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COMPENSATORY ERYTHROPOIESIS IN THE RAT: RESPONSES TO ANEMIAS INDUCED BY HEMOLYSIS, HEMORRHAGE AND ACUTE MYELOGENOUS LEUKEMIA

by

Ronnie Sima Horowitz

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.

1975

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.

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ABSTRACT

The rat, does not normally exhibit a high level of splenic erythropoiesis. Nevertheless, under certain pathological conditions, such as the primary anemias due to hemorrhage and chemically-induced hemolysis, and the anemia secondary to Acute Myelogenous Leukemia (AML) described in the present study, the spleen manifests a significant erythropoietic capacity as monitored by cytological (per cent benzidine-positive cells) and biochemical (δ -Aminolevulinic Acid Synthetase: ALA-S) alterations.

Compensatory splenic erythropoiesis appears to be governed by the interaction of at least two factors: (a) the state of medullary erythropoiesis, and (b) permissiveness of the splenic microenvironment. In the anemia secondary to AML, (Shay Chloroleukemia: SCL), the spleen exhibits a progressive increase in the per cent benzidine-positive elements with concomitant increase in specific activity (pmoles ALA per mg protein per hour) ALA-S. However, the presence of a critical percentage of leukemic myeloblasts and/or their presence for a critical period of

time in the spleen appears to be incompatible with erythropoiesis in this organ in spite of a significant inhibition of medullary red cell production. This is not unlike the primary situation in the bone marrow, in which the presence of leukemic myeloblasts, even in small percentages, appears to alter the microenvironment such that erythropoiesis is progressively depressed.

In the two primary anemias under investigation, in which medullary erythropoiesis is stimulated to approximately two-times the normal level, a high degree of splenic erythropoiesis is in evidence. The difference between the splenic erythroid response in these primary anemias due to hemolysis and hemorrhage, appears to be a quantitative, rather than a qualitative one, i.e., the more potent stimulus of anemic stress induced by phenylhydrazine (PH), elicits a proportionately greater response in the spleen both in terms of increased percentages of erythroid elements and specific activity ALA-S.

A comparison of specific activities of normal, hemorrhagic and hemolytic anemic rats with those of leukemic animals in which the per cent benzidine-positive cells were equivalent, suggests that the mechanism of compensatory

erythropoiesis in the leukemic spleen is not identical with that seen under conditions of primary anemia. Despite the increase in specific activity of splenic ALA-S concurrent with the progressive increase in per cent benzidine-positive elements, there appears to be a consistent depression of specific activity ALA-S in leukemic spleens when compared to normal, hemolytic anemic and hemorrhagic anemic rats with equivalent percentages of benzidine-positive cells. The erythropoietic pathogenesis associated with AML may be related to a biochemical lesion involving the functional capacity of ALA-S.

INTRODUCTION

I. SPLENIC ERYTHROPOIESIS:

This study focuses on erythropoietic compensation by the spleen under conditions of primary and secondary anemia in the rat. The primary anemias are of two different types: hemolytic and hemorrhagic. The secondary anemia is a consequence of an acute myelogenous leukemia, the Shay Chloroleukemia. Data obtained from the three pathologies and comparison of them with normal hematological and physiological parameters may help to define a predictable pattern of compensatory splenic erythroid compensation in various disease states.

The spleen in the adult rat, is not associated with a high degree of erythropoiesis under normal conditions (Fruhman and Gordon, 1952; Garcia, 1957; Handler and Handler, 1970b). Clearly, however, it retains the erythropoietic capacity once operative during fetal life, for in certain pathological states, the spleen becomes highly erythropoietic and manifests an elevated percentage of benzidine-positive cells. Primary anemias in the rat, such as those induced by phenylhydrazine (Azen and Schilling, 1963)

and bleeding (Baxter, *et al*., 1955), exhibit splenic erythroid compensation as evidenced by increased percentage of immature erythroblasts. A similar compensatory response has characterized a wide range of human pathologies (Blaustein and Diggs, 1963), including polycythemia vera, acute infection, leukemia (Fruhman, 1970) and the anemia secondary to myeloid metaplasia (Argano, *et al*., 1969; Szur, *et al*., 1973) as well as the rodent AML studied in this laboratory (Handler and Handler, 1970b).

II. PATHOGENESIS OF SHAY CHLOROLEUKEMIA:

The pathology accompanying acute myelogenous leukemia (AML) in the rat provides a unique approach to the study of compensatory erythropoiesis.

The Shay Chloroleukemia, pathologically similar to human AML, was initially induced in rats by gastric instillation of 20-methylcholanthrene (Shay, *et. al*., 1951; 1952).

Intravenous inoculation of Shay Chloroleukemic cells (SCL) into the Long-Evans rat results in a fairly predictable alteration of medullary histology (Handler and Handler, 1970b). Leukemic myeloblasts may be detected in the bone marrow from 3 to 5 days after the initial injec-

tion of SCL cells. The bone marrow pathology is characterized by a progressive:

(a) decrease in total cellularity (Varsa, et al., 1965);

(b) increase in per cent leukemic myeloblasts (Handler, et al., 1968);

(c) decrease in normal myeloid and erythroid elements (Handler, et al., 1968).

Hepato-splenomegaly, particularly in late stages, is also apparent (Handler and Handler, 1970b).

The progressive increase in the per cent bone marrow myeloblasts remains the most consistent parameter of this leukemic pathology and thus, has been used in the staging of the leukemia (Varsa, et al., 1965; Handler and Handler, 1970b).

Infiltration and/or proliferation of leukemic myeloblasts in the spleen, however, follows a less predictable course than that in the bone marrow. An additional correlate of the leukemic condition appears to be elevated serum and urine levels of muramidase noted in such patients (Levi, et al., 1973). These findings may provide the basis for a potential assay of pathologic involvement.

III. SECONDARY ANEMIA DUE TO LEUKEMIA AND NEOPLASTIC DISORDERS:

The decreasing pattern of medullary erythropoiesis during the course of AML in the rat is associated with a progressive increase in leukemic myeloblast content in the bone marrow (Handler, et al., 1968). This inverse relationship is characteristic of the pathogenesis of chloro-

leukemic rats. Hematocrit, degree of reticulocytosis, peripheral red blood cell count and hepatosplenomegaly, however, cannot be consistently correlated with the degree of leukemic involvement. Histological evidence (Handler, et al., 1968) and ^{59}Fe-incorporation studies (Handler and Handler, 1970a; 1972), clearly implicate the spleen as the major site of extamedullary erythroid compensation during the course of the leukemia.

The changing hematological pattern, viz., reticulocytosis, anemia, seen with progressive development of the Shay Chloroleukemia in rats suggested a series of experiments to determine the plasma erythropoietin (EPO) values in these animals. An apparent elevation in circulating levels of EPO was observed during the course of the pathogenesis (Handler and Handler, unpublished) and is suggestive of a functioning EPO-generating system.

This is in agreement with findings in human leukemias in which high circulating levels of EPO have been reported (Krantz and Jacobson, 1970). In such systems it is assumed that the bone marrow of leukemic subjects is incapable of a significant response to increased concentrations of EPO (Thorling, 1965; Zaizov and Matoth, 1971; Krantz and Jacobson,

1970). Recent evidence, *in vitro*, suggests a further distinction underlying the failure mechanisms of erythropoiesis in patients with acute lymphocytic leukemia (ALL) and AML (Chiyoda, *et al*., 1974). Bone marrow from patients with ALL, though low in erythroblast content at the beginning of culture, was capable of sustained EPO-stimulated heme synthesis, indicating a retention of the inherent capacity to respond to exogenous EPO. Similar trials with bone marrow from subjects with AML, however, suggest that these cells are refractory to EPO-induced heme synthesis (Chiyoda, *et al*., 1974).

This evidence implicating the stem cell compartment as one of the possible sites of the erythropoietic lesion in leukemia is further corroborated in other systems: rat (Hoelzer and Harriss, 1973); mouse (Lajtha, 1973) and human AML (Killmann, 1970).

The data concerning EPO titers in patients with neoplastic disorders is inconclusive. Reports of both elevated and unchanged EPO levels have been reported (Krantz and Jacobson, 1970). Inconsistencies in this regard must be interpreted with caution, however, as anemias associated with neoplasms may be influenced by other factors, e.g.,

malnutrition, marrow replacement by tumor, vitamin and mineral deficiency, hemorrhage or hemolysis (Krantz and Jacobson, 1970).

In apparent contradiction to the findings in leukemias cited above in which EPO levels were elevated above normal, plasma EPO assays of mice infected with Rauscher leukemia virus (RLV) and Friend leukemia virus (FLV) indicate a severe depression (Ebert, et al., 1972) or a total absence of EPO (Mirand, 1967) in the blood. It has been suggested that either these murine leukemia viruses induce erythropoiesis independent of normal EPO production (Mirand, 1967) or that the levels of EPO produced in response to viral-induced erythropoiesis are below the limits of detection (Ebert, et al., 1972). Ebert, et al., (1972) were able to demonstrate the presence of detectable levels of EPO in RLV-infected mice. Nevertheless, the degree of EPO noted in the plasma of such mice was inadequate for the degree of anemia encountered. Attempts to enhance plasma EPO in RLV-infected mice by bleeding were not successful suggesting a possible inhibition of the EPO-generating mechanism. However, on the basis of recent studies of plasma EPO levels and the rate of plasma clearance of exogenously administered EPO, OKunewick

and Erhard (1974) have provided an alternative hypothesis concerning EPO involvement in Rauscher leukemia. These authors suggest that as a result of the leukemia, EPO production and clearance are accelerated to a nearly maximal level, such that little reserve synthetic capacity remains to permit response to an additional erythropoietic stress, e.g., bleeding (OKunewick and Erhard, 1974). These findings stand to be corroborated. Nevertheless, they appear to explain results obtained by Mirand and Ebert and are entirely consistent with alterations in hematopoiesis observed during the leukemia, e.g., reticulocytosis, anemia (OKunewick and Erhard, 1974).

Another critical parameter in the assessment of the effectiveness of erythropoiesis in various pathological states, is the relative activity of δ-aminolevulinic acid synthetase (ALA-S). It has been postulated that ALA-S mediates the rate-limiting step in the biosynthesis of heme, viz., generation of ALA from the condensation of glycine and succinate (Granick and Urata, 1963; Granick and Sassa, 1971), and as such, provides an opportunity to relate various conditions of ineffective erythropoiesis (including

anemias of multiple aetiology), to a specific biochemical lesion.

In mice infected with RLV, the specific activity of splenic ALA-S is depressed below normal levels, despite the proliferation of erythroid elements. The increased net activity of ALA-S reflects an existing splenomegaly. In spleens of FLV-infected mice, however, ALA-S activity has been demonstrated to be elevated above normal levels (Ebert, et al., 1969).

IV. PRIMARY ANEMIA DUE TO HEMOLYSIS:

Induction of acute hemolytic anemia in rodents by phenylhydrazine results in severe, sustained anemia characterized by:

(a) decline in peripheral red blood cells (Hodgson, et al., 1972);

(b) reticulocytosis (Hodgson, et al., 1972); Iyengar and Chandra, 1974; Gauger, et al., 1973; Azen and Schilling, 1963);

(c) decline in hematocrit (Ebert, et al., 1972; Jandl, et al., 1965; Chen and Weiss, 1973);

(d) splenomegaly (Jandl et al., 1965; Chen and Weiss, 1973).

Phenylhydrazine hydrochloride (PH) is a known oxidative agent capable of irreversible oxidation of hemoglobin. This results in the denaturation of the hemoglobin molecule.

Such precipitated matter is considered to be identical to the so-called Heinz bodies found within the red blood cell under these conditions (Jandl, 1963; Jandl, _et al_., 1960; Allen, _et al_., 1961; Emerson, _et al_., 1949; Lemberg, _et al_., 1949).

With low and moderate doses of PH, detection, sequestration and ultimate destruction of these abnormal red blood cells occurs in the spleen, making hemolysis essentially an extravascular phenomenon (Iyengar and Chandra, 1974; Cruz, 1941; Azen and Schilling, 1963; Rifkind, 1964). More massive doeses, however, appear to induce an intravascular hemolysis via a direct lytic effect on erythrocytes (Cruz, 1941; Bratley, _et al._, 1931; Beuler, _et al_., 1954).

Medullary and splenic erythroid compensation associated with hemolytic anemia is evidenced by erythroid hyperplasia (Krantz and Jacobson, 1970), increase in splenic specific and net activities of ALA-S (Ebert, _et al_., 1972) and appears to be mediated by normal EPO mechanisms (Krantz and Jacobson, 1970; Eskuche, _et al_., 1962; Ebert, _et al_., 1972).

V. PRIMARY ANEMIA DUE TO HEMORRHAGE:

The anemia resulting from hemorrhage, quite obviously results in a decrease in the number of peripheral red blood

cells, decline in hematocrit and reticulocytosis. In addition, the spleen undergoes an array of alterations suggestive of compensatory erythropoiesis (Boggs, et al., 1969) which include:

(a) erythroid hyperplasia (Boggs, et al., 1969; Hershko, et al., 1970; Krantz and Jacobson, 1970; Fruhman, 1970);

(b) increase in ^{59}Fe-incorporation (Bozzini, et al., 1970);

(c) reticulocytosis (Hershko, et al., 1970).

As in the case of hemolytic anemia, erythropoiesis appears to operate via normal EPO mechanisms.

VI. RATIONALE:

Shay Chloroleukemia in rats provides an excellent model system in which to study the interaction of the leukemic process both on the decline of medullary erythropoiesis and on splenic erythroid compensation. The cause of erythroid hypoplasia in the bone marrow of rats with AML is unknown. Methodological limitations however, prohibited the monitoring of ALA-S in the marrow. Enzyme assays of spleens and livers of leukemic animals were performed in order to assess the relative effectiveness of compensatory erythropoiesis as mediated by this rate-limiting enzyme. Furthermore, it is suggested that some insight

into the shut-down of medullary erythropoiesis will be gained through the study of similar interactions of erythroid and leukemic elements in the spleen.

The mechanisms of compensatory erythropoiesis in primary anemias due to hemorrhage or hemolysis appear to be better understood than those underlying the anemia secondary to AML, insofar as the former appear to be modulated via established EPO-ERC (Erythropoietin-Responsive Cell) interactions. It is expected, therefore, that a comparative analysis of histological and hematological parameters, including the assay of ALA-S activities in animals with these primary and secondary anemias will shed some light on aspects of the biochemical mechanism of compensatory erythropoiesis.

MATERIALS AND METHODS

I. ANIMAL BREEDING AND MAINTENANCE:

Experimental animals used are Long-Evans, black hooded rats (200-300g) purchased as adults.[1,2*] Rats are maintained on a diet of Purina Laboratory Chow[3] and water *ad libitum*.

II. MAINTENANCE OF SHAY CHLOROLEUKEMIA TUMOR CELLS:

A. Tumor Induction -

Subcutaneous injection of 20 x 10^6 Shay Chloroleukemic (SCL) cells into 40-60g male rats[1] results in a well-localized tumor at the site of injection. The tumor is populated primarily by leukemic myeloblasts, characteristic of the SCL, which are capable of inducing acute myelogenous leukemia (AML) in rats under proper experimental conditions described below (Section III). Viable leukemic myeloblasts are generally found in well-vascularized, non-necrotic tumors. Those tumors bearing massive necrotic areas which had progressed through the skin are not chosen for experimental use.

B. Preparation of Leukemic Myeloblast Cell Suspension-

All procedures used in the preparation of leu-

*See Appendix: Footnotes

kemic myeloblast cell suspensions are performed under sterile conditions under a vertical laminar flow hood.[4] Glassware is made sterile by dry heat (220°C; 3 hr.). The tumor mass with its leukemic myeloblasts and its attending connective tissue is minced and placed in 30 ml homogenizing flask containing normal, non-pyrogenic saline. A hand homogenizer, loosely fitted with a ground-down teflon pestle, is gently moved up and down through the flask resulting in the liberation of intact cells from the connective tissue matrix. The suspension is filtered 2 times through glass wool fiber which retains the debris and connective tissue, permits the passage of leukemic myeloblasts and thus, results in a free suspension of SCL cells. A hemocytometer[5] cell count is then taken using 2% acetic acid as a diluent, in order to assess the concentration of nucleated cells.

