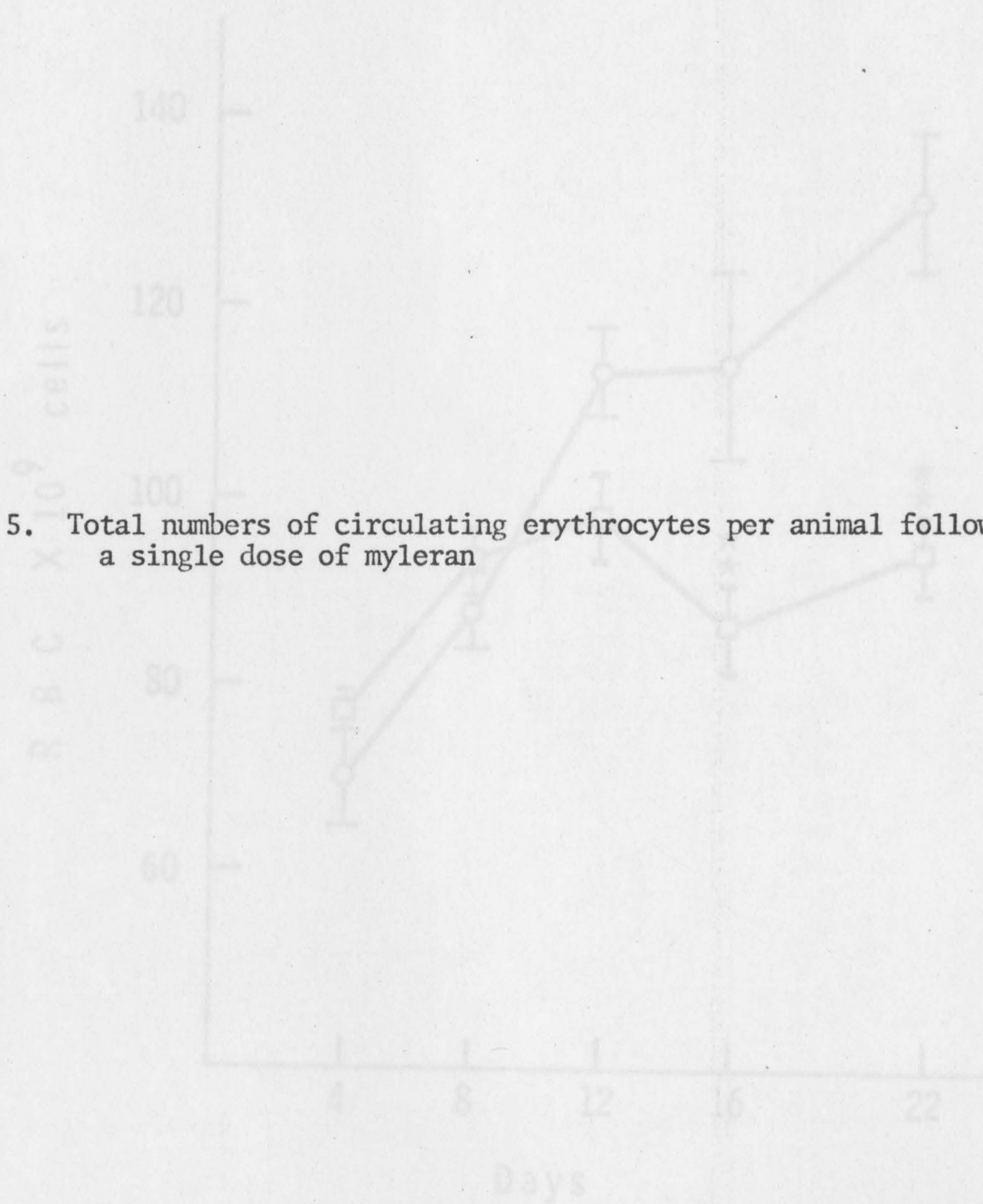
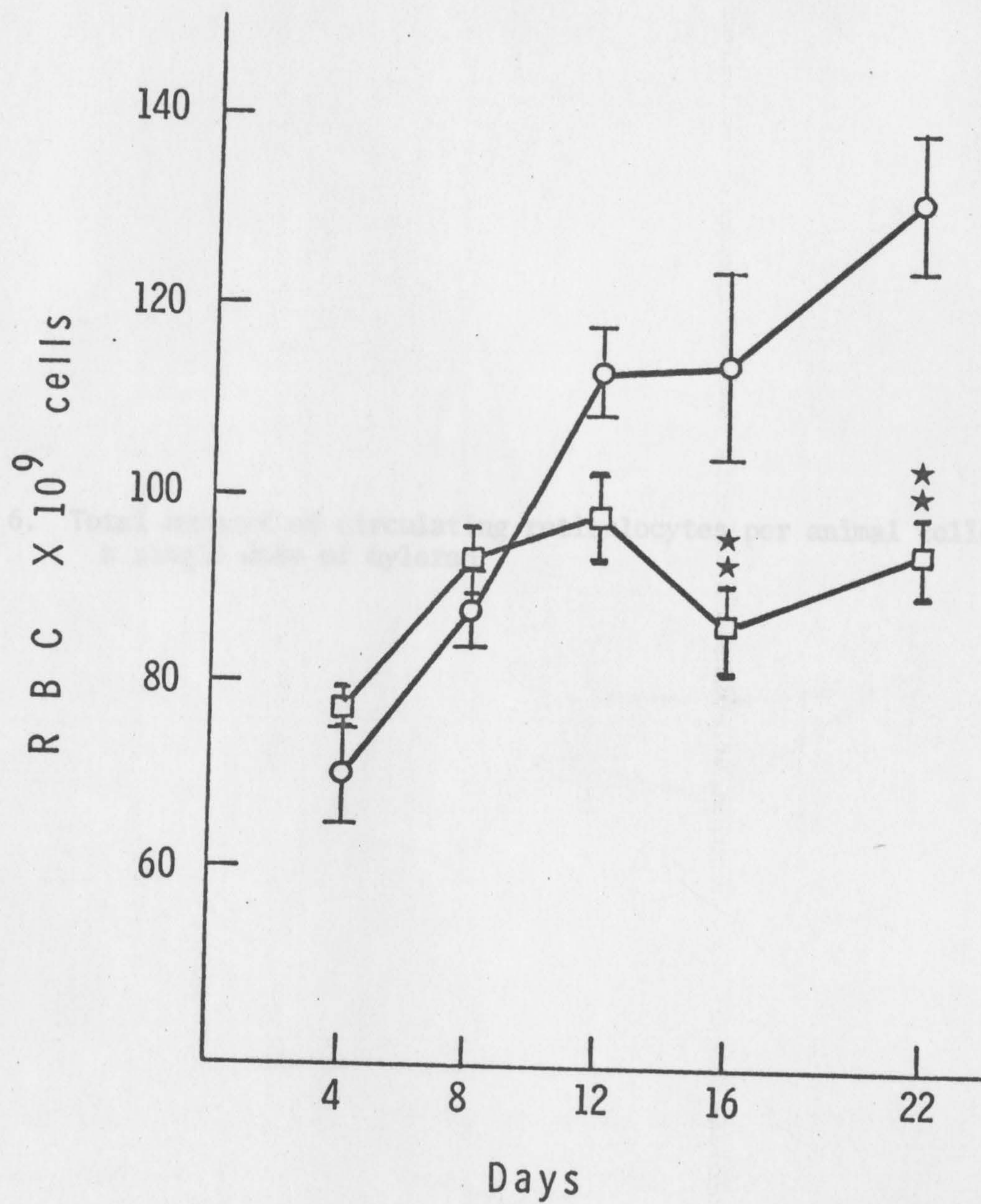


Figure 5. Total numbers of circulating erythrocytes per animal following a single dose of myleran