III. INDUCTION OF ACUTE MYELOGENOUS LEUKEMIA IN THE LONG-EVANS RAT:

15-20 x 10^6 SCL cells in suspension, obtained from the tumor as outlined above (Section IIB) are injected intravenously via the jugular vein into 180-200g rats.[1] The appearance of leukemic myeloblasts in the femoral bone marrow, has been used in this laboratory as a convenient and reliable parameter for staging of leukemic progression in the rat.

Such staging is expressed as:

$$\frac{\text{number leukemic myeloblasts}}{\text{total number of nucleated cells}} \times 100\%$$

Bone marrow slides are brushed directly from the femur using rat serum-NCTC 109 mixture as diluent, dried and immediately fixed with methanol. Slides are routinely stained with 3,3' - Dimethoxybenzidine[6] and counterstained with Wright's[7]-Giemsa[8] (See Appendix A). Reference to benzidine positive cells in the present study are restricted exclusively to nucleated benzidine-positive cells, i.e., immature erythroblasts.

The average life expectancy of the rat after I.V. injection ranges from 9-11 days.

IV. INDUCTION OF HEMOLYTIC ANEMIA IN THE LONG-EVANS RAT:

Hemolytic anemia is induced in 250-300g rats[2] by intraperitoneal injection of phenylhydrazine hydrochloride[7] in sterile saline (50mg/kg body weight). Three injections are administered over a 5 day period on days 0, 1 and 3. Food is removed on day 4 and animals are routinely sacrificed on day 5. Hematocrits are monitored on days 0, 1, 3 and 5 from tailblood samples using heparinized microhematocrit tubes[9] and centrifuges.[10] Peripheral red blood cells are quantitat-

ed with Coulter Counter.[11] Circulating reticulocytes counted on day of assay are stained with new methylene blue (Appendix B).

Controls are injected with normal saline following an identical schedule.

V. INDUCTION OF HEMORRHAGIC ANEMIA IN THE LONG-EVANS RAT:

The anemia is induced in 250-300g rats[2] by the removal of 3 ml of blood via cardiac puncture over a 6 day period. Rats are bled on days 0, 2 and 4. Food is removed on day 5 and the animals are routinely sacrificed for assay on day 6. Tailblood samples for hematocrits are taken on days 0, 2, 4 and 6.

VI. DETERMINATION OF ALA-S ACTIVITY:

A. ALA-S Assay: General Procedure -

The procedure used for the determination of the rate of ALA production in spleen and liver homogenates is derived from a technique developed by Urata and Granick, (1963) and modified by Marver, _et al._, (1966b) and Ebert, _et al._, (1972). The present modification involves the miniaturization of the assay to one-quarter of the original volume.

Rats are killed by cervical fracture brought about by

a sharp blow to the back of the head caudal to the medulla region. The spleen and liver are quickly removed and placed in iced saline. A fresh cut is made in the tissue and smears are drawn directly from the cut surface, dried immediately under an air-blower and fixed with methanol. Due to the dyssynchrony of leukemic stages among animals injected with the same tumor suspension and dosage, pooling in leukemic experiments was not possible. Pooling of tissues from phenylhydrazinized rats was possible and was performed routinely. Equal weights of tissue (2g) from spleens and livers of 3 animals are respectively pooled and homogenized.

The following procedures are performed in ice: The appropriate amount of spleen or liver is minced and placed in separate glass homogenizing flasks. Homogenizing solution, pH 7.4, (Appendix C) is added in quantities sufficient to produce a 25% homogenate (1:3; g. tissue:homogenizing solution).

The tissues are homogenized using an electric-driven stirrer[12] with tightly-fitted teflon pestle attached. The flasks are replaced in ice and transferred by pipet to 400 ml beakers. The total volume is noted in order to confirm

that a 25% homogenate was produced. The ice bucket, containing beakers with spleen and liver homogenates, are put on a mechanical shaker[13] and aliquots are pipeted directly into 50 ml Erlenmeyer flasks containing incubation medium (Appendix D). This procedure greatly reduced sampling error due to settling. Incubation was carried out over a 40 minute period at 37°C in a Dubnoff Metabolic Shaking Incubator.[14] The presence of EDTA in the incubation medium under optimal conditions, inhibits the catalytic conversion of ALA to porphobilinogen, by the enzyme ALA dehydrase, and thus permits the accumulation of ALA synthesized during the incubation period. The incubation period is terminated by the addition of 1 ml 25% TCA.

Controls consisted of identical aliquots of homogenate samples pipeted immediately into flasks containing incubation mixture plus 1 ml of 25% TCA. The amount of ALA in the controls thus reflected pre-existing quantities of ALA in the tissues before incubation.

The TCA supernatant containing ALA was obtained by a 7 minute centrifugation[15] at approximately 1500 rpm. The supernatant is buffered with 1M sodium acetate buffer, .

(pH 4.6)[7], and is stored frozen for 24 hours in 25 ml Erlenmeyer flasks.

B. Formation of the ALA-pyrrole -

The frozen TCA supernatant is thawed and diluted with 0.25 ml 2.5 N NaOH[7], 0.07 ml acetylacetone[7] and boiled for 10 minutes in a copper bath. In general, this procedure facilitates the conversion of aminoketone to pyrrole with an unsubstituted α -position, and as such, facilitates the conversion of ALA to ALA-pyrrole.

C. Preparation of Resin -

AG1-X8, 200-400 mesh chloride from (purified Dowex)[16] is converted to the acetate form by overnight washing of 100 g. of resin in 3500 ml of a saturated sodium acetate solution. The resin is then washed on a Buchner funnel with distilled water until neutral and is used directly, or stored in distilled water for a miximum of 2 weeks.

D. Column Chromatography: Separation of ALA and Aminoacetone (AA) -

In addition to ALA, a second aminoketone of importance in this system is aminoacetone. It can be synthesized enzymatically by the condensation of glycine and acetyl-CoA (Marver, et al., 1966b) and is often found in the TCA super-

natant. Thus, any procedure designed to measure ALA levels must include separation and elimination of AA from ALA.

Chromatography columns are 5 ml disposable glass pipets (ID: 0.5 cm). The pyrroles, (total volume:approximately 9 ml), are passed through a 0.5 cm x 5.0 cm AG1 acetate resin. The column is washed with 2.5 ml n-butanol, using slight pressure. Next, the column was primed with 2.5 ml 1M acetic acid, again, using slight pressure. The ALA-pyrrole is finally eluted with 2.5 ml methanol:acetic acid (2:1) with no pressure.

E. Spectrophotometry: Colorimetric Determination of ALA -

The basis of the colorimetric procedure rests on the reactivity of the unsubstituted α -position characteristic of the ALA and AA pyrroles with modified Ehrlich's Reagent (Appendix E). The degree of color development has been shown to be linear to the concentration of ALA pyrrole (Mauzerall and Granick, 1956).

The final eluate is mixed with vortex action and a 1 ml aliquot is added to an equal volume of modified Ehrlich's Reagent. A minimum of 15 minutes is allowed to elapse in order to provide sufficient time for optimal color development.

Samples are read with Gilford Spectrophotometer[17], λ = 553nm, using 1.5 ml capacity, semi-micro cuvettes. Spectrophotometer blank consists of equal volumes methanol: acetic acid (2:1) and Ehrlich's Reagent.

F. Calculation of the Rate of ALA Synthesis -

1. The molar extinction coefficient is calculated from 10^{-4}M stock synthetic ALA[18] as well as from 10^{-5}M and 10^{-6}M solutions diluted from the original stock. It is then converted to the ALA-pyrrole and processed as described in Section V (B-E). The formula employed for this calculation is:

$$E_{1cm}^{553} = \frac{\text{Optical Density}}{\text{concentration, M/L}} ,$$

where E_{1cm}^{553} is the molar extinction coefficient when read at λ = 553 nm through a 1 cm pathlength and concentration is expressed in moles per liter. Under these conditions described, $E_{1cm}^{553} = 5.70 \times 10^4$.

2. The concentration of ALA recovered in the final eluate, X, is derived at by the use of the formula:

$$X = \frac{OD \times 2}{E_{1cm}^{553}} ,$$

where X is the concentration of ALA expressed in moles/liter, OD is the optical density and E_{1cm}^{553} remains as defined above

in Section V (F. 1). Calculation of the number of moles of ALA pyrrole recovered in the final eluate, Y, is derived from the following relationship:

$$Y = 2.6X,$$

since the average volume recovered from each column is 2.6 ml.

The value thus obtained is expressed as number of moles per aliquot homogenate per 40 minutes. In order to standardize presentation of results, all data is expressed as number of moles ALA/mg protein/hr. Conversion from "aliquot homogenate" to "mg protein" requires the determination of protein content per unit volume homogenate. This is accomplished by means of Biuret procedure.

VII. <u>BIURET PROCEDURE:</u>

A. Standard Curve: Spectrophotometric Determination of Stock Bovine Serum Albumin (BSA) Concentration -

A stock solution of BSA[19] (2 g/100 ml) is prepared by weight. A 1:20 dilution of the stock was made resulting in a 1 mg/ml solution and determination of the actual concentration of BSA, Y, is calculated spectrophotometrically. Samples are read at 260 nm, 280 nm and 320 nm and the determina-

tions were made according to the following relationships:

$$X = (OD_{280}) - (OD_{320}) \times 20$$

and

$$Y = \frac{X}{6.7} \times 10,$$ where 6.7 is the extinction coefficient, E^{550}_{1cm}, of BSA (Handbook of Biochemistry, 1970).

0.01 - 0.50 ml of stock BSA (2 g/100 ml) is added to sample tubes containing sufficient water to bring the volume to 1.0 ml. An additional 4.0 ml of Biuret reagent is added, the solution mixed well and a half-hour was allowed to elapse before samples are read (λ = 550nm). A standard curve (OD v. mg/ml) is constructed using the data obtained from the spectrophotometric determination of BSA concentration.

B. Determination of Protein Content in Spleen and Liver Homogenates -

The technique for protein determination used in the present study is a modification of the method described in Methods in Enzymology (Appendix F).

A 25% tissue homogenate is prepared exactly as described in Section VI(A). With the sample shaking in ice, a 0.1 ml aliquot of homogenate is rapidly pipeted in a 12 ml graduated, conical centrifuge tube containing 0.5 ml 10%

TCA. Duplicate samples are pipeted with an automatic pipeter[20] calibrated to deliver 0.1 ml. The samples were then stored at -20°C until a later date when batch Biuret determinations are made.

To the thawed TCA homogenates the following reactants are added in the order presented:

1.0 ml Biuret Reagent; "vortex" to facilitate dissolving of TCA precipitate;
1.0 ml water;
3.0 ml Biuret Reagent; mix well.

The solution is permitted to stand for thirty minutes to permit maximal color development and appears turbid at this time. 1.5 ml diethyl ether is added to each sample tube, capped, mixed and centrifuged at room temperature for 3 minutes in order to extract lipids from the aqueous phase. The upper ether phase, containing the lipids is pipeted off and discarded. The aqueous phase is no longer turbid and an aliquot of this layer is read directly with Gilford Spectrophotometer (λ = 550nm). Spectrophotometric blank consists of 1.0 ml Biuret reagent and 4.0 ml water. The concentration of protein (mg/ml) is then determined by inspection of the standard curve.

VIII. PAPER CHROMATOGRAPHY:

A. In order to confirm that the Ehrlich's-positive

product isolated from column chromatography is indeed ALA, ascending paper chromatography is performed. Since an ER-positive reaction is given by any pyrrole with an unsubstituted -position, it is important to note the presence of an additional ER-positive contaminant in the TCA supernatant which is originally loaded on the column, i.e., aminoacetone.

The advantage of the column chromatography method, as modified by Marver, et al., (1966b), lies partly in the relative simplicity with which the aminoacetone is eliminated from the system and a relatively pure ALA-fraction is concentrated. AA-pyrrole is eluted in the first butanol wash whereas the bulk of the ALA-pyrrole is retained by the column at this time. After priming with 1M acetic acid, washing with methanol:acetic acid (2:1) results in the elution of ALA pyrrole.

The above is confirmed by ascending paper chromatography using 10 N NH_4OH:n-butanol:water (1:49:50) solvent system.

B. Synthetic ALA (10^{-2}M)[18] was converted to its pyrrole form and processed through the resin as described in

Section VI(D) above. The final eluate is evaporated to dryness in a vacuum oven (60°C;4 hr.)[19] and the remaining precipitate is redissolved in 0.5 ml methanol. The resulting solution is spotted 5 times on Whatman No. 1 chromatography paper strips (5 cm x 20 cm) and dried immediately with an airblower.

The eluate obtained from experimental sources (spleens of phenylhydrazine-induced and leukemic rats) is similarly processed and spotted at the origin 2 cm from the standard ALA spot. The strips are attached to an overhead bar, hung in a chromatography jar (10 in. x 10½ in.) containing 300 ml solvent and allowed to run for 4 hours. Strips are removed, the solvent front outlined in pencil and paper is dried in air under a fume hood.

Differential staining of the chromatogram together with the known R_f values for AA and ALA in this solvent system permits the confirmation of the fraction purity.

Once dried, the strips were hung and sprayed with ninhydrin[19] in order to detect the presence of amino acid residues, e.g., aminoacetone. The chromatogram is then heated in an oven for 3 minutes at 105°C. Any visible spots are immediately outlined in pencil. The strips are counter-

stained with Ehrlich's reagent spray and all ER-positive spots, (identifiable by pink color), are similarly outlined to insure accurate determination of R_f values in the event of fading. R_f is calculated from the following relationship:

$$R_f = \frac{\text{distance from origin to center of spot x, cm}}{\text{distance from origin to solvent front, cm}}$$

IX. COMPUTER ANALYSIS:

In an effort to maximize the analytical potential of the data, particularly in the leukemic experiments, several computer programs were run. The data is organized as described in Appendix G. Each leukemic experiment has 11 variables which were considered potentially important in the analysis of the leukemic pathogenesis:

Var 001 - day leukemia
Var 002 - % spleen benzidine-positive cells
Var 003 - Specific Activity splenic ALA-S
Var 004 - Net Activity splenic ALA-S
Var 005 - spleen weight
Var 006 - % spleen myeloblasts
Var 007 - % bone marrow myeloblasts
Var 008 - % bone marrow benzidine-positive cells
Var 009 - Specific Activity hepatic ALA-S
Var 010 - Net Activity hepatic ALA-S
Var 011 - liver weight

The following subprograms were run:

1. Scattergram (SG)
2. Multiple Regression Analysis (MRA)
3. Automatic Interaction Detector (AID)
4. Curve Fit Analysis (CFA)

A brief discussion of the four programs follows:

SG, MRA and CFA are subprograms belonging to an integrated system of computer programs known as the Statistical Package for the Social Sciences (SPSS).

1. SG: This subprogram produces a graph which presents the relationship between two variables entered. In addition, standard statistical analysis is provided optionally and includes: (a) Pearson's R; (b) R squared; (c) significance of R; (d) standard error; (e) intercept with the vertical axis; (f) slope. A further advantage of this subprogram is its ability to provide for missing data without nullification of the entire experiment. SG's of all variable pairs for leukemic data were run in 6 sets:

1. All data (subfiles 1-4, inclusive)
2. Subfiles 2, 3 and 4
3. Subfile 1 only
4. Subfile 2 only
5. Subfile 3 only
6. Subfile 4 only

An integer printed on the graph indicates the coincidence of that number of experiments at that precise value.

2. MRA: This subprogram permits the study of the linear relationship between a set of independent variables and a number of dependent variables while taking into account the interrelationships between the independent variables. MRA produces a linear combination of independent variables

which will correlate as highly as possible with the dependent variable. The resultant equation which relates these variables can be used to predict the values of the dependent variable. For MRA of leukemic data, per cent benzidine-positive cells was chosen as the dependent variable.

3. AID: This subprogram belongs to a program package known as Organized Set of Integrated Routines for Investigation with Statistics (OSIRIS) and is similar to the stepwise regression program. AID subdivides the data into a series of subgroups which maximize the ability to predict values of the dependent variable (per cent spleen benzidine-positive cells) and is most effective with large sample sizes, i.e., several thousand. The limitation of sample size of the leukemic data (n = 85), makes AID output not especially reliable.

4. CFA: Inspection of the SG's often suggested interrelationships between variable, which though not linear, showed definitive algebraic patterns. Several SG's which were clearly non-linear, were transformed to semi-logarithmic form in an attempt to linearize the original SG. In most cases linearization did not occur, suggesting that the two variables were not related in this manner.

(ref.: SPSS and OSIRIS manuals)

RESULTS

I. DETERMINATION OF PROTEIN CONTENT IN SPLEEN, LIVER AND TUMOR CELL HOMOGENATES

A. Normal Spleen and Liver - A total of twelve normal rats were used in the determination of protein content of normal spleen and liver homogenates. The mean protein content of normal spleen ($\pm$ standard error) is equal to 14.0% ($\pm$ 0.2) or 7.14 mg wet weight spleen per mg protein. The mean protein content of normal liver ($\pm$ S.E.) is equal to 16.9%($\pm$0.6) or 5.92 mg wet weight liver per mg protein.

B. Leukemic Spleen and Liver Homogenates - In order to determine the effect of the leukemic state and of the relative myeloblast content on the per cent protein in spleen and liver, Biuret determinations were performed on 39 leukemic animals whose per cent splenic myeloblasts ranged from less that 3% to greater than 40%, and whose hepatic myeloblast content appeared to be less than 10%,

No correlation is seen between per cent splenic and hepatic myeloblasts and protein content ($r_{spleen} = -0.099$). The mean per cent protein of leukemic spleen ($\pm$ SE) equals 14.4% ($\pm$ 0.3) or 6.94 mg wet weight spleen/mg protein, whereas the mean per cent of leukemic livers ($\pm$ SE) equals 17.6%

(± 0.4) or 5.68 mg wet weight liver/mg protein.

C. Tumor Cell Homogenates - A total of 8 tumor-bearing rats were used to determine the protein content of tumor cell homogenates. The mean per cent protein content (± SE) is 14.7%(± 0.7) or 6.80 mg wet weight tumor per mg protein.

The results of protein determinations of normal, leukemic and tumor cell homogenates are summarized in Table 1.

II. INDUCTION OF ACUTE MYELOGENOUS LEUKEMIA IN THE LONG-EVANS RAT

A. Histological Parameters - Histological analysis of bone marrow of rats with acute myelogenous leukemia has proved to be a convenient method for following the course and correlative effects of the disease. The leukemia is generally characterized by the appearance of leukemic myeloblasts in the bone marrow (femora) within 3 - 5 days after intravenous injection of SCL cells. A fairly predictable pattern of decreasing erythroid proliferation can be correlated with an increasing per cent of leukemic myeloblasts in the bone marrow (Fig. 1).*

It will be noted that with the exception of a cluster of points in the lower-left corner of Fig. 1 (low per cent bone marrow benzidine-positive cells with correspondingly

*See Appendix G

low per cent bone marrow myeloblasts) representing ineffective erythropoiesis with a minor degree of bone marrow myeloblast proliferation, there appears to be a strong negative correlation between per cent bone marrow myeloblasts and per cent bone marrow benzidine-positive cells.

With progressive shut-down of erythropoiesis in the bone marrow, there is a corresponding increase in the per cent benzidine-positive cells in the spleen (Fig. 2). This splenic erythroid compensation, however, does not exhibit linear correlation with decreasing medullary erythropoiesis. Instead, with the exception of 5 unusual cases to be discussed (sub-population 2A), splenic compensation is not generally in evidence until the per cent medullary benzidine-positive cells approaches 12% as compared to a mean normal value of 16.8% (2B). At this time, there is a population of animals which begins to exhibit erythroid compensation indicated by benzidine-positive cells in the spleen, ranging from 7-15% (2C). It is seen from inspection of Fig. 2, that a significant population of rats whose medullary erythropoiesis is reduced (benzidine-positive cells ranging from 0-12%), does not respond to this loss of erythropoietic capacity by enhanced splenic erythropoiesis (sub-population 2E). Nevertheless, as per cent bone

marrow benzidine-positive cells declines from 6-0%, there is a continuing increase in per cent benzidine-positive elements among some animals ranging from 20-50% (2D). Fig. 2A illustrates the relative linearity between the two variables when the ranges of sub-populations 2B-D are plotted.

Fig. 3 illustrates the relationship between per cent spleen myeloblasts and per cent splenic benzidine-positive cells. This figure includes all experiments with the exception of those in subfile 1, i.e., those where per cent spleen benzidine-positive cells range from 0-2.9%. Justification for removal of this subfile rests on its apparent non-uniform composition. Many cases in subfile 1 represent animals where the per cent myeloblast and benzidine-positive cells in spleen and bone marrow are almost normal, and thus may characterize a pre-leukemic or early leukemic state. Others, quite clearly show signs of severe pathology, as evidenced by one or a combination of abnormal values of per cent bone marrow myeloblast, and/or benzidine-positive cells. Fig. 3A illustrates the contribution of subfile 1 to Fig. 3. The essential addition, as evidenced by a cluster of experiments which fall in the lower-left corner of the graph, represent animals whose per cent spleen myeloblast content is less than 7% with cor-

responding spleen compensation ranging from 0-3% benzidine-positive cells. These animals may be in an early leukemic condition and have not, as yet, developed spleen compensation.

Fig. 4 attempts to assess the relationship between per cent bone marrow and spleen myeloblasts. The graph is suggestive of 2 populations of leukemic animals. In the primary population, there appears to be linearity with respect to bone marrow and spleen myeloblasts. In addition, however, there exists a substantial sub-population, composed of 15 cases in which spleen myeloblasts remain relatively low despite high per cent bone marrow myeloblasts (sub-population 4A).

Another relationship investigated, though possibly an indirect one, is that between per cent bone marrow myeloblasts and per cent spleen benzidine-positive cells (Fig. 5). With the exception of approximately 15 cases (sub-population 5A) identical to those in sub-population 4A, a pattern of splenic compensation emerges that is similar to the shape of the curves seen in Figs. 1, 2 and 3A above. In addition, two classes of animals exist: (1) derived from subfile 1, shows a minor degree of compensation at per cent bone marrow myelo-

blasts ranging from 0-10%; and (2) derived from subfiles 2, 3 and 4: exhibits high degree of splenic compensation ranging from 10-40%. Fig. 5A illustrates the identical relationship minus subfile 1. Justification for removal of this class from the bulk of experiments presented, rests again, on the experimental composition of subfile 1, i.e., consists of "apparently" normal, preleukemic or early leukemic animals which may not conform to the same pathological alterations imposed by a highly leukemic state.

Another, perhaps indirect, but nevertheless interesting relationship is that between per cent spleen myeloblasts and per cent bone marrow benzidine-positive cells (Fig. 6). In general, an inverse relationship exists between these two variables with a tendency toward a modified "hyperbolic-shaped" curve. A sub-population can be identified in the lower-left corner of the graph, which is contributed by subfiles 3 and 4 and which represents animals whose medullary erythropoiesis is practically non-existent and whose spleens have low myeloblast content and exhibit a marked splenic erythroid compensation.

B. Physiological Parameters -

1. Spleen weight -

Figure 7 illustrates the linear relationship between spleen weight and per cent spleen myeloblasts ($r = 0.738$; $p = 0.00001$). Though obviously an indirect effect, a similar linear correlation ($r = 0.735$; $p = 0.00001$) is evident between spleen weight and per cent bone marrow myeloblasts (Fig. 8). An interesting lack of correlation is seen between spleen weight and per cent spleen benzidine-positive cells ($r = 0.130$; Fig. 9). However, the significance of this finding is questionable ($p = 0.130$).

2. Liver weight - Since no direct histological examination of the liver was performed, only indirect correlations with liver weight are reported. Nevertheless, there seems to be a significant correlation between liver weight and per cent bone marrow myeloblasts (Fig. 10; $r = 0.692$; $p = 0.00001$). Figure 11 presents the effect of increasing per cent splenic myeloblasts on liver weight ($r = 0.505$; $p = 0.00001$).

C. ALA-S Activity -

1. Spleen - Methodological limitations of this ALA-S procedure do not permit the assay of spleens of less than 1g. Thus, in early and some exceptional, late-stage

leukemic animals whose spleens were still normal in weight could not be monitored for ALA-S activity.

In addition to the compensatory relationship between spleen and bone marrow erythropoiesis, there is yet another important parameter influencing splenic erythroid compensation: splenic leukemic myeloblasts.

Figs. 12 and 13 illustrate the degree of linearity which exists between the specific activity (SA: pmoles per mg protein per hour) and net activity (NA: mμmoles per spleen per hour) of splenic ALA-S and per cent spleen benzidine-positive cells ($r = 0.864$; $p = 0.00001$ and $r = 0.726$; $p = 0.00001$, respectively. In addition, the activities of splenic ALA-S in normal, hemorrhagic and hemolytic anemic rats are presented and will be discussed below. Fig. 14 presents data whose representation is not unlike that seen in 3A (per cent splenic myeloblasts v. per cent splenic benzidine-positive cells). This follows logically from the linear relationship described above in Fig. 12. It is clear from this analysis that very high levels of splenic compensation, as measured by SA splenic ALA-S, occurs only when per cent spleen myeloblasts is low. Fig. 15 depicts the relationship between spleen NA and per cent spleen myeloblasts. With the excep-

tion of 1 case, NA's greater than approximately 75 mμ moles/spleen/hr, are only achieved when the per cent spleen myeloblasts is negligible. The converse is not true, however, i.e., not all cases in which the per cent spleen myeloblasts is low show high NA's. Table 2 shows the relationship between per cent myeloblasts, benzidine-positive cells and the corresponding ALA-S activities of the spleen. It is clear from this presentation that with a few exceptions, the per cent benzidine-positive cells and the corresponding SA of ALA-S are depressed under conditions in which the per cent spleen myeloblasts is high: range per cent spleen myeloblast: 11.3--63.6, with only 4 cases out of 23 less than 20%. Furthermore, the SA of ALA-S in spleen where per cent benzidine-positive cells is very low (subfile 1) and thus approaching normal values (0.2.9%) is less than that of normal spleens.

Figure 16 (per cent bone marrow myeloblasts v. spleen SA), clearly shows no linear relationship. Nevertheless, one can isolate 2 sub-populations. The major component of the curve approximates a logarithmic relationship between these two variables, i.e., as per cent bone marrow myeloblasts increases, there is a corresponding increase in

spleen SA until the SA approaches 130 pmoles/mg protein/hr (almost 2-times normal SA), beyond which point there is no significant increase in per cent bone marrow myeloblasts despite increasing values of SA. In the second population, (sub-population 16A) which is noted along the abscissa in Fig. 16, a high spleen SA is seen with no corresponding increase in per cent bone marrow myeloblasts. This population derives from subfiles 3 and 4 and is identical to that previously discussed in Figs. 3A and 4, i.e., this class is characterized by: low per cent bone marrow myeloblasts, low per cent bone marrow benzidine-positive cells, low per cent splenic myeloblasts and high per cent splenic benzidine-positive cells.

The picture of splenic NA v. per cent bone marrow myeloblasts (Fig. 17) is more complex than that of the corresponding SA curve. The relatively small degree of splenomegaly seen in subfiles 3 and 4, depress the corresponding values of NA, significantly in some cases, so as to remove them from the sub-population in Fig. 16 described above. Thus, of the approximate 15 cases in sub-population 16A, only 8 remain as a sub-population in Fig. 17. The remaining cases have shifted into the general population.

2. Liver - Fig. 18 depicts the relationship between bone marrow myeloblasts and liver SA. No discernible pattern is evident from those data. Clearly, the relationship between these two variables is, at best, an indirect one. Yet, the corresponding curve of liver NA v. per cent bone marrow myeloblasts exhibits some degree of linearity not observable with SA (Fig. 19; $r = 0.506$; $p = 0.00004$).

Figs. 20 and 21 (per cent bone marrow benzidine-positive cells v. hepatic SA and NA, respectively), like that seen in Fig. 18, exhibit no recognizable or correlatable pattern.

3. Tumor - No ALA-S activity was recorded in tumor cell homogenates prepared from subcutaneous tumors containing an essentially pure population of leukemic myeloblasts.

III. INDUCTION OF HEMOLYTIC ANEMIA IN THE LONG-EVANS RAT

A. Physiological Parameters - Injection of normal rats with phenylhydrazine (PH), induces a severe, hemolytic anemia as evidenced by decreased hematocrit, decreased peripheral red blood cell count and reticulocytosis. The pattern seen is one of increasing reticulocyte numbers in the circulation and is shown in Fig. 22. Hematocrits were monitored on days 0, 1, 3 and 5. The pattern of hematocrit

over the couxse of experimental period is shown in Fig. 23. Following a sharp decline on day 1, 24 hours after the initial PH injection, the hematocrit continues to fall and by day 5, shows clear evidence of recovery. On day 5, the day of assay, the mean hematocrit of experimental rats was 31.6 ± 0.3, whereas the normal hematocrit averaged 47.2 ± 0.7.

Fig. 24 demonstrates the progressive decrease in peripheral RBC which occurs with continuing PH treatment. Splenomegaly, apparent on the day of assay (day 5), represents a 3-fold increase over normal values of spleen weight. Hepatomegaly was not evident (Table 3). It should be noted that data presented in Figs. 22 and 24 were derived from experimental conditions that were not identical to those of assay animals, i.e., the regimen of PH-treatment is 4 injections administered every other day. Nevertheless, though not comparable to schedule of PH-injection used throughout the assay protocol, the data is included in order to provide some notion of the peripheral blood picture under conditions of hemolytic anemia. This profile is not considered to be substantially different from those changes in degree of peripheral reticulocytosis and RBC

count that would arise as a result of standard PH treatment.

B. Histological Parameters - The per cent benzidine-positive cells in spleen and bone marrow were monitored on day of assay. A value, approximately 2-times normal per cent bone marrow benzidine-positive elements, was noted under these conditions (31.6 ± 1.2%). Splenic erythroid compensation is highly significant in this hemolytic anemia as evidenced by an almost 75-fold increase in per cent benzidine-positive elements (29.5 ± 1.0%).

Table 3 summarizes and compares mean per cent benzidine-positive cells in bone marrow and spleen, hematocrits and spleen and liver weights in PH-induced and normal rats.

C. ALA-S Activity -

1. Spleen - The increased erythropoietic capacity of the spleen in PH-treated rats, as evidenced by a 75-fold increase in per cent spleen benzidine-positive cells over normal values was further substantiated by a more than 10-fold increase in the SA of ALA-S. In addition, a consistent splenomegaly contributed to the NA in PH-treated rats which was 35-times greater than that observed in normal rats' (Table 4).

2. Liver - The SA of ALA-S in PH-treated rats was within normal limits. No hepatomegaly was evident. Thus, the NA of ALA-S similarly, remained normal (Table 4).

IV. <u>INDUCTION OF HEMORRHAGIC ANEMIA IN THE LONG-EVANS RAT</u>

A. Physiological Parameters - Thrice-bled rats, as described above, evidenced a moderate anemia as measured by a decline in hematocrit from a mean normal value of 45.7 ± 0.7 to a mean equal to 37.7 ± 0.8. Contrary to the findings seen with PH-induced anemia, only a slight splenomegaly occurred with hemorrhagic anemia (Table 3). Fig. 25 shows the pattern of hematocrits over the 6-day induction period. The initial pattern of hematocrits is similar to that of PH-treated rats: a precipitous drop 24 hours after initial injection. However, in the case of hemorrhagic anemia, this decline is followed by a plateau until finally, on day 6, a recovery is seen. Hepatomegaly does not occur under these conditions.

B. Histological Parameters - The mean per cent benzidine-positive cells in the bone marrow, 29.9 ± 1.2, seen on day of assay, is similar to that observed in PH studies and is nearly twice the values observed in normal rats.

Spleen compensation, though not as marked as in the

hemolytic anemia, is still quite substantial, with the per cent benzidine-positive cells almost 25-times that of normal animals (Table 3).

C. ALA-S Activity -

1. Spleen - The mean splenic SA of ALA-S of animals with hemorrhagic anemia is 275 pmoles ALA/mg protein/hr which represents an almost 5-fold increase over normal. As a significant splenomegaly did not develop under these conditions, the NA was approximately 5-times normal (Table 4).

2. Liver - As in the case of hemolytic anemia, the hepatic SA and NA were within the normal range (Table 4).

RESULTS APPENDIX

I. PROTEIN DETERMINATION

A. Spectrophotometric Determination of Bovine Serum Albumin (BSA) Concentration in Stock Solution

The concentration of the stock BSA is equal to 17.58 mg/ml as determined by spectrophotometric analysis. The spectrophotometric record of BSA stock solution and the resultant calculation of the actual concentration of BSA is shown in Table 5. This value is used to convert the optical densities of standard solutions to mg/ml in the preparation of the standard curve.