140
120
100
80
60
R B C X 10⁹ cells
4
8
12
16
22
Days

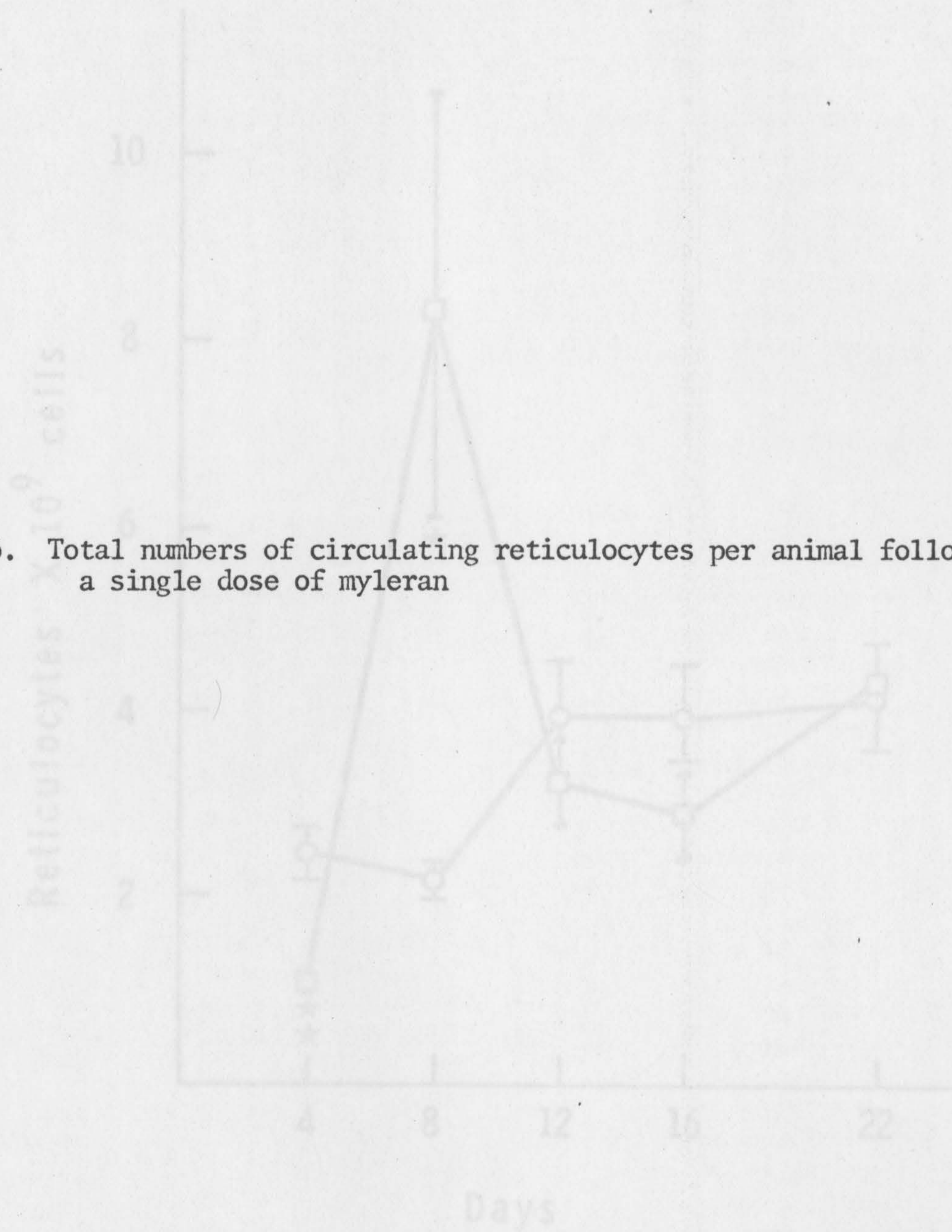
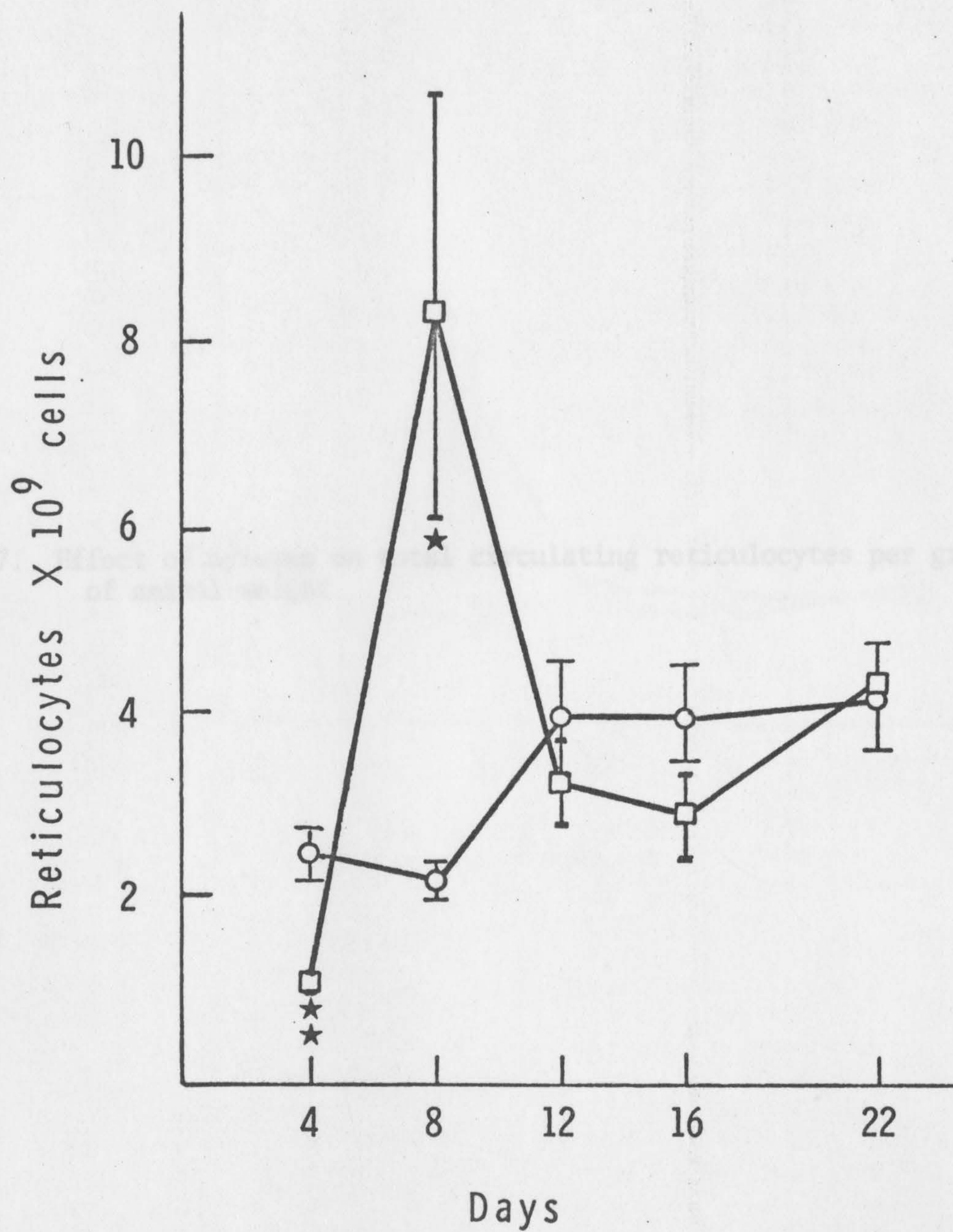


Figure 6. Total numbers of circulating reticulocytes per animal following a single dose of myleran


10
8
6
4
2
Reticulocytes X 10^9 cells
4
8
12
16
22
Days

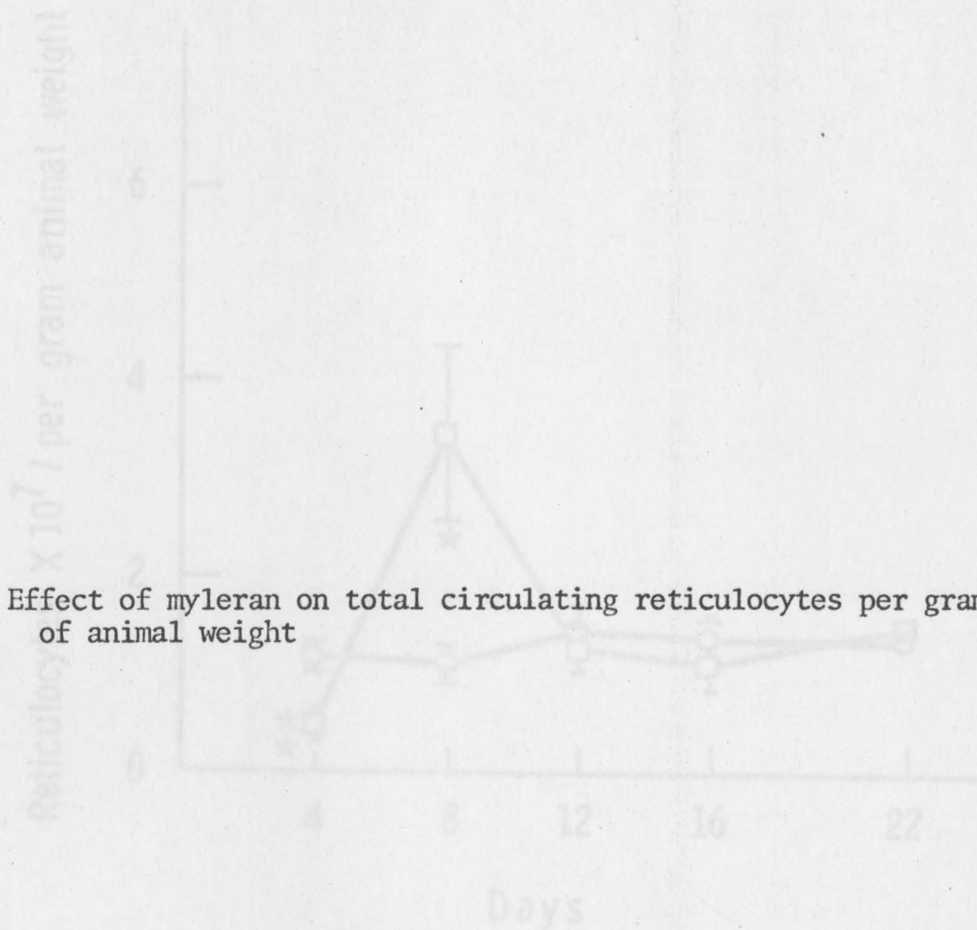
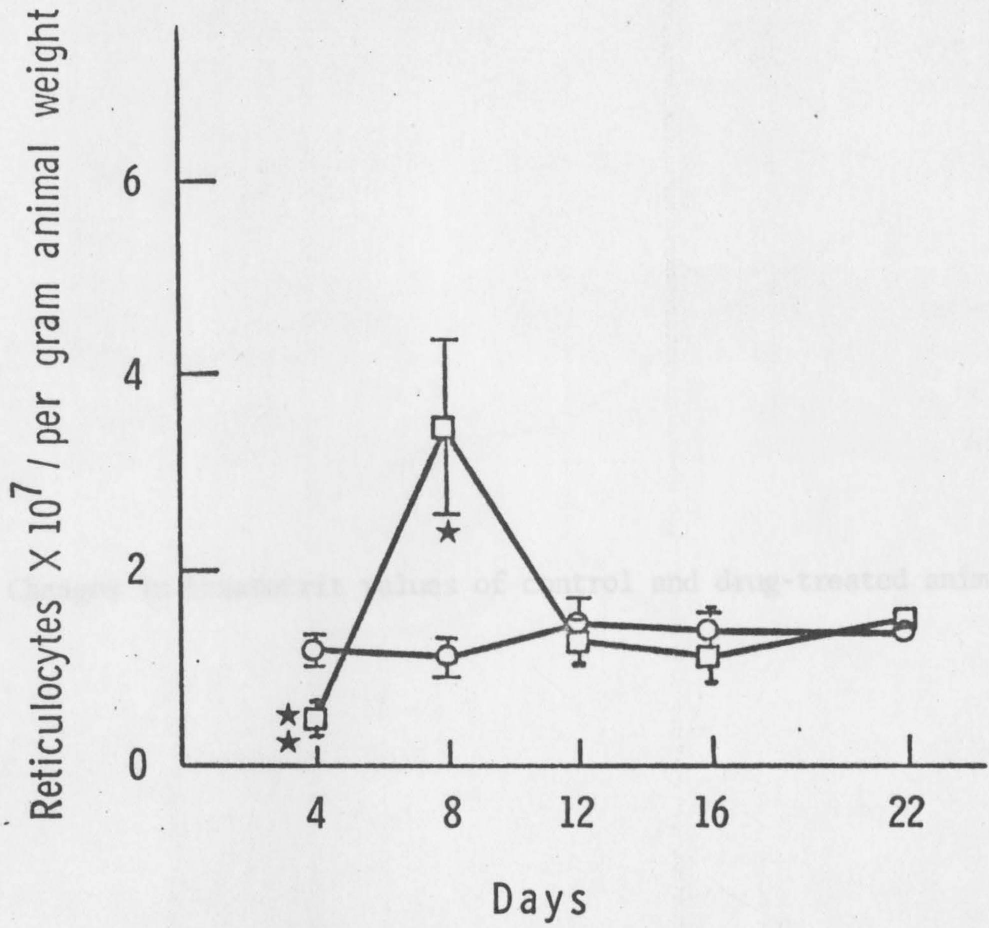