B. Preparation of the Standard Curve

Table 6 presents the ODs (A_{550nm}) of 11 standard solutions using the BSA concentration determined above. Fig. 26 is the standard curve derived from this data.

II. PAPER CHROMATOGRAPHY

Synthetic ALA-pyrrole, prepared as described above was routinely co-chromatogramed with experimental samples and served as standard reference for ALA-pyrrole. The results of the chromatography and differential staining are shown in Table 7.

The reported R_f value for the synthetic ALA-pyrrole with this hydrated NH_4-butanol system is equal to 0.2 (Mauzerall and Granick, 1956) and is confirmed in these studies. The results from experimental samples indicate the presence of a spot which co-chromatograms with synthetic ALA-pyrrole (R_f = 0.2) and is both Ehrlich's Reagent (ER) positive and ninhydrin negative. This strongly suggests the presence of ALA-pyrrole in the experimental samples. The absence of an ER+ aminoacetone (AA) fraction (ER+, NIN-; R_f = 0.93) in the final eluate is significant in that it indicates that the ER+ activities assayed with tissue homogenates are primarily, if not solely, the result of the interaction of ER with ALA-pyrrole and not with an ER+ contaminant.

III. OPTIMALIZATION OF ALA-S ACTIVITY

A. Spleen - Optimal incubation conditions for ALA-S assays in spleen were determined using phenylhydrazine-induced spleens since these tissues were found to have the highest enzyme activities and were, on the average, over 3 times the weight of normal spleens.

The following parameters were optimized: (1) EDTA concentration, (2) volume homogenate (25%), (3) time incubation. The results are presented in Figs. 27a, 28a and 29a, respectively.

1. Figure 27a shows the effect of increasing EDTA concentration of ALA-S activity. Optimal generation of ALA is seen with 30 mM EDTA.

2. Figure 28a describes the linear relationship between the volume of 25% homogenate and ALA-S activity. 2.0 ml was observed to be the opitmal volume and was used in all subsequent experiments.

3. Figure 29a demonstrates ALA-S activity as a function of time of incubation from 0 to 50 minutes. Maximal accumulation of ALA is observed when samples were incubated for 40 minutes.

B. Liver - Normal liver was used in determining the optimal conditions for ALA-S activity. The same three parameters optimalized for spleen activity are monitored in the liver:

1. EDTA concentration: Optimal EDTA concentration in the liver was found to be identical to that of the spleen: 30mM (Figure 27b).

2. Volume Homogenate: Contrary to the results found in the spleen, the effect of increasing volume homogenate was not linear. Optimal values were observed when the volume homogenate was equal to 1.2 ml (Figure 28b).

3. Time Incubation: A 40 minute incubation period resulted in optimal generation of ALA under conditions described (Figure 29b).

C. Tumor - The low levels of ALA-S activity in tumor cells necessitated the use of a 50% cell homogenate rather than the 25% homogenate used with spleen and liver assays. The optimal conditions for ALA accumulation in tumor cell homogenates are identical to those observed for liver homogenates and were used in all tumor cell assays (Figures 27c, 28c, 29c).

IV. MINIATURIZATION OF ALA-S ASSAY

The procedure for measuring ALA accumulation as reported by Ebert, et al., (1972) requires the use of twice the volume and thus twice the amount of tissue for equivalent activities than reported in these studies.

Since leukemic spleens assayed for ALA-S activity could not be pooled due to the disparity of the leukemic states, it was necessary to increase the general sensitivity of the procedure in order to permit the use of single spleens which would otherwise be too small to assay. This modification was accomplished by miniaturization to 25% of original volumes used in Ebert's technique. Figure 30 compares the optical densities resulting from phenylhydrazine-induced ALA-S activity of spleens using the complete and ¼-volume methods.

DISCUSSION

I. COMPENSATORY ERYTHROPOIESIS:

A. Relationship of Bone Marrow and Spleen - Stimuli to erythropoiesis in rats, such as the anemia due to hemolysis (Fruhman, 1970), hemorrhage (Baxter, et al., 1955) and the injection of EPO itself (Fruhman, 1970; Kurtides, 1965) have been shown to induce an erythropoietic hyperplasia in hematopoietic tissue, including bone marrow and spleen (Fruhman, 1970).

The histological profile of erythropoiesis in hemolytic and hemorrhagic anemias yields interesting results. The mean per cent benzidine-positive cells in the bone marrow of rats with both types of primary anemias increased from a mean of 16.8% (± 0.9) to approximately 30%. The compensatory responses of the spleens, however, were not equivalent. Hemolytic anemia induced a mean per cent benzidine-positive cell value 3-times that achieved in hemorrhagic anemia: 29.5% (± 1.0) and 10.5% (± 1.0), respectively. The fact that medullary erythropoiesis in both primary anemias, as monitored by histological analysis, were equivalent, despite differential splenic compensation, suggests the existence of a relative upper limit of approxi-

mately 30% benzidine-positive cells in this organ under these conditions. Jacobs and Finch, (1971) suggest a similar phenomenon in rabbits. They report a uniform erythroid proliferation in the bone marrow of differentially anemic rabbits as measured by mean per cent hemoglobin levels. Consequently, they have postulated the existence of a ceiling on cellular proliferation in acute anemia, which may be regulated by limitation of stem cell recruitment. This conclusion is consistent with Jacobson's,(1960) comment regarding compensatory erythropoiesis in the mouse: "In the mouse, the spleen happens to be a marvelous organ because if you can push erythropoiesis in the animal beyond the bone marrow capacity, erythropoiesis will increase in the spleen."

Fruhman, (1970) expands on this characterization of the spleen, in full agreement with results presented in this study, by stating that marrow capacity need not be "pushed" in order to induce splenic erythropoiesis. He further points out, that high degrees of erythropoietic activity are noted in the spleen when medullary erythropoiesis is rather minimal. This is precisely the situation encountered during this investigation. In the primary anemias,

marrow capacity was apparently maximally responsive while the spleens were compensating to different extents. In the case of AML, however, where the pathology includes medullary inhibition of erythropoiesis, significant levels of splenic compensation are similarly noted.

In an effort to further understand the extraordinary phenomenon of splenic erythroid compensation in the rat, a discussion of erythropoiesis in the mouse follows. Special attention is given to essential differences between the two rodent systems.

In the normal adult mouse, splenic erythropoiesis contributes significantly to the total erythropoietic capacity of the animal. (Dunn, 1954; Albert, et al., 1966; Brodsky, et al., 1966; Fruhman, 1966a; Boggs, et al., 1969; Bozzini, et al., 1970; Fruhman, 1970).

Erythropoiesis in the normal adult rat spleen is not considered to be a large contributor to the total erythropoietic output (Fruhman and Gordon, 1952; Garcia, 1957; Handler and Handler, 1970b). Nevertheless, it is capable of substantial levels of compensatory red blood cell production during conditions of primary anemic stress (Azen and Schilling, 1963; Baxter, 1955), as well as during the

pathogenesis of AML, the latter, no doubt, the result of secondary anemia associated with the leukemia (Handler and Handler, 1970b).

Methods used to study the erythropoietic contribution of the spleen include, primarly, splenectomy, ligation of splenic blood vessels, spleen transplants, with and without maintaining integrity of the vasculature (Crosby, 1959).

The splenic response to varying degrees of anemia in the mouse, appears to operate via the activation of different erythropoietic mechanisms (Fogh, 1974).

In rather mild anemias caused by slight X-irradiation, splenectomy was found to have no effect on the rate of erythropoietic recovery (Hodgson, et al., 1968). Hematological recovery from more severe anemias, as measured by increasing hematocrit, like those produced by PH-injection (Hodgson, et al., 1968), intense bleeding or hypoxia (Boggs, et al., 1969; Schooley, 1970) were found to be reduced in splenectomized mice when compared to non-splenectomized controls. Fruhman,(1970) similarly reported that splenectomized mice treated with PH, had decreased hematocrits, decreased per cent reticulocytes and a slower rate of hematological recovery. Thus, it appears that splenic eryth-

roid compensation may play its most significant role only when anemic stress is quite severe (Fogh, 1974). Similarly substantial radiation treatment (Brecher, et al., 1948; Jacobson, et al., 1949) and post-starvation refeeding (Fruhman, 1966b) are both associated with significant levels of erythroid hyperplasia.

The literature concerning the relationship between splenic and medullary erythropoietic response in such moderate and severe anemias has been contradictory. In vivo ^{59}Fe-incorporation studies in hematopoietic tissue of erythropoietic-stimulated animals, indicate that the radioiron content in the spleen is significantly greater than that in the bone marrow Aggio and Garcia, 1969; Boggs, et al., 1969; Bozzini, et al., 1970; Fruhman, 1970), leading to the conclusion that the spleen is the primary organ involved in the erythropoietic response and that the bone marrow is non-effective in this regard. A closer analysis indicates some inherent problems in the interpretation of the above results. Cytological studies of the marrow in highly stimulated mice, reveal a pronounced erythroid hyperplasia. This is difficult to reconcile with the depressed levels of ^{59}Fe-incorporation which is measured in ery-

thropoietic-stimulated mice even when compared to normal bone marrow (Aggio, et al., 1974; Fruhman, 1970; Kubanek, et al., 1968; Turner, et al., 1967). This is especially true in view of in vitro investigations of ^{59}Fe-incorporation into bone marrow and spleen which suggest that erythropoiesis is stimulated in both hematopoietic organs (Fruhman, 1970). Aggio, et al.,(1973), have proposed a possible source of error in the in vivo experiments. Apparently, the bone marrow competes unsuccessfully with the spleen and circulating reticulocytes for free Fe in erythropoietic-stimulated mice. Preferential uptake of Fe by spleen and reticulocytes over the bone marrow has been substantiated in experiments in which the splenic vessels were occluded (Aggio, et al., 1974). Under such conditions, in comparison with animals with equivalent degrees of anemia with non-occluded spleens, in vivo ^{59}Fe-incorporation in the marrow increases, indicating, in fact, active medullary erythropoiesis. It is further suggested by Aggio, et al.,(1973), that under anemic conditions, EPO may be responsible for creating a micro-environment which favors preferential iron uptake in the spleen by the reported vasodilation effect on splenic vasculature (McCuskey, et al.,

1972a; 1972b). This would result in increased blood flow through the spleen, a condition not as prominent in the bone marrow (Aggio, 1973).

Clearly, such considerations are important in interpretation of the results of all in vivo experiments of this type. On the basis of the data presented, therefore, Fruhman, (1970) has concluded that medullary and splenic response to certain erythropoietic stimuli, e.g., bleeding, result in equivalent activities of erythropoiesis in these two hematopoietic organs.

Nevertheless, it appears that there are conditions in which splenic and medullary erythroid compensation are not equally active (Fruhman, 1970). Such cases may be seen during recovery from moderate to large doses of X-irradiation and possibly in the refeeding period following acute starvation (Fruhman, 1970).

A third pattern of erythropoietic response in the mouse involves the shunting of erythropoiesis from the marrow to the spleen (Fruhman, 1970). This is encountered during the recovery period following injection of killed bacteria and bacterial endotoxin. Evidence is presented which suggests that when challenged with leukopoietic

stimuli, the bone marrow preferentially devotes its hematopoietic potential to proliferation of white blood cells and that medullary erythropoiesis is greatly inhibited. At the same time, the spleen becomes highly erythropoietic (Fruhman, 1970). These findings are compatible with the existence of a common, pluri-potential stem cell for which differentiation into erythroid and myeloid lines reflects a competitive relationship (Fruhman, 1970). The intimate and at times subtle relationship between hematopoiesis in general, and erythropoiesis in particular, in bone marrow and spleen, accentuates the need for concurrent examination of both organs in the investigation of blood cell production.

II. SPLENIC COMPENSATORY ERYTHROPOIESIS IN PRIMARY ANEMIAS:

A. Hemolytic Anemia - The anemia, induced by phenylhydrazine-injection, appears to operate via two distinct mechanisms which are dose-dependent. Massive doses of PH appear to have a direct lytic effect on red blood cells inducing a severe and essentially intravascular hemolysis (Cruz, 1941; Bratley, et al., 1931; Beutler, et al., 1954). More moderate doses, including that used in this study, appear to induce hemolysis via more indirect means (Cruz, 1941;

Azen and Schilling, 1963; Rifkind, 1964). Under these conditions, PH exerts its hemolytic effect through detection, sequestration and destruction in the spleen and is thus an extravascular phenomenon (Iyengar and Chandra, 1974; Chiyoda, et al., 1974).

Injection of PH results in a prolonged, intense erythropoietic stimulus with a progressive hemolytic curve (Iyengar and Chandra, 1974) The peripheral blood picture seen over the experimental period includes: decreased hematocrit, decreased red blood cell and reticulocytosis and is evidence of a severe, sustained hemolytic crisis.

The splenomegaly accompanying hemolytic anemia may be the result of a multiplicity of factors (Chen and Weiss, 1973):

1. Electron microscopic analysis of spleens in PH-treated animals shows clear evidence of damaged erythrocytes accumulating in the interendothelial slits of splenic sinuses. Heinz bodies, the hemoglobin-denaturation derivative, induced by PH, are thought to obstruct and impede the passage of red blood cells through the slits, with the resultant blockage of efferent blood flow from the spleen. This results in the accumulation of blood cells in the spleen and might be a primary cause of the splenomegaly

seen in this anemia (Chen and Weiss, 1973);

2. General proliferative response to phenylhydrazine-induced hemolysis involving all cells native to the spleen has been confirmed by a sharp increase in splenic DNA content and histological examination following PH administration (Jandl, et al., 1965).

3. Increased number of macrophages in splenic cords as a consequence of damaged red blood cells causing obstruction of slits (Chen and Weiss, 1973);

4. Interference with passage of fluids across the spleen into lymphatic vessels (Janout and Weiss, 1972), thus interfering with lymphatic drainage (Chen and Weiss, 1973);

5. Compensatory erythropoiesis (Chen and Weiss, 1973);

6. Direct effect of PH on endothelial cells comprising the slits with consequent malfunction and blockage of sinuses.

B. Hemorrhagic Anemia - Rats bled 3 times according to the regimen described in Materials and Methods, are characterized by decreased number red blood cells, decreased hematocrit and reticulocytosis. Splenomegaly is not evident despite active erythropoietic compensation by the spleen. It appears, therefore, that erythropoiesis in the spleen, at least to the degree found in this hemorrhagic anemia, does not contribute significantly to the increase in spleen size. This may be generalized to the case of PH-induced

hemolytic anemia, and may thus eliminate splenic erythropoiesis as a major contributor to the splenomegaly associated with hemolytic anemia. This must be considered with caution, however, as the extent of splenic compensation in PH-induced anemia is greater than that in hemorrhagic anemia.

It is evident that removal of blood from the circulation induces neither intra-nor extra-vascular hemolysis, nor does it deform or degrade the remaining cells in circulation. Thus, the spleen is not recruited, to any greater extent than normal, to sequester and destroy effete red blood cells and, consequently, may not undergo splenomegaly. Indeed, it should be noted, that spleens of rabbits subjected to chronic bleeding episodes undergo atrophy as a result of this treatment (deLangen, 1943). It is assumed from calculations based on the lifespan of the RBC that under conditions, less than 1% of circulating erythrocytes would die of "old age". This would essentially eliminate the role of the spleen in destruction of effete RBC and would result in hypoplasia of cells of the RES and splenic atrophy (Crosby, 1959).

III. <u>ROLE OF THE LIVER IN COMPENSATORY ERYTHROPOIESIS</u>:

The direct role of the liver in compensatory erythropoiesis appears to be negligible. No significant levels of benzidine-positive cells were in evidence upon scanning liver slides, despite the presence of a potent stimulus for such activity in both hemolytic and hemorrhagic anemias. Hepatomegaly was not in evidence in either type of primary anemia. Despite an apparent absence of erythropoietic activity, the liver is by no means quiescent during the anemic phase. The liver is reported to possess a fairly high level of erythrophagocytic activity by reticulo-endothelial (RE) cells under conditions of PH-induced anemia (Iyengar and Chandra, 1974). However, that such enhanced activity is not associated with hepatomegaly does not necessarily reflect the relative proliferative capacity of the RE cells, for indeed RE hyperplasia has been reported to occur following PH treatment (Jandl, <u>et al</u>., 1965). However, such proliferation is apparently not sufficient to result in an increase in liver weight.