Figure 7. Effect of myleran on total circulating reticulocytes per gram of animal weight


Reticulocytes X 10^7 / per gram animal weight
6
4
2
0
4
8
12
16
22
Days

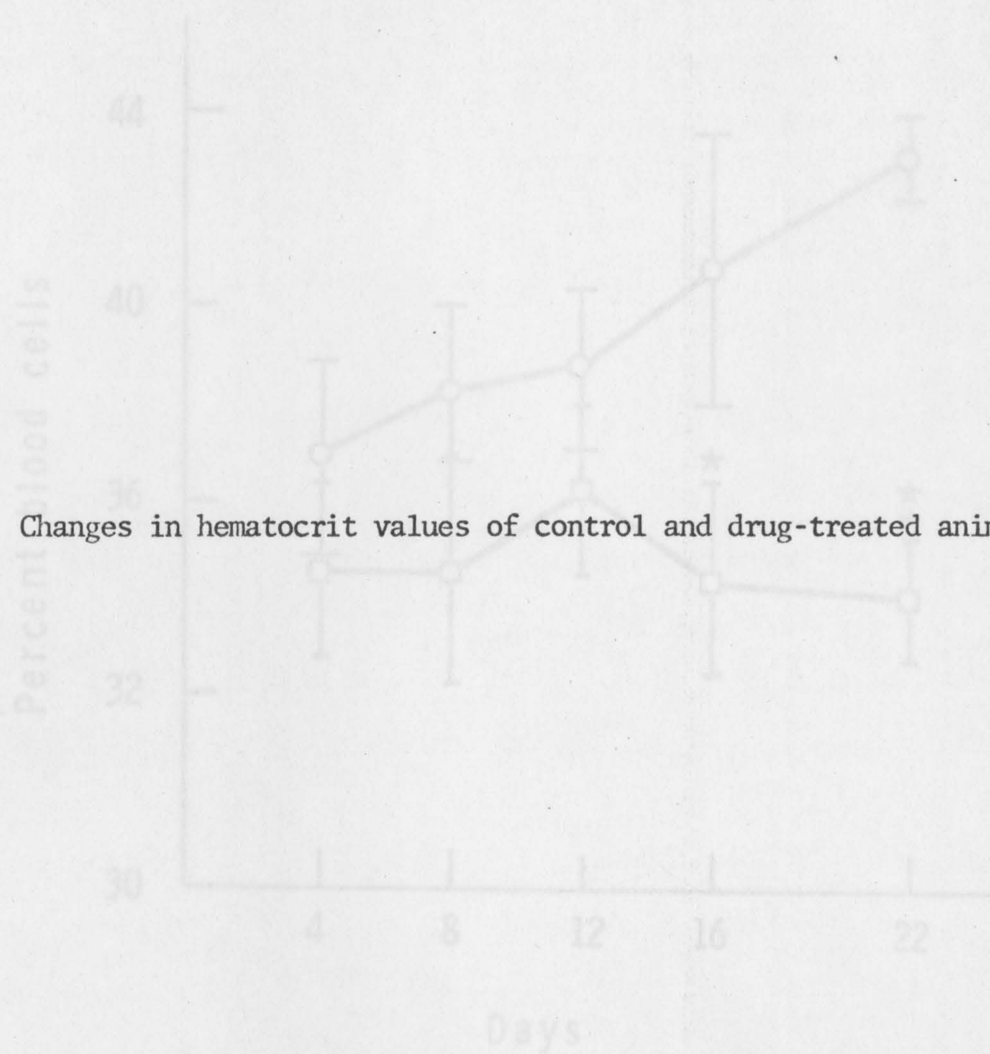
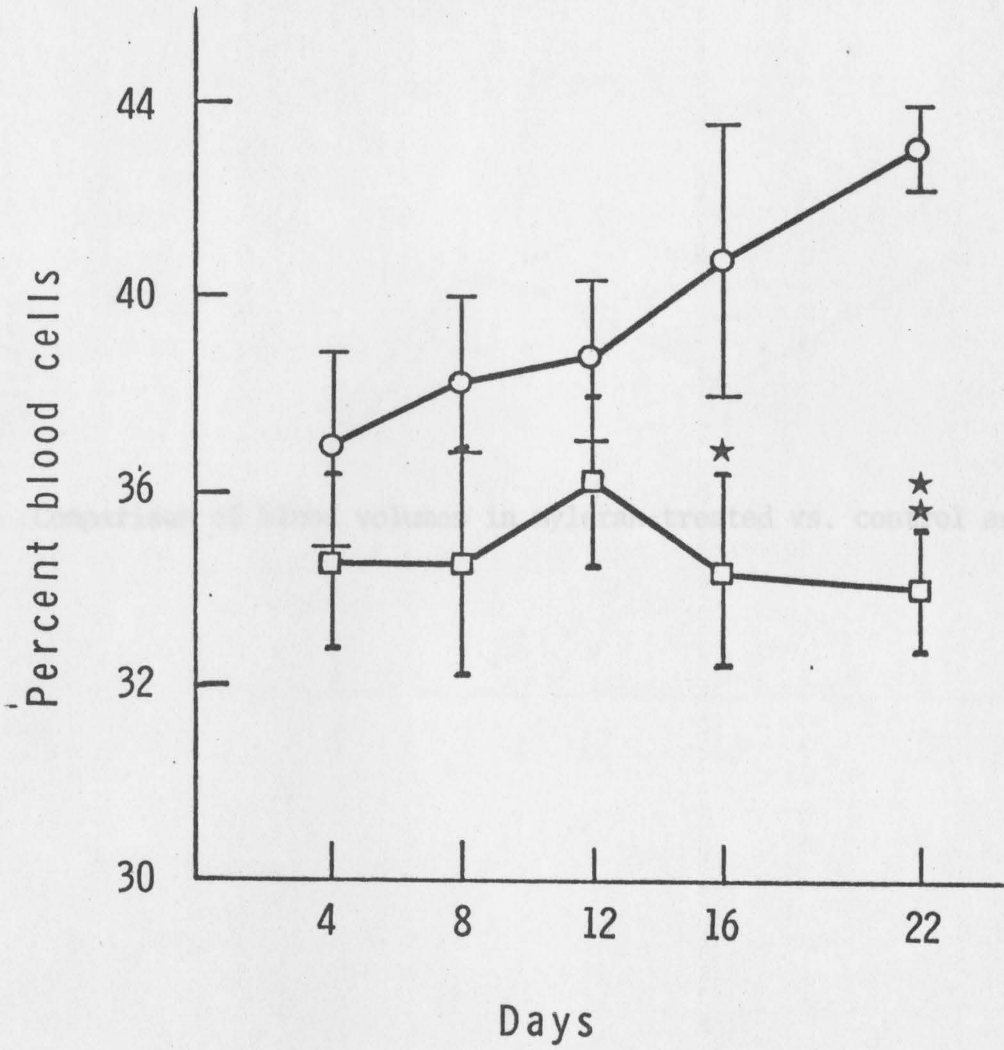