The degree to which the liver participates in erythrophagocytosis may be dependent on the dosage of PH administered. Rifkind, (1964) reports that rabbits receiving low doses of PH (5mg/kg), demonstrated no hepatic sequestration or destruction of red blood cells. Raising the dosage, however, did result in erythrophagocytic activity by Kupffer cells. He further noted, that this mechanism of erythro-

cyte destruction by phagocytosis appeared to be identical to that occurring concurrently in the spleen.

There appears to be agreement that there is a differential sensitivity in spleen and liver toward the removal capacity of damaged red blood cells, i.e., it is primarily the spleen that destroys moderately damaged red blood cells (Crome and Mollison, 1964; Hugh Jones, et al., 1957; Harris, et al., 1957), whereas the total RE system, the liver being the major component, is recruited into performing this function only when damage to the RBC is severe (Jandl and Kaplan, 1960; Mollison and Hugh Jones, 1958). Furthermore, the same relationship appears to hold for PH-induced damage and the site of sequestration (Azen and Schilling, 1963). With dosages of PH approximately equivalent to those used in the present study, Jandl, et al., (1965) report that hepatic sequestration of red cells was less pronounced than in the spleen, though erythrophagocytosis in littoral cells is more prominent and occurs earlier than in the spleen.

Jacobsen, et al., (1956) suggest an indirect role of the liver in compensatory erythropoiesis during PH-induced anemia. These investigators reported the presence of central lobular degradation of the liver in PH-treated rabbits. This

damage was thought to be responsible for enhanced levels of EPO found under these conditions compared to those of bled animals. The mechanism for such enhancement has been postulated to be the result of decreased production or activity of a hepatic inhibitor of EPO (Jacobsen, et al., 1956). Not all studies, however, report damage to the liver under conditions of PH-treatment (Krantz and Jacobson, 1970; Borsook, 1959; Lowy, et al., 1959; Stohlman and Brecher, 1957). Indeed, in such cases, EPO levels were still found to be greater than those with damaged livers.

IV. ERYTHROPOIESIS IN ACUTE MYELOGENOUS LEUKEMIA:

A. Relationship Between Medullary and Splenic Erythropoiesis and the Leukemic Process -

It appears that the leukemic animal exhibits compensatory erythropoiesis primarily in response to the decline in medullary red blood cell production. Nevertheless, it is equally apparent that the relationship between the per cent bone marrow benzidine-positive cells and per cent splenic benzidine-positive cells is not a simple inverse, linear one (Fig. 2). In order to analyze the elements which contribute to the deviation from linearity, the experiments are divided into various sub-populations as follows: With the exception of 4 cases, to be treated separately, (sub-population 2A), a decline in bone marrow benzidine-positive cells from the mean normal value of 16.8 ($\pm$ 0.9) to approximately 12% is not associated with significant splenic erythroid compensation

(sub-population 2B). Between 12→8% bone marrow benzidine-positive cells, there is a second population which exhibits splenic compensation to the extent of 7-15% spleen benzidine-positive cells (sub-population 2C). A third group, composed of animals from subfile 4, and thus exhibiting an extremely high degree of compensation (25-50% spleen benzidine-positive cells), is seen when per cent bone marrow benzidine-positive cells is approaching negligible values (6→0%; sub-population 2D). A graph presenting the range of these 3 sub-populations, 2B, 2C, 2D is plotted in Figure 2A and illustrates a fairly linear relationship. However, it is obvious from inspection of Figure 2, that a significant number of animals exist in which medullary erythropoiesis is inhibited, but which do not exhibit sufficiently high splenic erythroid compensation (sub-population 2E). Analysis of this group, reveals them to consist of approximately 15 leukemic animals belonging to subfile 2 (per cent spleen benzidine-positive cells range: 3.0-9.9%), all with relatively high per cent spleen myeloblast content: Total number of cases in sub-population 2E = 23; range per cent spleen myeloblast: 11.3-63.6, with only 4 cases less than 20%.

This finding prompts a further analysis of the results summarized in Figure 3 (per cent spleen benzidine-positive cells v. per cent spleen myeloblasts). This curve clearly suggests that no increase in per cent splenic benzidine-positive cells above 10% occurs when per cent spleen myeloblasts exceeds approximately 15%, and further, that proliferation of benzidine-positive cells to levels greater than 20%, coexists exclusively with conditions in which per cent spleen myeloblast content is less than 4%.

Thus far, the results presented in Figures 2 and 3, suggest the interaction of at least two factors governing compensatory erythropoiesis in the spleen: (a) signal; (b) permissiveness of the microenvironment. The first step is related to the state and efficiency of medullary erythropoiesis. Presumably, the progressive shutdown of erythropoiesis in the bone marrow triggers the spleen, (via direct or indirect mechanism), to initiate its compensatory function. Superimposed on this, however, is the relative permissiveness of the splenic microenvironment to proliferation of benzidine-positive cells. Apparently, the presence of a critical per cent spleen myeloblasts, or, their presence for a critical period of time, is sufficient to alter the splenic microen-

vironment so as to inhibit potential proliferation of benzidine-positive cells, despite the fact that medullary erythropoiesis may be severely inhibited.

Splenic unresponsiveness to the presumed presence of the bone marrow-initiated "trigger-factor" may not be unlike the primary situation in the bone marrow itself. The progressive decline in per cent bone marrow benzidine-positive cells observed during leukemia, has led to the speculation that the presence of leukemic myeloblasts in the bone marrow, even in small percentages results in an altered microenvironment which does not support effective erythropoiesis (Handler and Handler, 1972; Hoelzer and Harriss, 1973).

A primary symptom of acute leukemia is a progressive anemia which is not the result of appreciable hemolysis or hemorrhage (Chiyoda, *et al.*, 1974). It has been postulated, that at some point during the leukemic process, medullary erythroid cells become refractory to high circulating levels of endogenous EPO induced by the anemia (Thorling, 1965; Zaizov and Matoth, 1971; Chiyoda, *et al.*, 1974).

Zaizov and Matoth, (1971) have also reported subnormal levels of urinary EPO during the course of AML in children. Similarly, Ebert, *et al.*, (1972) have reported levels of EPO in RLV-infected mice to be inappropriately low for the degree of anemia encountered. Attempts to increase plasma erythropoietin concentrations by superimposition of hemor-

rhagic treatment in RLV-infected mice did not result in further EPO production and is suggestive of a failure of EPO synthesis. However, recent evidence suggests that EPO is both being produced and cleared at a much higher rate than normal (OKunewick and Erhard, 1974). Indeed it is suggested that the failure to induce increased levels of EPO in the leukemic mouse by bleeding is the result of a nearly maximally-stimulated EPO synthesis (OKunewick and Erhard, 1974).

Another suggestion forwarded to account for depressed levels of erythropoiesis which occurs secondary to leukemia, focuses on the possible depletion of the medullary erythron (including the ERC) (Chiyoda, et al., 1974; Nathan and Berlin, 1963).

Studies of EPO-stimulated heme synthesis, in vitro, by bone marrow from patients with AML, suggest the presence of a specific suppressive mechanism (Chiyoda, et al., 1974). However, it remains to be seen whether the mode of inhibition operates via decreased number of ERC's or by a depressed responsiveness of the ERC's to EPO (Chiyoda, et al., 1974). Substantiation of decreased marrow responsiveness to EPO in malignancies is offered by Zucker, et al., (1974). In addition, Handler, et al., (1974), have recently reported a depression in the EPO-stimulated RNA synthesis in leukemic bone marrow. Furthermore, preliminary experiments in this laboratory indicate a decline in the number of CFU-E (ERC?)

as measured by the plasma clot method (Axelrad, et al., 1974) concomitant with the progressive leukemic state (Handler and Handler, unpublished).

Two additional factors should be noted:

1. that the above three hypotheses relating to the mechanism of erythropoietic decline need not be mutually exclusive, i.e., it is possible that the numbers of ERC's are reduced concurrent with an increase or decrease in EPO production;

2. that contradiction in the literature cited above regarding production or underproduction of EPO may be accounted for by differential synthesis of EPO in different types of leukemia and/or the presence of EPO-inactivators (inhibitors) which might mask production of EPO at different times.

In the SCL used in the present study, two lines of evidence favor the hypothesis that the anemia, secondary to the AML, is the result of an altered microenvironment and not the malfunction of the EPO-generating system:

1. The presence of small numbers of leukemic myeloblasts in bone marrow, has been shown to inhibit heme synthesis as measured by ^{59}Fe incorporation into heme by femoral bone marrow, in vitro (Handler and Handler, 1972);

2. There is an apparent elevation of circulating EPO levels during the course of the leukemic pathogenesis (Handler and Handler, unpublished observations).

It may be further postulated, that the mechanism of erythropoietic inhibition in the bone marrow and that reported in the present study in the spleen, during the course of leukemia may be quite analogous.

The nature of the "trigger-factor" of splenic erythropoiesis in this sytem, has not been identified. Clearly, though, EPO is a prime candidate. It is possible to speculate that at an early stage in the leukemic process, when the initial medullary erythropoietic decline occurs, endogenous levels of EPO increase (Thorling, 1965; Zaizov and Matoth, 1971; Handler and Handler, unpublished observations). At this early time, the splenic microenvironment is apparently unaltered and thus remains responsive to normal erythropoietic stimuli, resulting in proliferation and splenic erythroid compensation. Once proliferation of leukemic myeloblasts occurs in the spleen, however, and is of a quality and/or quantity sufficient to generate a change in the microenvironment, the ERC becomes refractory to EPO, and differentiation and proliferation does not occur. Studies designed

to test this hypothesis are being carried out in this laboratory at the present time, and indeed, appear to corroborate the above hypothesis. Preliminary results utilizing the plasma clot procedure for the assay of CFU-E (ERC?) (Axelrad, et al., 1974) suggest a huge increase in the numbers of CFU-E during the course of the pathogenesis. However, at late stages when the per cent spleen myeloblast content is high, the number of CFU-E is severely depressed (Handler and Handler, unpublished).

In considering the relationship between medullary erythropoiesis and myeloblast proliferation, an interesting sub-population is noted, consisting of approximately 11 cases, in which medullary erythropoiesis is severely depressed despite low per cent bone marrow myeloblast (less than 6%; Fig. 1). This exceptional group, makes an extremely important contribution toward the development of a model for the mechanism of erythropoietic inhibition during leukemia. The existence of these animals, provides evidence against a "crowding-out" theory of erythropoietic decline during leukemia, in which the mechanical encroachment of proliferating myeloblasts in the bone marrow, with the consequent displacement of RBC precursors, is considered to be

responsible for the lack of erythropoietic response (Zaizov and Matoth, 1971). Clearly, this cannot be the case for this population, at least, since the per cent bone marrow myeloblasts is indeed quite low. Rather, such cases implicate a more subtle alteration of the microenvironment as the primary influence on erythropoietic shut-down. Other studies of anemia associated with leukemia suggest that some alteration in the proliferative capacity of erythroid precursors (ERC?) results in decreased erythropoiesis (Troup, et al., 1960; Wickra, et al., 1968; Zaizov and Matoth, 1971). This may have clinical significance on the aetiology of idiopathic anemias in that a percentage of such pathologies may represent a preleukemic condition.

An additional factor gaining increasing attention with regard to the erythropoietic inhibition during leukemia focuses on the activity of muramidase in the serum of such patients. Much evidence has accumulated indicating the presence of abnormally high serum levels of this hydrolytic enzyme in AML, acute myelomonocytic leukemia and chronic granulocytic leukemia (Levi, et al., 1973). In addition, it has been reported, that increased serum muramidase activities are associated with decreased numbers of peripheral mature granulocytes in patients with megaloblastic anemia (Perillie, et al., 1967). Furthermore, the inadequate bone marrow production of erythroid elements may be the result of interaction of muramidase with the erythron (Perillie, et al., 1967). This may not be unlike

the leukemic condition in which the erythropoietic lesion in AML may be the result of a similar enzymatic action on erythroid precursors (ERC or earlier precursors). The results discussed thus far with regard to ERC sensitivity are entirely consistent with this hypothesis. It is conceivable that muramidase renders the ERC unresponsive to EPO and/or causes a decreased differentiation into, or proliferation of ERC, thus resulting in reduced numbers of such cells. Indeed, the increased muramidase activity associated with leukemia may be a local influence on hematopoietic tissue as well, and may constitute, at least in part, the alteration in microenvironment considered to be responsible for erythropoietic inhibition during leukemia.

It has been noted, that for the general population of leukemic animals, a linear relationship exists between per cent bone marrow and spleen myeloblasts (Fig. 4). The sub-population seen along the abscissa and which deviates from the linearity described above is composed of animals whose bone marrows are characterized by the presence of a high percentage of leukemic myeloblasts yet whose spleens

do not exhibit a correspondingly high percentages of leukemic myeloblasts (sub-population 4A). These approximately 15 cases are derived from subfiles 3 and 4 and thus, possess highly erythropoietic spleens. Small percentage of myeloblasts in the spleen, does not interfere with the favorable microenvironment in which considerable erythropoiesis may proceed. Deviation of sub-population 4A from the general pattern seen in Figure 4, is related to the process which governs the rate and degree of leukemic myeloblast infiltration and/or proliferation in the spleen.

The degree to which the spleen supports proliferation of leukemic elements, and the rate of such growth, may be related to the immunological competence of the individual animal.

The aetiology of splenomegaly associated with SCL (Handler and Handler, 1970b) is not definitively understood. Spleen enlargement may be due to one, or indeed, a combination of several factors: (a) increased red blood cell pooling; (b) increased cell proliferation, including: (1) RE hyperplasia; (2) erythroid hyperplasia; (3) leukemic myeloblast proliferation.

(a) Pooling of red blood cells in the spleen, is a normal function of this organ (Blaustein and Diggs, 1963). Furthermore, many pathological states are associated with enhanced RBC sequestration and pool size (Markoe and OKunewick, 1973; Toghill and Green, 1971; Christensen, 1973), and as such, is a possible cause of clinical and experimental splenomegaly. Nevertheless, RLV-induced splenomegaly is not considered to be the result of the increased pool size (Markoe and OKunewick, 1973).

(b) Though a linear relationship has been demonstrated both between non-nucleated and nucleated cells, and spleen size in RLV-infected mice, the former variable has been eliminated as the primary causative agent of splenomegaly (Markoe and OKunewick, 1973). Cell proliferation, consequent to RLV-infection is well documented (Axelrad and Steeves, 1964; Chirigos, et al., 1967; Mirand, et al., 1968; Markoe and OKunewick, 1973). On the basis of equivalent ratios of total cells/nucleated cells in normal and leukemic mice, despite a 30-fold increase in spleen size during the course of the pathology, Markoe and OKunewick,(1973) have proposed that the splenomegaly associated with RLV is

the result of a uniform increase in the number of proliferating cells in the spleen as a direct consequence of viral infection.

1. RE hyperplasia has been implicated as a likely cause of splenomegaly in PH-induced hemolytic anemia, as has been noted in the discussion on this primary anemia (Chen and Weiss, 1973), and cannot be eliminated as a possible cause of splenomegaly in AML.

2. It is possible that the splenomegaly of leukemic animals is the result of compensatory erythropoiesis attendant with the pathology. Fruhman, (1970) has suggested that erythroid hyperplasia, is often overlooked in histological examination of spleens in many pathologies, and, in fact, may be responsible for the increase in spleen weight. Snodgrass, et al., (1973) and Profitt, et al., (1972) have similarly suggested that Lactose Dehydrogenase Virus (LDV) infection (common viral contaminant of murine leukemia viruses), enhances the RLV-induced splenomegaly by a detectable splenic erythroid proliferation. Erythroid hyperplasia may also be a causative factor in the splenomegaly associated with hemolytic anemia. However, data from the present study (Figure 9) suggest no positive correlation

between per cent splenic benzidine-positive cells and spleen weight (r = 0.130). This can be appreciated since the spleen size of animals belonging to subfiles 3 and 4, (highly erythropoietic), is generally less than that from subfile 2. Due to the inherent methodological limitations in dealing with relative, i.e., % benzidine-positive cells, (to be discussed below), the absence of a positive correlation cannot eliminate absolutely, erythroid hyperplasia, as the causative factor in splenomegaly.

3. In the absence of any compelling evidence to the contrary, leukemic myeloblast proliferation in the spleen, as encountered in the present study, is considered to be the primary cause of splenomegaly in the SCL rat. A positive correlation (r = 0.738; p = 0.00001) is associated with these two variables and is consistent with the general hypothesis of Markoe and OKunewick (1973) which suggests that the increased cell proliferation is aetiologically related to the splenomegaly associated with murine leukemia.

V. ALA-S:

A. General Introduction to ALA-S - The condensation of glycine and succinyl-CoA to yield ALA, via the action of ALA-S, appears to be the first and rate-limiting step

in the biosynthesis of heme (Urata and Granick, 1963; Tschudy, et al., 1964; Granick, 1966; Marver, et al., 1966; 1968). Thus, the control of the biosynthesis of this enzyme is apparently the primary element in the control of heme synthesis (Granick and Sassa, 1971). Furthermore, there is evidence to suggest that control ALA-S biosynthesis is regulated both through transcriptional and translational means (Sassa and Granick, 1970).

An early clue to the relationship between ALA-S activity and the synthesis of heme was noted in the clinical syndrome known as acute intermittent porphyria (AIP). This disease, transmitted along classical Mendelian genetic lines, is characterized by: increased production of ALA by the liver and consequent excretion of ALA with porphobilinogen (PBG) in the urine or excretion in the form of porphyrins, the intermediates of heme synthesis (Granick and Sassa, 1971; DeMatteis, 1967; Granick and Levere, 1964; Schmid, 1966). Tschudy, et al., (1965) and Nakao, et al., (1966b) have shown that the biochemical lesion in AIP involves a 5-10-fold increase in levels of ALA-S. Furthermore, AIP does not become evident until after puberty,

suggesting a hormonal influence on expression of the disease (Granick and Sassa, 1971).

Subsequently, a whole range of chemical inducers of experimental porphyria has been discovered and exploited in expanding our understanding of the role of ALA-S in heme synthesis. These inducing chemicals are derived from four general classes of agents: (a) barbituates, including allyl isopropyl acetamide (AIA) and theophylline; (b) collodines, including 3, 5-diethoxycarbonyl, 4-dihydrocollodine (DDC); (c) steroids; (d) miscellaneous, including certain insecticides and fungicides (Granick, 1966). All inducers of ALA-S appear to have in common the following properties: they are relatively water-insoluble, lipid soluble molucules which are generally non-planar (Granick and Sassa, 1971; Marks, 1969).

The mechanism of induction of ALA-S appears to vary according to tissue and inducing agent. The chemicals belonging to classes (a), (b) and (d) above induce the enzyme only in liver (Granick and Sassa, 1971). In this case the mechanism of induction appears to involve, primarily, a translational mechanism (Granick and Sassa, 1971). Steroid induction, on the other hand, occurs in erythroid

cells of chick blastoderm as well as in liver. Furthermore, induction by steroids in liver and erythropoietic tissue appears to operate via transcriptional mechanisms.

B. ALA-S in Malignancy - Tumor cell homogenates assayed for ALA-S activity by colorimetric methods in this study showed no enhanced activity above controls. This may be due to: (a) insufficient sensitivity of colorimetric technique in measuring low levels of ALA-S activity, or (b) inability of leukemic myeloblasts to synthesize ALA. The present investigation cannot distinguish between these two alternatives. Nevertheless, preliminary experiments in this laboratory using radiochemical techniques suggest the presence of ALA-S activity in SCL cells (Amrutavalli, unpublished). Evidence from other systems may be of assistance in clarifying the role of ALA-S in malignancy.

Evidence presented by Walters, et al., (1967) suggest a failure by immature leukemic cells to demonstrate: (a) ALA-S activity; (b) utilization glycine as a substrate for heme synthesis. They further report the existence of an ^{59}Fe-incorporation capacity in leukemic myeloblasts from patients with AML, when ALA itself is supplied as the substrate for heme synthesis. This is confirmed by Vannotti

and Jeunet, (1963) who report the synthesis of porphyrins by immature leukemic cells from ALA. Thus, it is possible either: (a) that conditions for heme synthesis using glycine as substrate were not suitably optimalized, (though similar incubation conditions proved adequate for heme synthesis when ALA and protoporphyrin were used as substrates), and that the inability to register activity of ALA-S was a consequence of the relatively low sensitivity of the colorimetric procedure, or (b) that these leukemic myeloblasts do not possess the capacity to induce ALA-S.

In apparent contradiction to the findings by Walters, et al., (1967) Takaku, et al., (1968) have found that leukocytes from patients with AML are indeed able to utilize ^{14}C-glycine as a substrate for heme. The authors suggest that this capacity to synthesize heme via ALA-S is a function of the immaturity, rather than the leukemic nature of the cell.

Bonkowsky, et al., (1973) using the whole homogenate method, report the absence of inducibility of ALA-S by AIA concurrent with low levels of the enzyme in a series of hepatomas, suggesting that the biochemical lesion of heme synthesis in the tumor cells is associated with ALA-S.

The fact that these authors were able to identify low levels of ALA-S in tumor homogenates lends more credence to the latter alternative above, which suggests that the failure to record ALA-S activity may not be a function of methodological limitations. Similarly, it appears that incubation of SCL cells with DDC during the radiochemical assay for ALA-S, does not result in enhanced enzyme induction (Amrutavalli, unpublished).

Reports of de novo porphyrin synthesis in chloromas have been published (Schultz and Schwartz, 1956). More recently, Handler and Handler, (1972) have reported significant levels of de novo synthesis of heme in vitro, by SCL cells.

A unifying concept relating the apparent contradiction in the literature concerning inducibility of ALA-S and biosynthesis of heme in various tumors and leukemic cells may involve the repression of ALA-S by heme, a phenomenon previously reported (Granick, 1966; Hayashi, et al., 1969; Potter, et al., 1970). Bonkowsky, et al.,(1973) suggest that at least in the case of solid tumor homogenates, high levels of endogenous heme may be present as a result of: (a) tumor necrosis; (b) breakdown of red

blood cells circulating through the tumor; (c) some other unknown mechanism. This heme may be responsible for repression of ALA-S induction in the tumors. A similar explanation may apply to the results found in the present study, as high levels of hemin were reported to exist in chloroma tissue (Schultz, et al., 1954). One cannot rule out, however, based on preliminary experiments using radioactive assay procedures, (Amrutavalli, unpublished), the additional consideration, that indeed, the levels of ALA-S activity were below the limits of detection of the present assay system.

Blood containing leukemic myeloblasts derived from patients with acute leukemia (Walters, et al., 1967) was drawn and leukocytes were isolated from red blood cells by two methods. Nevertheless, some contamination of the leukocyte fraction by red blood cells and heme, derived from hemolyzed red blood cells, is no doubt present, and, when the leukocyte fraction is homogenized for assay, may be sufficient to repress induction of ALA-S.

In the in vitro heme synthesis studies (Handler and Handler, 1972), the assay is performed on leukemic cell suspensions, i.e., intact cells. The preparation of a

tumor cell suspension from a solid tumor mass, is in itself a purifying procedure, which may eliminate or dilute considerably the pre-existing, non-cellular heme derived from the tumor. Furthermore, the presence of a cellular membrane may impede the transport of remaining heme to a degree such that heme repression of ALA-S-induction does not occur.

The above argument cannot be made to reconcile the discrepancy between the lack of ALA-S activity found in the present study and the de novo porphyrin synthesis reported by Schultz and Schwartz, (1956) in chloromas, since: (a) the method of porphyrin study, like that of the ALA-S assay, require the preparation of tissue homogenate, and, (b) ^{14}C-glycine, the substrate of ALA-S, was used as the source of radioactivity later found in porphyrin. Recovery of significant amounts of radio-labeled-porphyrin within the experimental period, strongly implies that ALA-S was present and active in the chloroma.

Two explanations are offered to explain the apparent contradiction. The first, already noted, involves the relative insensitivity of the colorimetric ALA-S assay. The second, involves the differences reported to exist between prophyrin synthesis in individual chloromas (Kelenyi, et al.,

1961). It is possible that the ALA-S activity in the SCL tumor, maintained in this laboratory is less than that originally reported in the chloroma. There is suggestive evidence to support this contention: (a) the SCL tumor, presently maintained in this laboratory, no longer possesses the characteristic green color of the chloroma studied by Kelenyi, et al., (1961); (b) Furthermore, Kelenyi, et al., (1961) reported a positive correlation between the green color of the tissue and the porphyrin content. Thus, the SCL tumor, examined for ALA-S activity in the present study, may indeed have different rates of synthesis and levels of porphyrin. More conclusive determination and clarification in the present as well as other malignant cell systems, must await corroboration of preliminary results provided by the more sensitive, radiochemical methods.

C. ALA-S in Hematological Pathologies - In recent years, several investigations on ALA-S activities in various pathological states have been conducted. Spleens of RLV-infected mice (Ebert, et al., 1972) and bone marrow from patients with sideroblastic anemia (Tanaka and Bottomley, 1974; Aoki, et al., 1974; Takaku, et al., 1972), iron-de-

ficiency anemia and β-thalassemia (Takaku, et al., 1972) all exhibit depressed levels of specific activity of ALA-S.

D. Comparative Responses of ALA-S in Leukemia and Primary Anemia

1. Specific Activity Splenic ALA-S - The linearity (r = 0.864) which exists between SA of splenic ALA-S and per cent spleen benzidine-positive cells suggests that the increase in activity assayed, is the result of an increased proliferation of benzidine-positive cells (Figure 12). Comparison of SA of normal and leukemic spleens, both with equivalent per cent benzidine-positive cells should yield information regarding relative rates of ALA generation under these conditions. The mean basal levels of SA in leukemic animals with minimal per cent spleen benzidine-positive cells (subfile 1: 0-2.9%) is less than the mean SA of ALA-S in normal spleens with equivalent per cent benzidine-positive cells, suggesting that for equivalent per cent benzidine-positive cell content, the rate of synthesis of ALA per mg protein of leukemic spleen is less than that of normal spleen (Figure 12).

Estimation of the comparative rates of ALA synthesis in leukemic spleens and in spleens of animals with hemoly-

tic anemia leads to the following consideration: It is apparent that after a regimen of PH-treatment, a severe anemia, with compensatory splenic erythropoiesis results. Thus, a comparison of SA of splenic ALA-S in leukemic and hemolytic anemic animals, whose per cent benzidine-positive cells are equivalent, should provide insight into the comparative rates of ALA synthesis under various pathological conditions. The mean per cent benzidine-positive cells in the spleens of animals with hemolytic anemia equals 29.5% (± 1.0) with a corresponding mean SA equal to 627.2 pmoles/mg prot./hr. (± 39.4). Leukemic rats with equivalent mean per cent splenic benzidine-positive cells, however, produced ALA at a rate of approximately 400 pmoles/mg prot./hr.

Similar considerations maybe made for the case of hemorrhagic anemia. Under these conditions, the mean per cent splenic benzidine-positive cells equals 10.5% (± 1.0) with a corresponding SA of 275.0 pmoles/mg prot./hr. (± 40.2). The value of SA for leukemic spleens with the equivalent degree of compensation, (derived from the equation of the line in Figure 12) equals 114.0 pmoles/mg prot./hr.

Thus, the SA of splenic ALA-S in leukemic rats appears to be consistently less than that in spleens from normal, hemolytic anemic and hemorrhagic anemic animals with equivalent degrees of compensatory erythropoiesis as measured by benzidine-positive elements. Indeed, it is possible to project a line uniting the data presented above from normal, hemolytic anemic and hemorrhagic anemic rats (Figure 12). The coincidence of these three values on a single line invites the speculation that: (a) there is a distinct and significant relationship between the basal levels of erythropoiesis in the normal animal and the compensation phenomena of hemolytic and hemorrhagic anemic rats. The existence of a line uniting distinct groups of experimental systems seems to suggest, indirectly, that the fundamental difference between normal splenic erythropoiesis and erythroid compensation in hemolytic and hemorrhagic anemias is a quantitative, rather than qualitative one, and (b) an early, 1-step biochemical alteration within the compensatory erythroid cell in the leukemic spleen results in an overall and almost constant depression in SA of ALA-S over the entire range of compensation. Ebert, _et al_., (1972)

reports a similar depression of SA of splenic ALA-S in mice infected with RLV.

2. Net Activity Splenic ALA-S - The importance of NA (nmoles ALA/spleen/hr.) in the consideration of erythroid compensation may be substantial in that it reflects the nature of the erythropoietic response by the organ as a whole, and as such, may illustrate an overall physiological significance not noted with SA alone.

The NA is a mathematical derivative of SA for which the number of moles ALA are reported on a total organ basis rather than on a mg protein basis, and is calculated from knowledge of the protein content of the organ.

The NA of splenic ALA-S in hemorrhagic anemia is greater than 6 times that seen in normal spleens. The NA of splenic ALA-S in hemolytic anemia is approximately 38 times that of normal. The great difference in NA between these two primary anemias reflects both the greater degree SA and splenomegaly of hemolytic anemia over hemorrhagic anemia.

The relationship between per cent benzidine-positive cells and NA of splenic ALA-S is seen in Figure 13. A positive correlation exists in which r = 0.726. Nevertheless, inspection of the graph suggests a slight increase in

slope beginning at approximately 90 nmoles ALA/spleen/hr., and roughly corresponding to experiments in subfiles 3 and 4. This reflects a general decrease in spleen size in animals belonging to these subfiles.

Comparative NA's of leukemic and primary anemic spleens with equivalent per cent benzidine-positive cells again reflects the varying degrees of splenomegaly under these conditions, NA of splenic ALA-S in hemorrhagic anemia equals 46.3 nmoles ALA/spleen/hr. ($\pm$ 9.6) and is not significantly different from that seen in leukemic spleens (approximately 35 nmoles ALA/spleen/hr.) with equivalent per cent benzidine-positive cells. This results from the contribution of the balancing factors of decreasing SA with increasing spleen weight in leukemic animals versus large increases in SA with insignificant splenomegaly in animals with hemorrhagic anemia.

The situation comparing NA's in hemolytic anemia and leukemia, with equivalent per cent benzidine-positive cells, is different from that noted for hemorrhagic anemia, for splenomegaly in hemolytic anemia is quite apparent. Thus, for equivalent per cent benzidine-positive cells in hemolytic anemia and leukemia (approximately 30%), the NA of splenic ALA-S in hemolytic anemia is nearly 2 times that seen

in the leukemic state (257.4 nmoles ALA/spleen/hr. in hemolytic anemia versus approximately 130 nmoles ALA/spleen/hr. in leukemia).

The implication of NA as discussed above assumes uniform distribution of erythropoietic cells in the spleen. A given SA, expressed as number of moles ALA/mg prot./hr. converted to NA, expressed as number of moles ALA/spleen/hr., assumes that each mg protein in the spleen contains equivalent amounts of erythropoietic, and thus ALA-generating tissue. This is probably not the case, especially in PH-induced anemia in which splenomegaly is quite possibly the result of RE-hyperplasia and not compensatory erythropoiesis, in spite of the high degree of red blood cell production in the spleens of such animals. Furthermore, the spleen, being a highly compartmentalized organ is most likely not undergoing erythropoiesis according to a uniform or random distribution. Favorable microenvironments most likely enforce preferential growth of erythropoietic elements in rather localized areas (Curry, et al., 1967; Wolf and Trentin, 1968). Further evidence in support of compartmentalization of erythroid growth comes from work by Suzuki, et al., (1973) in which the spleen was fragmented into 3 parts: (a) "capsule"; (b) the outer part,

including "capsule"; (c) the inner part. They concluded that in polycythemic mice, the capsule, which evidenced a higher rate of heme synthesis/DNA than other parts, most likely contained a larger number of ERC's. This follows from studies in polycythemic mice in which Fe-incorporation into heme after addition of EPO, represents the reaction of ERC's to EPO (Suzuki, et al., 1973; Miura, et al., 1968; Nakao, et al., 1966a).

Thus, while the results obtained in the present study regarding increased NA's of splenic ALA-S agree with those reported by Ebert, et al., (1972) in RLV-infected mice, the significance of such determinations cannot be accurately assessed without detailed histological analysis, perhaps involving serial sections, of spleen.