Figure 8. Changes in hematocrit values of control and drug-treated animals


44
40
36
32
30
Percent blood cells
4
8
12
16
22
Days

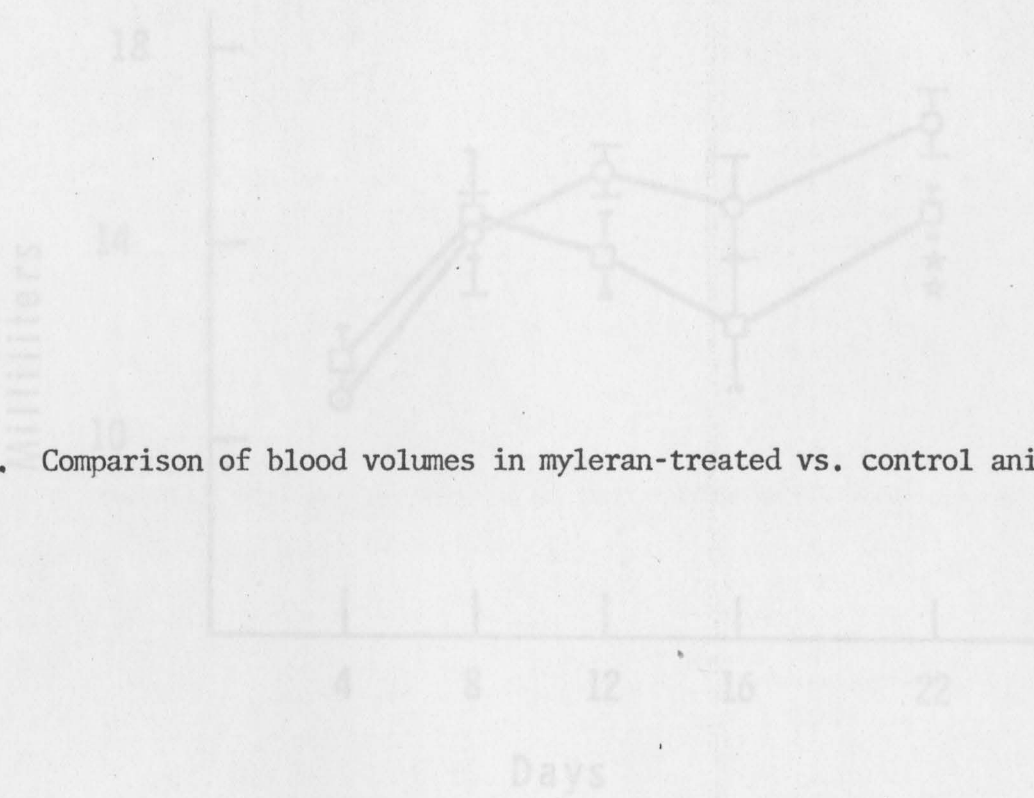
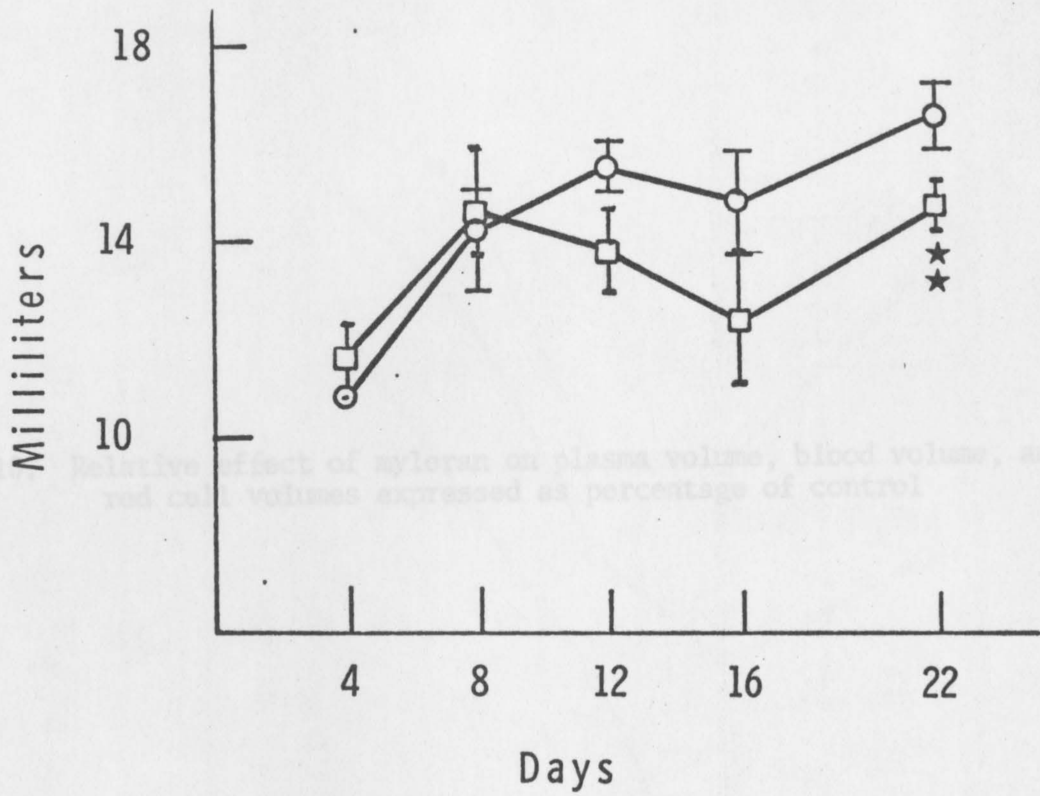


Figure 9. Comparison of blood volumes in myleran-treated vs. control animals


18
14
10
Milliliters
4
8
12
16
22
Days

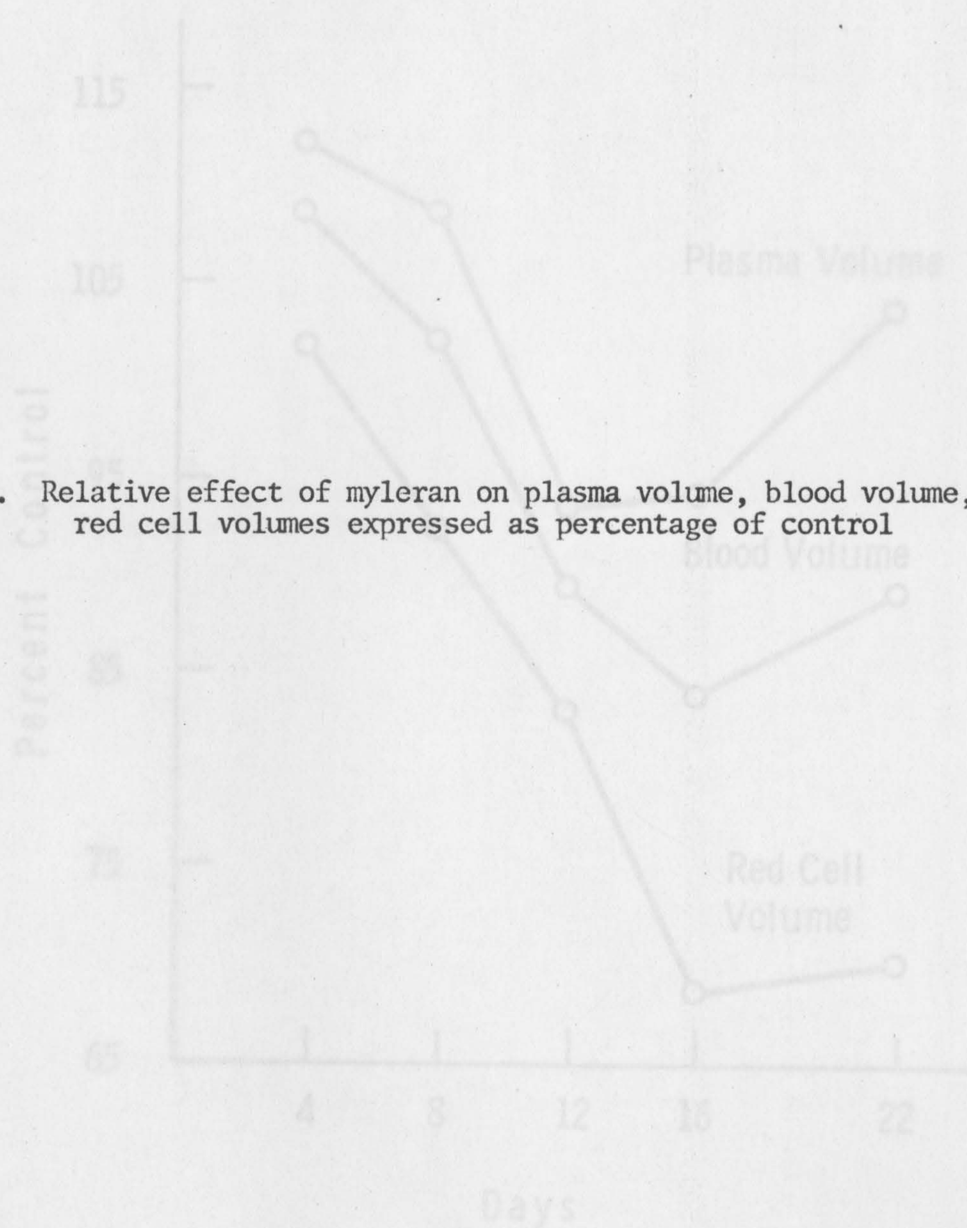
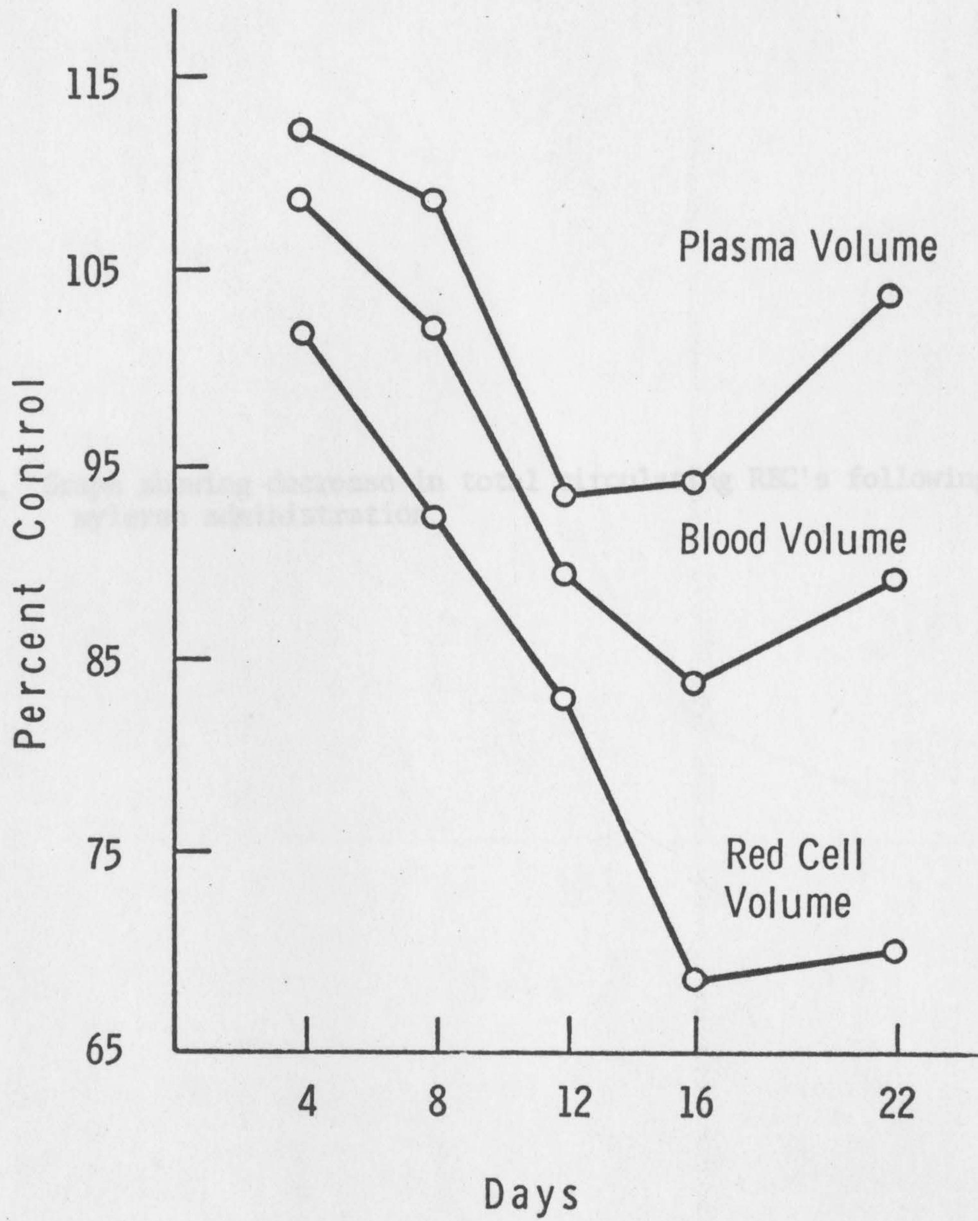


Figure 10. Relative effect of myleran on plasma volume, blood volume, and red cell volumes expressed as percentage of control



115
105
95
85
75
65
Percent Control
Plasma Volume
Blood Volume
Red Cell
Volume
4
8
12
16
22
Days

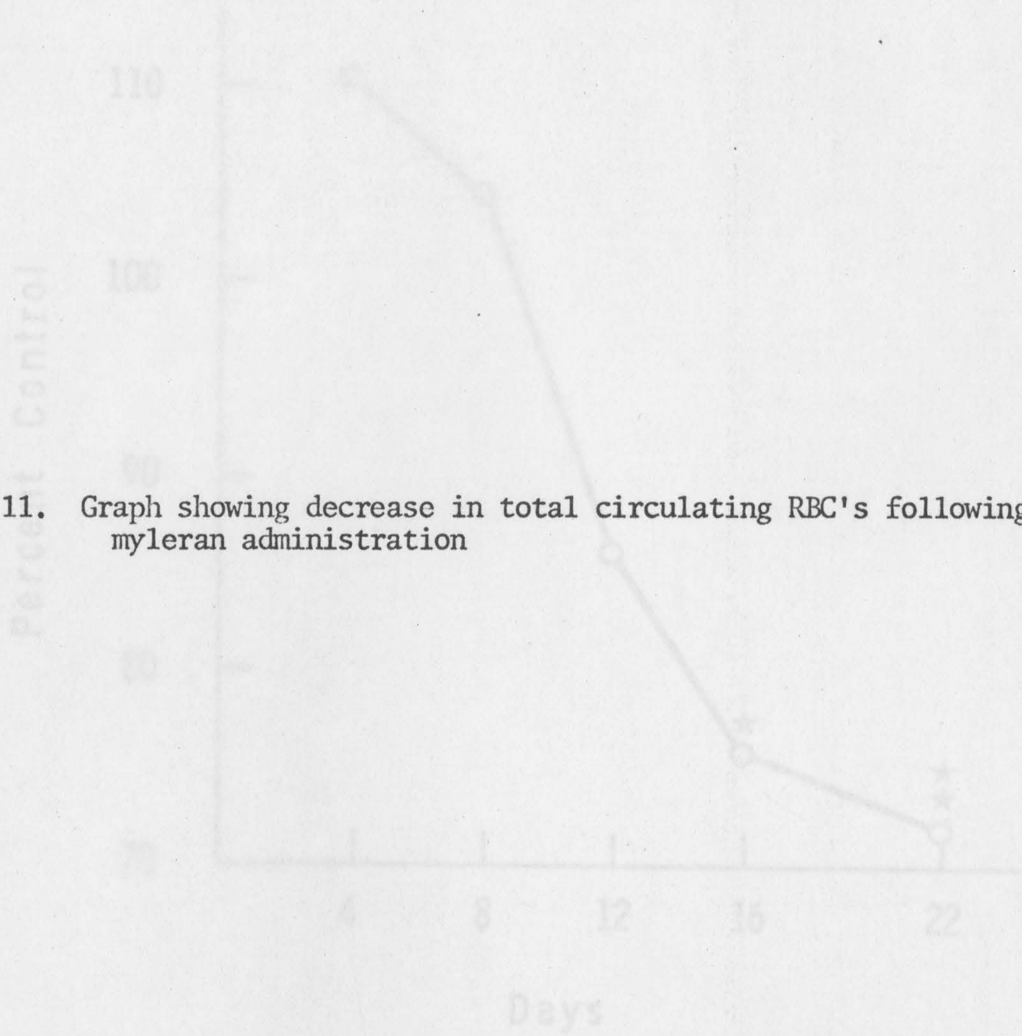
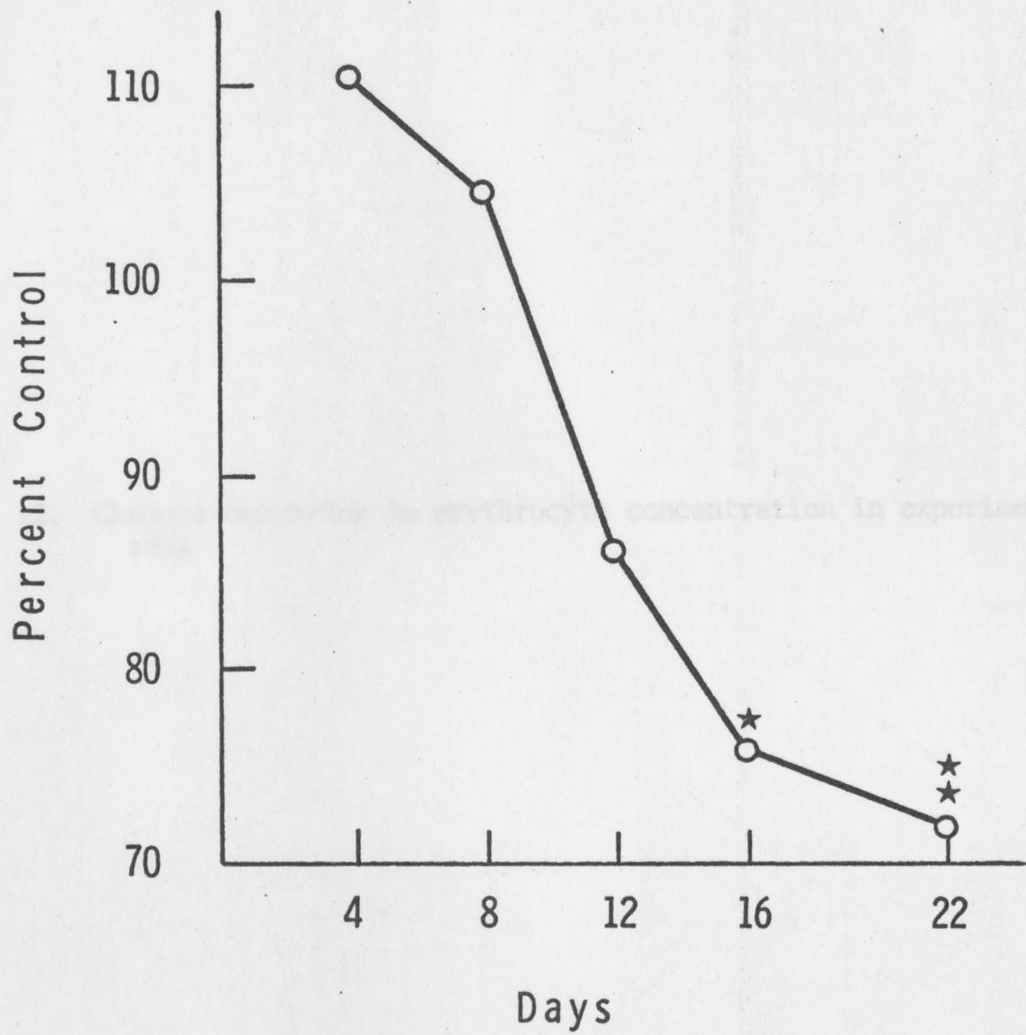