The term "microenvironment" is clearly a broad, all-encompassing one and, in general, reflects the state of ignorance in the field regarding specific mechanisms of induction of cell differentiation and proliferation. The popularization of the concept of microenvironment in the literature of hematopoietic differentiation, stems from a series of extraordinarily careful and extensive studies by Curry and Trentin, (1967) into the histological nature of bone

marrow and spleen colonies. Such colonies arise following intravenous injection of low doses of bone marrow cells into lethally irradiated mice, and, based on chromosomal analysis, are believed to be derived from a single (Becker, et al., 1963), pluripotent stem cell (Ford, et al., 1959). The general conclusions drawn from these studies suggest that:

(1) the bone marrow is preferentially favorable to the differentiation of neutrophilic elements, whereas the spleen becomes populated predominantly, though not exclusively, with erythroid colonies. The authors postulate the existence of a Hematopoietic Inductive Microenvironment (HIM) within the two organs which dictates the preferential selective differentiation of erythroid and neutrophilic colonies at different hematopoietic sites;

(2) there is selective localization of erythroid and granulocytic colonies within the spleen itself. Erythroid colonies are found anywhere in the red pulp but rarely in empty lymphoid follicles; granulocytic colonies are noted in subcapsular region, along trabeculae and may develop within empty lymphoid follicles. Megakaryocyte colonies are usually found subcapsular. This is suggestive of a microenvironmental influence within the spleen -- Splenic Hematopoietic Microenvironment (SHIM) -- which is selectively favorable to erythroid and granuloid colonies at different sites within the spleen;

(3) all spleen colonies are initially composed of one hematopoietic type, e.g., erythroid, granucytic. However, at later periods, mixed colonies are noted. This information, along with histological sections of predominantly erythroid, though mixed colonies, led the authors to postulate the following: A colony, increase in mitotic rate and colony growth in the

> presence of systemic EPO. As the differentiating cells of the expanding colonies encroach upon a SHIM that is granulocytic in nature, they differentiate accordingly and thus the colony becomes mixed (Curry and Trentin, 1967).

Speculation as to the nature of the HIM, requires that it be relatively resistant to lethal doses of irradiation compared to hematopoietic cells. It has been suggested that these sites are dictated by the distribution of reticular cells or other radioresistant cell types (Curry and Trentin, 1967) and that induction by these cells may not be unlike the mesenchymal-induced differentiation of endothelial cells into thymic lymphocytes (Auerbach, 1961). Indeed, the use of Millipore filters in these experiments supports the view that lymphoid differentiation may occur in the absence of direct cellular intervention by mesenchyme (Auerbach, 1961).

A further extention of the microenvironment concept has arisen of late, in connection with the effect of the presence of leukemic cells on normal erythropoiesis. Experimental observations, including heme synthesis, *in vitro* (Handler and Handler, 1972), histological analysis (Handler and Handler, 1970b), EPO-stimulated RNA synthesis (Handler, *et al*., 1974) and ALA-S activity (present study) all confirm the decline in erythropoietic activity in the presence

of leukemic myeloblasts in bone marrow and/or spleen. Indeed, inhibition of erythropoiesis is often noted in the presence of a low percentage leukemic elements. The nature of the erythropoietic lesion in acute leukemia, in general, and in SCL, in particular, as it relates to the changing microenvironment, is a subject of intense investigation at the present time. Preliminary results derived from erythroid colony assay indicate a decline in the numbers of EPO stimulated erythroid colonies in leukemic bone marrow and spleen (Handler and Handler, unpublished observations). This is considered to be reflective of either a decreased number of ERC's or a lowered responsiveness of such cells to EPO in AML.

It is clear that the intact marrow and spleen architecture is not required for this inhibitory effect, since all of the experimental procedures described above are not performed *in vivo*, and thus perturb the natural state either by homogenization or cell suspension in tissue culture. Nevertheless, it is not known whether this inhibitory influence of leukemic myeloblasts on erythropoiesis requires the direct interaction of leukemic cells or is the result of the elaboration of substances from these cells which

specifically inhibits erythroid differentiation via modification of the ERC. The recently-described erythroid colony assay (Axelrad, et al., 1974), should contribute greatly to the understanding of this critical question. The use of tumor-conditioned media in the red cell colony assay, for example, may be a useful approach in determining the requirement of direct cell-cell interaction in the erythropoietic inhibition during AML, and may thus assist in the dissection of the "microenvironment" into its constituent mechanistic elements.

Absolute quantitation of cells in the spleen are replete with technical problems whose results are, at best, questionable (Lajtha, 1967). Tissue sections, smears and fresh-cut imprints all present relative values of cell type. The rich connective tissue stroma characteristic of spleen, prevents complete dispersal of cells, thus making the accurate use of electronic particle counters impossible (Fruhman, 1970).

The inability to obtain absolute numbers of cells in the spleen imposes certain limitations on interpretation which might otherwise be made. For example, in analysis of Figure 12 (SA splenic ALA-S v. percent benzidine-positive

cells) no definitive statement can be made regarding the SA ALA-S/spleen benzidine-positive cell, i.e., rate of generation of ALA per erythroblast, since the reciprocal of the slope of the line in Figure 12 represents SA ALA-S/ per cent spleen benzidine-positive cells, itself a relative quantity.

The above considerations are made assuming a normal proliferating erythron (first-level approximation). Indeed it is possible that:

(1) within a single leukemic spleen, e.g., early leukemic state, the population of benzidine-positive cells may be composed, primarily, of early erythroblasts;

(2) at a later time in the leukemic pathogenesis, i.e., myeloblast proliferation has been established, and no new erythroid differentiation occurs, the benzidine-positive population of the spleen is composed, primarily, of late-stage erythroid cells;

(3) further, the rates of ALA synthesis will vary between the various differentiating compartments.

Thus, comparison of rate of ALA generation, on a per cell basis, is further complicated by the possible existence of non-uniform populations between animals in different leukemic states. Similarly, comparison of SA's of leukemic, and hemorrhagic or hemolytic anemic animals is

made difficult by the possible dissimilar nature of the erythroid compartments predominating in these circumstances.

E. Induction of Hepatic ALA-S and it Relationship to Splenic Induction -

Hepatic ALA-S activities assayed in the present study of hemolytic and hemorrhagic anemias were not found to be significantly different from normal values. This is in strong contrast to the highly active splenic ALA-S recorded in these primary anemias.

The mean hepatic SA of ALA-S in individual subfiles composed of data from leukemic systems is not significantly different from the average normal level of activity 81.4 (± 9.0). The values for the four subfiles are: 74.6 (± 19.6), 68.1 (± 17.1), 69.9 (± 15.1), 67.1 (± 26.9), for subfiles 1-4 respectively. Thus, the pathogenesis of SCL, like the case of primary anemia, does not appear to induce significant alterations in hepatic ALA-S activity. Further evidence corroborating the dissociation of the leukemic process and ALA-S induction, is provided by studies which indicate a normal capacity for DDC-induced ALA-S activity in the livers of rats with SCL (Amrutavalli, unpublished).

Wada, _et al_., (1967) have reported that hypertransfusion results in a decreased ALA-S activity in liver and spleen. Administration of EPO to such polycythemic mice, leads to gradual increase in splenic ALA-S activity concurrent with erythroid hyperplasia (Wada, _et al_., 1967). The liver, however, does not respond to the addition of exogenous erythropoietic stimulus, and its levels of ALA-S activity remained low. It was suggested by these authors that depressed levels of ALA-S in the liver, may be due to repression of ALA-S induction by hemin as a result of large numbers of RBC injected into the animals. Hemin has been found to inhibit, to some extent, DDC-induction of ALA-S in liver (Granick and Sassa, 1971; Wada, _et al_., 1967) without significantly interfering with the increasing enzyme activity in hypoxic mice (Wada, _et al_., 1967).

In the liver, heme is required for the synthesis of hemo-proteins other than hemoglobin, i.e., the mitochondrial cytochromes and catalase (Granick and Sassa, 1971). The incorporation of ALA into 6 hepatic cytochromes is well documented in the literature (Aschenbrenner, _et al_., 1970; Druyan, _et al_., 1969; Levin and Kunzman, 1969). Further-

more, ALA-S is thought to be involved in mitochondrial biogenesis (Beattie and Stuchell, 1970; Beattie, 1971). The changes in ALA-S activity, therefore, are no doubt, related to changes in general liver and mitochondrial metabolism (Granick and Sassa, 1971) and not to the erythropoietic state of the animal.

CONCLUSIONS

The present study is able to confirm and elaborate on several findings previously reported from this laboratory. These include:

A. Inhibition of medullary erythropoiesis during the pathogenesis of the Shay Chloroleukemia in rats: The histological profile of bone marrow reveals, in general, a decreasing per cent benzidine-positive cell content which is negatively correlated with the per cent myeloblasts. The relationship, however, not strictly linear, but rather is suggestive of a hyperbolic function.

B. Occurence of compensatory splenic erythropoiesis during the pathogenesis of the Shay Choloroleukemia in rats: Compensatory splenic erythropoiesis is evidenced by the quantitation of spleen smears and ALA-S activity.

C. Non-erythropoietic nature of the liver during the pathogenesis of Shay Chloroleukemia in rats: Histological (per cent benzidine-positive cells) and enzymatic (ALA-S) analyses of the liver during the course of the AML suggest no significant erythropoietic activity.

Furthermore, the following observations regarding the phenomenon of splenic erythropoiesis are presented:

I. Acute Myelogenous Leukemia (AML):

A. The emergence of a pattern of compensatory splenic erythropoiesis and its inhibition during the pathogenesis of AML:

1. Erythropoietic compensation in the spleen appears to be governed by the interaction of at least two factors:

(a) state of medullary erythropoiesis;

(b) permissiveness of splenic microenvironment.

It has been found that splenic erythroid compensation is incompatible with a critical per cent splenic myeloblast content. It is suggested that not unlike the bone marrow, the presence of a critical per cent leukemic myeloblasts in the spleen, and/or their presence for a critical period of time, is sufficient to alter the microenvironment such that compensatory erythropoiesis cannot occur.

B. Histological and Physiological Parameters:

1. Spleen - The degree of splenic compensation ranged up to almost 50% benzidine-positive cells from the mean normal value of 0.4%. The degree of splenomegaly was correlated with per cent splenic myeloblasts and this varied accordingly during the pathogenesis.

2. Liver - Scans of liver smears revealed no significant erythropoietic content.

C. Detection and Measurement of ALA-S Activity in the Spleen of Rats with AML:

1. Specific activity of splenic ALA-S (pmoles/mg protein/hour) appears to be linear to the per cent benzidine-positive cells in the spleen.

II. Primary Anemias Due to Hemorrhage and Hemolysis:

A. Histological and Physiological Parameters:

1. Bone Marrow - Medullary erythropoiesis as monitored by per cent benzidine-positive cells in both hemolytic and hemorrhagic anemias is twice the normal value.

2. Spleen - Histological analysis reveals an almost 75-and 25-fold increase in per cent benzidine-positive cells in hemolytic and hemorrhagic anemias, respectively. Spleen weight increased 3-fold when compared to normal values in phenylhydrazine-induced anemia. However, no significant splenomegaly developed in hemorrhagic anemia.

3. Liver - Histological liver scans revealed no significant erythropoiesis in this organ. No hepatomegaly was in evidence in the primary anemias.

B. Detection and Measurement of ALA-S Activities:

1. Spleen - ALA-S activities in hemolytic and hemorrhagic anemias were found to be approximately 10-and 5-times normal levels, respectively.

2. Liver - Specific activities ALA-S were found to be within normal range.

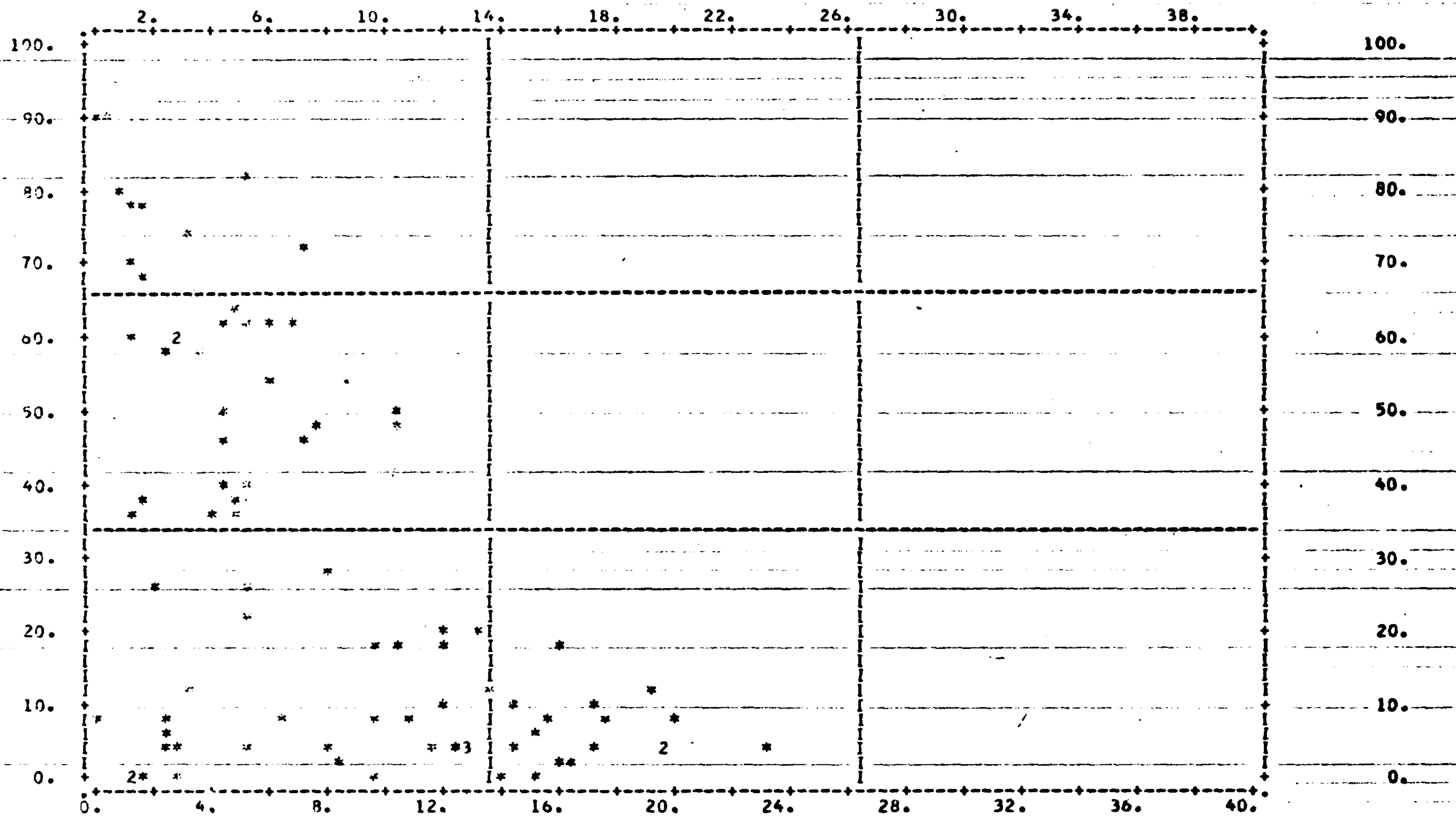
III. Insight into the General Phenomenon of Compensatory Erythropoiesis by Comparative Analysis of Primary and Secondary Anemias:

A. The consistent depression in specific activity splenic ALA-S in animals with secondary anemia due to AML when compared to activities of primary anemic animals with equivalent per cent benzidine-positive cells, suggests a possible early, 1-step biochemical alteration within the compensating erythroid cell in the leukemic rat.