Figure 11. Graph showing decrease in total circulating RBC's following myleran administration


110
100
90
80
70
Percent Control
4
8
12
16
22
Days

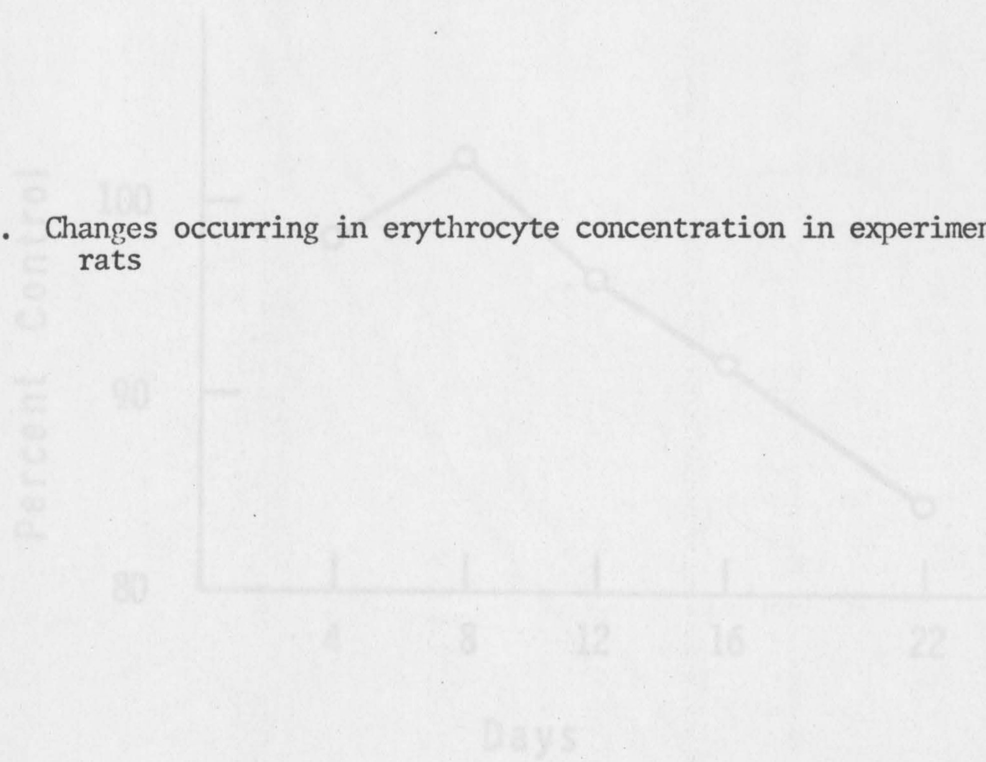
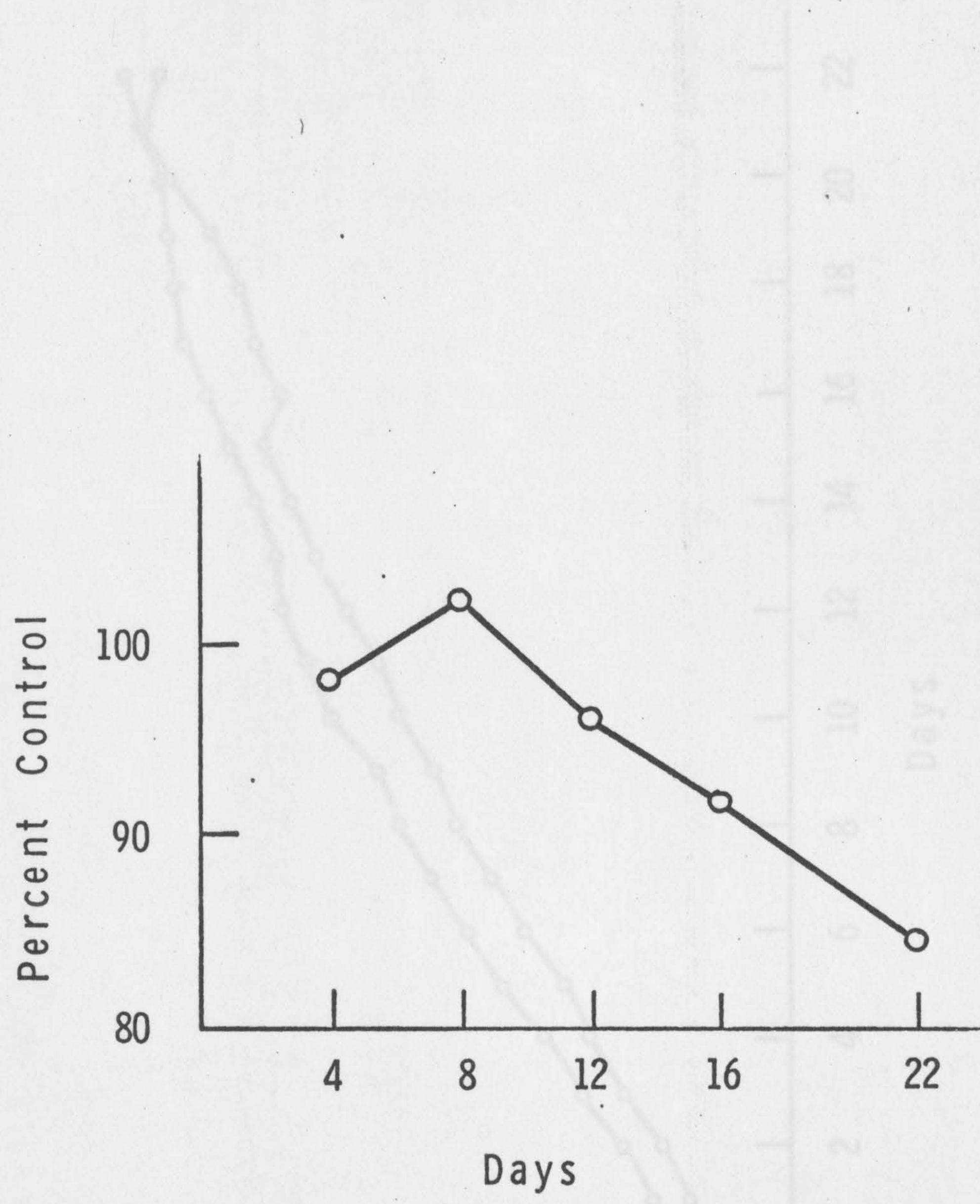
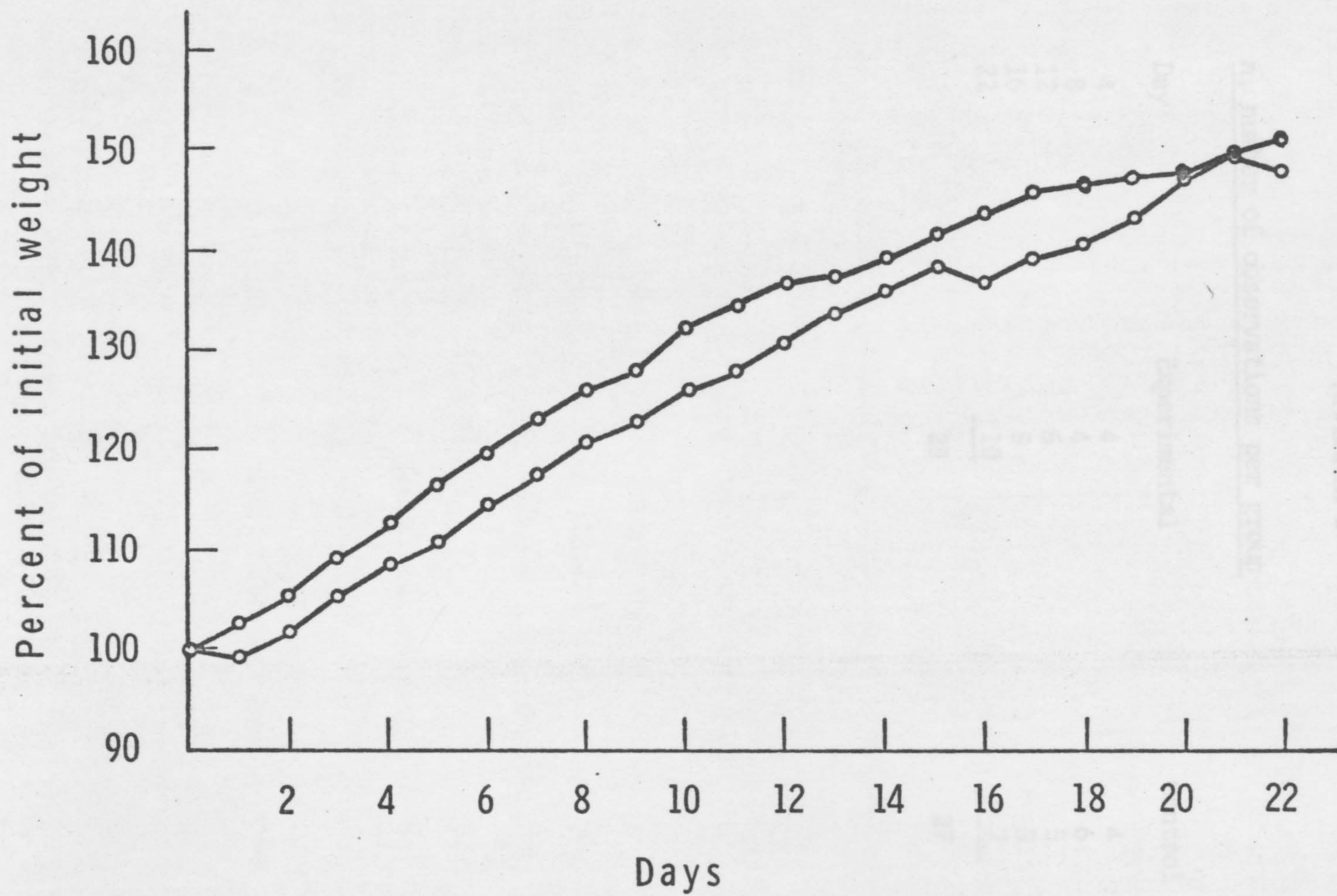


Figure 12. Changes occurring in erythrocyte concentration in experimental rats


Percent Control
100
90
80
4
8
12
16
22
Days

APPENDIX A



APPENDIX B

n, number of observations per group

Day	Experimental	Control
4	4	4
8	4	6
12	6	5
16	5	5
22	10	7
	29	27

BIBLIOGRAPHY

[1]ALLEN, R.C., D.J. MOORE and J.W. FISHER (1968) Preliminary purification of sheep and mouse erythropoietin by vertical flat bed discontinous electrophoresis in acrylamide gel.
Annuals of the New York Academy of Science 149:63.

[2]BECKER, A.J. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells.
Nature 197:452.

[3]BECKER, A.J., E.A. MCCULLOCH, L. SIMINOVITCH and J.E. TILL (1965) The effect of differing demands for blood cell production on DNA synthesis by hemopoietic colony-forming cells of mice.
Blood 26:296.

[4]BHUYAN, R.K. A method for a permanent preparation of blood and bone marrow reticulocytes.
Proceedings of the Anatomical Society of Great Britian and Ireland. 300-1.

[5]BRUCE, W.R. and E.A. MCCULLOCH (1964) The effect of erythropoietic stimulation on the hemopoietic colony-forming cells of mice.
Blood 23:216-32.

[6]CURRY, J.L., J.J. TRENTIN and N. WOLF Hemopoietic spleen colony stueies. II. Erythropoiesis.
Journal of Experimental Medicine 125:703-20.

[7]CURRY, J.L., and J.J. TRENTIN (1967) Hemopoietic spleen studies. I. Growth and differentiation
Developmental Biology 15:395-413.

[8]ELSON, L.A. (1955) A comparison of the effects of radiation and radiomimetic chemicals on the blood.
British Journal of Haematology 1:104.

[9]ELSON, L.A., D.A.G. GALTON and M. TILL (1958) The action of chlorambucil (CB. 1348) and busulphan (Myleran) on the haemopoietic organs of the rat.
British Journal of Haematology 4:355.

[10]ERSLEV, A.J. (1964) Erythropoietin in vitro. II. Effect on "stem cells."
Blood 24:331.

[11]ESPADA, J., A.A. LANGTON and M. DORADO (1972) Human erythropoietin: studies on purity and partial characterization.
Biochimica et Biophysica Acta 285:427-35.

[12]FILMANOWICZ, E. and C.W. GURNEY (1961) Studies on erythropoiesis: XVI Response to a single dose of erythropoietin in polycythemic mouse.
Journal of Laboratory and Clinical Medicine 57:65-72.

[13]FISCHER, S. (1962) Studies on the mechanism and site of action of erythropoietin. In: Erythropoiesis, edited by L.O. Jacobson and M. Doyle. Grune & Stratton, New York. 204-15.

[14]FISHER, J.W. (1972) Erythropoietin: Pharmacology, biogenesis, and control of production.
Pharmacological Reviews 24:459-508.

[15]FISHER, J.W., L.G. LAJTHA, A.S. BUTTOO, and D.D. PORTEOUS (1965) Direct effects of erythropoietin on the bone marrow of the isolated perfused hind limbs of rabbits.
British Journal of Haematology II:342-9.

[16]FISHER, J.W., B.L. ROH and K. SHENEP (1964) Influence of x-irradiation of the dog kidney on erythropoietin production.
Acta Haematologica 31:90.

[17]FOWLER, J.H., A.M. WU, J.E. TILL, E.A. MCCULLOCH and L. SIMINOVITCH (1967) The cellular composition of hemopoietic spleen colonies.

[18]GALLAGHER, N.I., G.L. SEIFERT, J.L. CALLINAN, A.A. MAES, and R.D.LANGE (1963) Influence of erythropoietin on erythropoiesis in polycythemic rats.
Journal of Laboratory and Clinical Medicine 61:258-65.

[19]GALLIEN-LARTIGUE, O. and S. GOLDWASSER (1965) On the mechanism of erythropoietin-induced differentiation: I: The effects of specific inhibitors on hemoglobin synthesis.
Biochimica et Biophysia Acta 103:319-24.

[20]GOLDWASSER, E. and C.K.H. KUNG (1971) Properties of purified erythropoietin
Federal Proceedings 30 (II) 1128.

[21]GORDON, A.S., G.W. COOPER, and E.D. ZANJANI (1967) The kidney and erythropoiesis
Seminars in Hematology 4:337.

[22]GORDON, A.S., S.J. PILIERO, S.J. KLEINBERG and H.H. FREEDMAN (1954) A plasma extract with erythropoietic activity
Society for Experimental Biology and Medicine (Proceedings) 86:255.

[23]GORDON, A.S. and E.D. ZANJANI (1970) Some aspects of erythropoietin physiology.
In: Regulation of Hemopoiesis, edited by A.S. Gordon. Appleton-Century-Crofts, New York.

[24]GORDON, A.S. and E.D. ZANJANI (1970) Hormones and erythropoiesis. In: Formation and Destruction of Blood Cells. Edited by T.J. Greenwalt and G.A. Janieson. J.B. Lippincott Company, Philadelphia 34-64.

[25]GORDON, A.S. and E.D. ZANJANI (1970) Mechanisms of erythropoietin production. In: Erythropoiesis-Regulatory mechanisms and developmental aspects. Edited by Y. Matoth. Academic Press, New York 141-9.

[26]GORDON, A.S. and E.D. ZANJANI (1970) The renal erythropoietic factor (erythrogenin) and erythropoietin (ESF). In: Hemopoietic Cellular Proliferation. Edited by F. Stohlman, Jr. Grune & Stratton, New York

[27]HANNA, I.R.A. (1967) Response to early erythroid precursors to bleeding. Nature 214:355.

[28]HARRISS, E.B. (1957) In vivo uptake of radioactive iron by erythroid cells of rat bone marrow. Advances in Radiobiology 333.

[29]HODGSON, G. (1966) Biochemical control of erythroid cell development. In: Current Topics in Developmental Biology, vol. 1. Edited by A. Monroy and A. A. Moscona. Academic Press, New York. 173.

[30]HODGSON, G. (1967) Synthesis of Rna and DNA at various intervals after erythropoietin injection in transfused mice. Society for Experimental Biology and Medicine 124:1045-7.

[31]HODGSON, G.S. and I. ESKUCHE (1962) Time Course of effects of erythropoiesis stimulating factor(s) (ESF) on Fe^{59} distribution. In: Erythropoiesis. Edited by L.O. Jacobson and M. Doyle. Grune & Stratton, New York. 222-7.

[32]HRINDA, M.E. and E. GOLDWASSER (1969) On the mechanism of erythropoietin-induced differentiation. VI. Induced accumulation of iron by marrow cells. Biochimica et Biophysica Acta 195:165-75.

[33]HRINDA, M.E. and E. GOLDWASSER (1966) Facilitated iron entyr into marrow cells: An induced permease-like activity. Federal Proceedings 25:284.

[34]HRINDA, M.E. and E. GOLDWASSER (1968) Studies on the control of hemoglobin formation in marrow cells. Annuals of the New York Academy of Science 149:412-5.

[35]JACOBSON, L.O., E. GOLDWASSER, W. FRIED, and L. PIZAK (1957) Role of the kidney in erythropoiesis. Nature 179:633.

[36]JACOBSON, L.O., E. GOLDWASSER, C.W. GURNEY, W. FRIED, and L. PIZAK (1959) Studies of erythropoietin: The hormone regulating red cell production. Annuals of the New York Academy of Science 77:551-3.

[37]JURASKOVA, V. and L. TKADLELEK (1965) Character of primary and secondary colonies of hematopoiesis in the spleen of irradiated mice. Nature 206:951-2

[38]KRANTZ, S.B. and E. GOLDWASSER (1965) On the mechanism of erythropoietin-induced differentiation: II: The effect on RNA synthesis. Biochimica et Biophysica Acta 103:325-32.

[39]KRANTZ, S.B., O. GALLIEN-LARTIGUE, and E. GOLDWASSER (1963) The effect of erythropoietin upon hemesynthesis by marrow cells in vitro. Journal of Biological Chemistry 238:4085-90.

[40]KRANTZ, S.B. and L.O. JACOBSON (1970) Erythropoietin and Regulation of Erythropoiesis. The University of Chicago Press, Chicago. 80.

[41]KURATOWSKA, Z., B. LEWARTOWSKI, and B. LIPINSKI (1964) Chemical and biologic properties of an erythropoietin-generating substance obtained from perfusates of isolated anoxic kidneys. Journal of Laboratory and Clinical Medicine 64:226.

[42]KURATOWSKA, Z.B., B. LEWARTOWSKI, and E. MICHALAK (1961) Studies on the production of erythropoietin by isolated perfused organs. Blood 18:527-34.

[43]LAJHA, L.G., POZZI, L.V., R. SCHOFIELD, and M. FOX (1969) Kinetic properties of haemopoietic stem cells.

[44]LEWIS, J.P., and F.E. TROBAUGH, Jr. (1964) Haemotopoietic Stem Cells. Nature 204:589-90.

[45]LUKOWSKY, W. and R.H. PAINTER (1968) Molecular weight of erythropoietin from anemic sheep plasma and human urine. Canadian Journal of Biochemistry 46:731.

[46]MATOTH, Y., and E. BEN-PORATH (1959) Effect of erythropoietin on the mitotic rate of erythroblasts in bone marrow cultures. Journal of Laboratory and Clinical Medicine 54:722-7.

[47]MCCULLOCH, E.A. and J.E. TILL (1971) Regulatory mechanisms acting on hemopoietic stem cells. American Journal of Pathology 65:601-19.

[48]MCDONALD, T.P., E.D. ZANJANI, T. JORDON, R.D. LANGE, and A.S. GORDON (1969) Studies of the renal erythropoietic factor using a hemagglutination-inhibitor system. Society for Experimental Biology and Medicine (Proceedings) 132:533-6.

[49]MIRAND.A.E. and T.C. PRENTICE (1957) Presence of plasma erythropoietin in hypoxic rats with or without kidney(s) and/or spleen. Society for Experimental Biology and Medicine (Proceedings) 96:49.

[50]NAKOO, K., S. SASSA, O. WADA, and F. TAKAKU (1968) Erymatic studies on erythroid differentiation and proliferation.
Annuals of the New York Academy of Science 144:224-8.

[51]NELHELES, T.F., R.G. SHEEHAN and H.J. MEYER (1968) Studies on the control of hemoglobin synthesis: Nucliuc acid synthesis and normoblast proliferation in the presence of erythropoietin.
Annuals of the New York Academy of Science 149:449-55.

[52]O'GRADY, L.F., J.P. LEWIS, and F.E. TROBAUGH, Jr. (1968) The effect of erythropoietin on differentiated erythroid precursors.
Journal of Laboratory and Clinical Medicine 71:693.

[53]ORLIC, D., A.S. GORDON, and J.A.G. RHODIN (1965) An ultrastructural study of erythropoietin-induced red cell formation in mouse spleen.
Journal of Ultrastructure Research 13:516-42.

[54]PIEBER-PERRETTA, M., W. RUDOLPH, M. PERRETTA, and G. HODGSON (1965) Effect of erythropoietine on (^{14}C) formate incorporation into rat bone marrow RNA.
Biochimica et Biophysica Acta 95:360-2.

[55]PORTEOUS, D.D. and LAJTHA, L.G. (1964) Repair processes in irradiated mouse hematopoietic tissue.
Annuals of the New York Academy of Science 114:115.

[56]PORTEIOUS, D.D. and L.G. LAJTHA (1968) Restoration of stem cell function after irradiation.
Annuals of the New York Academy of Science 149:151.

[57]POWSNER, E.R. and L. BERMAN (1967) Effect of erythropoietin on DNA synthesis by erythroblasts in vitro.
Blood 30:189-97.

[58]PRUDEN, E. and M.A. WINSTEAD (1964) Accuracy control of blood cell counts with coulter counter.
American Journal of Medical Technology 1-35.

[59]REISSMANN, K.R. and S. SAMORAPOOMPICHIT (1969) Effect of erythropoietin on regeneration of hemopoietic stem cells after 5-fluorouracil administration.
Journal of Laboratory and Clinical Medicine 73:544-50.

[60]ROSSE, W.F., R.J. BERRY and T.A. WALDMAN (1963) Some molecular characteristics of erythropoietin from different sources determined by inactivation by ionizing radiation.
Journal of Clinical Investigation 42:124.

[61]RUDOLPH, W. and M. PERRETTA (1967) Effects of erythropoietin on ^{14}C-formate uptake by spleen.
Society for Experimental Biology and Medicine 124:1041-5.

[62]SCHOOLEY, J.C. (1965) Responsiveness of hematopoietic tissue to erythropoietin in relation to the time of administration and duration of action of the hormone.
Blood 25:795.

[63]SCHOOLEY, J.C. (1966) The effect of erythropoietin on the growth and development of spleen colony-forming cells.
Journal of Cellular Physiology 68:249-62.

[64]SCHOOLEY, J.C. (1968) A summary of some studies of erythropoiesis using anti-erythropoietin immune serum.
Annuals of the New York Academy of Science 149:266-80.

[65]SCHOOLEY, J.C., E.D. ZANJANI, and A.S. GORDON (1970) Immunologic aspects of the biogenesis of erythropoietin.
Blood 35:276-81.

[66]SLAUNWHITE, W.R., Jr., E.A. MIRAND and T.C. PRENTICE (1957) Probable polypeptide nature of erythropoietin.
Society for Experimental Biology and Medicine 96:616.

[67]STEEL, R.G.D. and J.H. TORRIE (1960) Principles and Procedures of Statistics. Edited by McGraw-Hill, New York 72-3,447.

[68]STOHLMAN, F. Jr. (1967) Some aspects of erythykinetics. In: Seminars in Hematology 4:304-14.

[69]TILL, J.E. and E.A. MCCULLOCH (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.
Radiation Research 14:213-22.

[70]TOEPFER, J.R. (1969) Hemopoietic Function of the Lymphocyte in Rat Bone Marrow. Doctoral Dissertation. Kent State University.

[71]WU, A.M., J.E. TILL, L. SIMINOVITCH, and E.A. MCCULLOCH (1967) A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells.
Journal of Cellular Physiology 69:177-84.

[72]ZANJANI, E.D., J.F. CONTRERA, A.S. GORDON, G.W. COOPER, K.K. WONG, and R. KATZ (1967) The renal erythropoietic factor (REF). III. Enzymatic role in erythropoietin production.
Society for Experimental Biology and Medicine (Proceedings) 125:505-8.

[73]ZANJANI, E.D., G.W. COOPER, A.S. GORDON, K.K. WONG, and V.A. SCRIBNER (1967) The renal erythropoietic factor (REF). IV. Distribution in mammolian kidneys.
Society for Experimental Biology and Medicine (Proceedings) 126:540-2.

[74]ZANJANI, E.D., J.C. SCHOOLEY and A.S. GORDON (1968) Renal erythropoietic factor (REF). Neutralization of *in vitro* generated erythropoietic activity with anti-erythropoietin. *Life Science* 7:505.