Figure 1

Relationship Between the Per Cent Bone Marrow Myeloblasts and Bone Marrow Benzidine-Positive Cells in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR007 BONE-MARROW MYELOBLAST (ACROSS) VAR008 BONE-MARROW BEN +



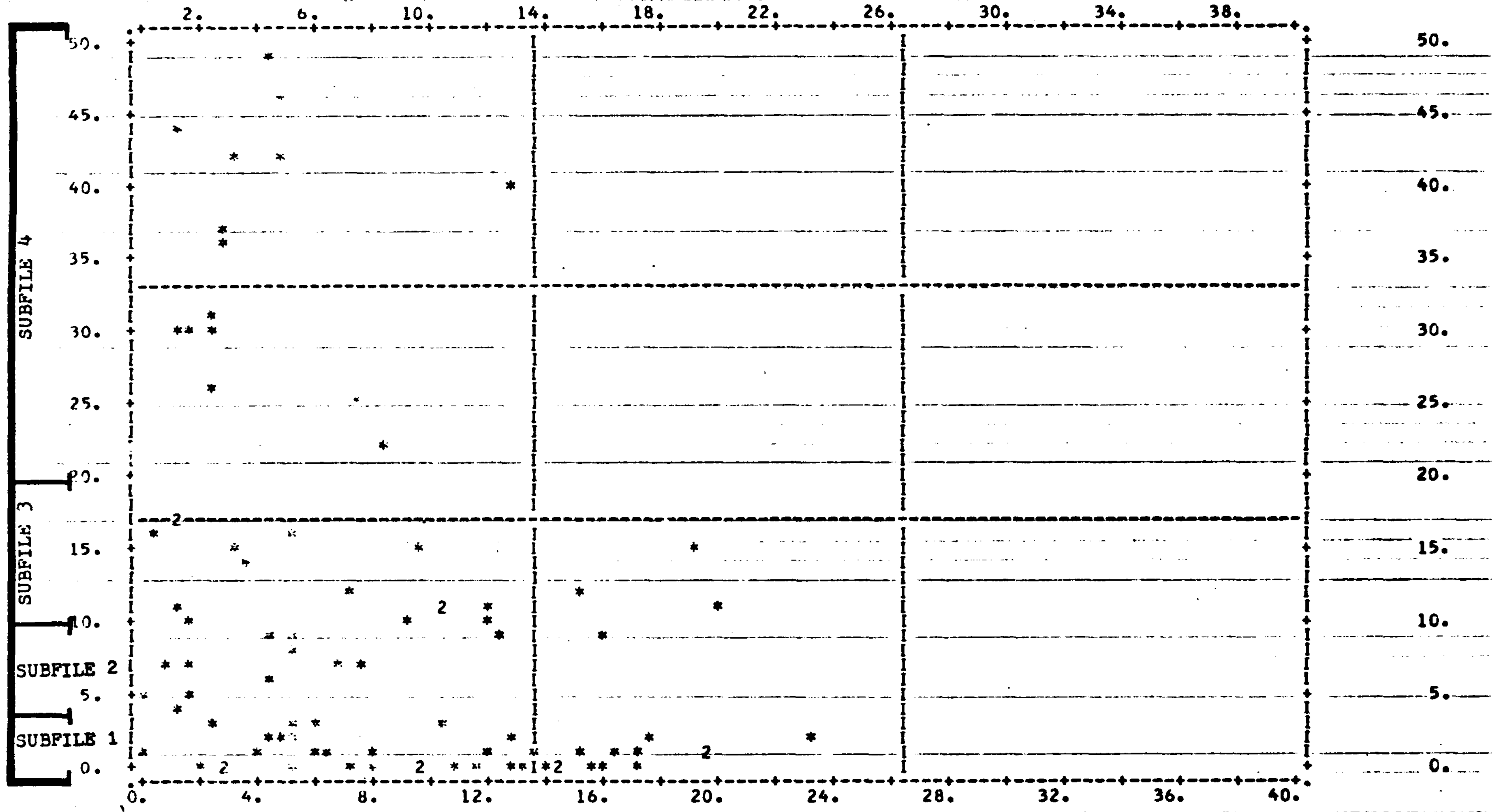
STATISTICS..

CORRELATION (R)-	-0.55392	R SQUARED -	0.30683	SIGNIFICANCE -	0.00001
STD ERR OF EST -	22.83878	INTERCEPT (A) -	50.09015	SLOPE (B) -	-2.54575

Figure 2

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Per Cent Bone Marrow Benzidine-Positive Cells in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR002 SPLEEN BEN + (ACROSS) VAR008 BONE-MARROW BEN +



STATISTICS..

CORRELATION (R)-	-0.35737	R SQUARED -	0.12771	SIGNIFICANCE -	0.00039
STD ERR OF EST -	12.16014	INTERCEPT (A) -	17.02792	SLOPE (B) -	-0.78403
PLOTTED VALUES -	85	EXCLUDED VALUES-	0	MISSING VALUES-	0

Figure 2A

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Per Cent Bone Marrow Benzidine-Positive Cells in Three Sub-Populations of Leukemic Rats: Subfiles 1-4.

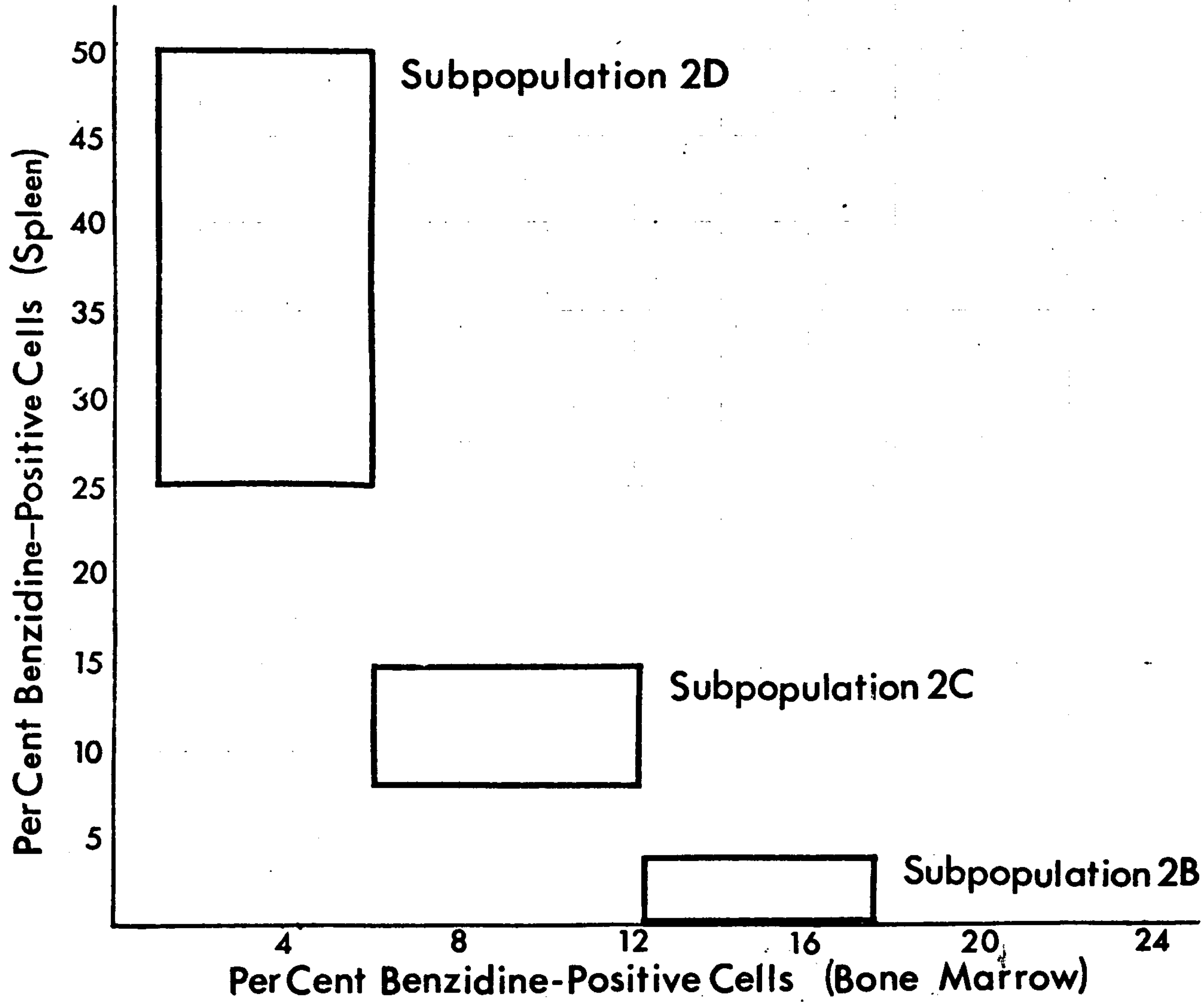
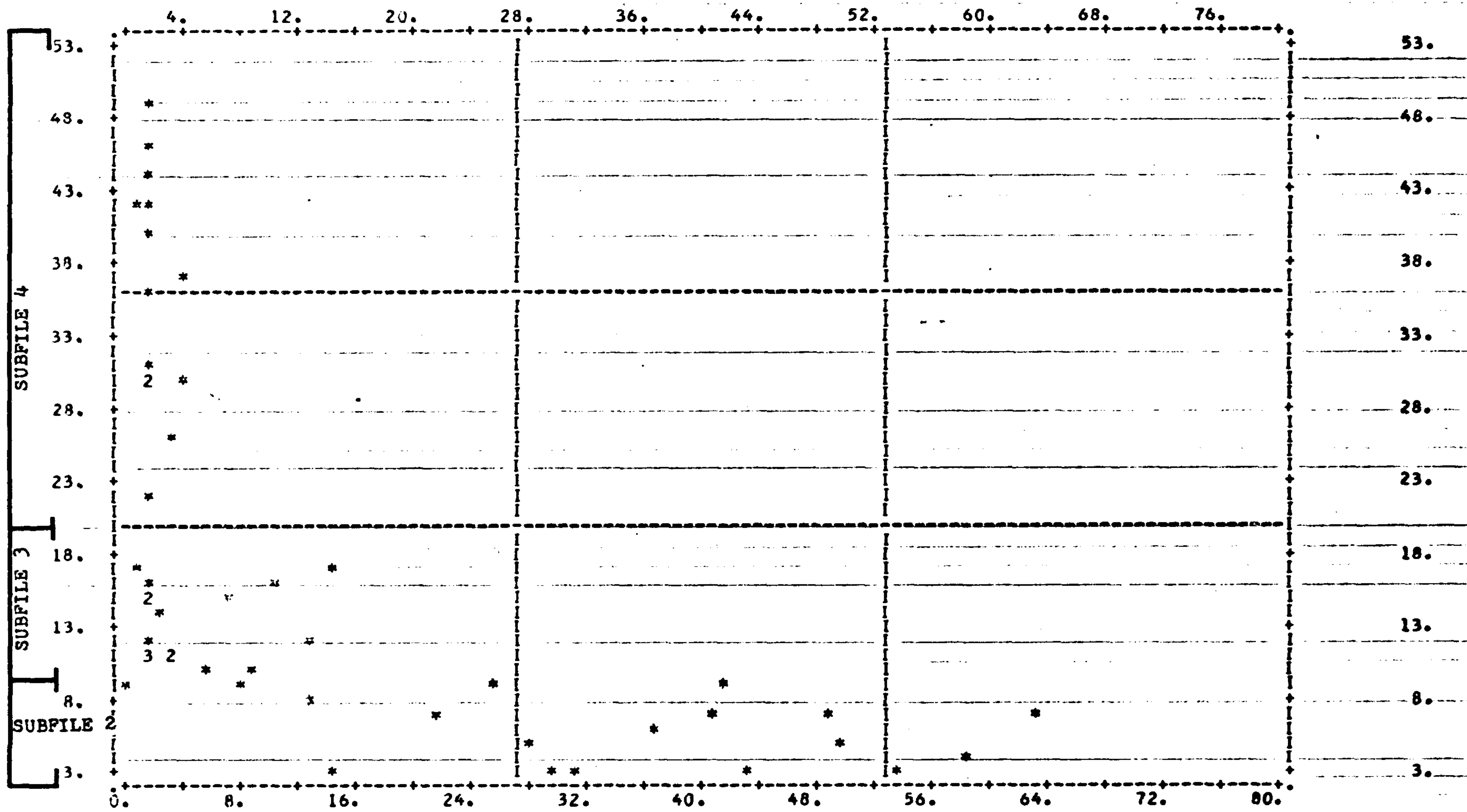

Subpopulation 2D
Subpopulation 2C
Subpopulation 2B
50
45
40
35
30
25
20
15
10
5
4
8
12
16
20
24
Per Cent Benzidine-Positive Cells (Spleen)
Per Cent Benzidine-Positive Cells (Bone Marrow)

Figure 3

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Per Cent Splenic Myeloblast in Leukemic Rats: Subfiles 2-4.

SUBFILE SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR002 SPLEEN BEN + (ACROSS) VAR006 SPLEEN MYELOBLAST

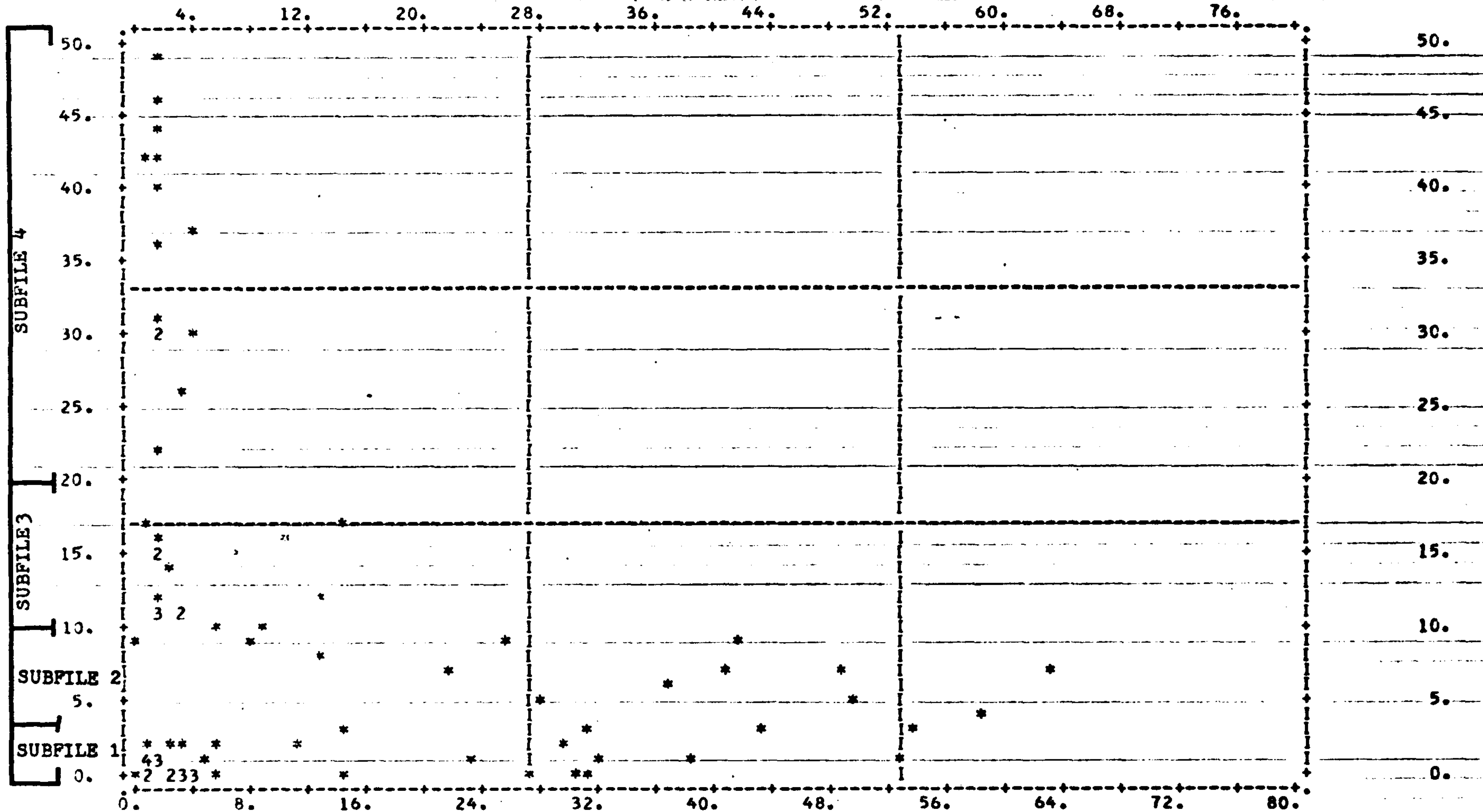


STATISTICS..

CORRELATION (R)-	-0.57758	R SQUARED -	0.33360	SIGNIFICANCE -	0.00001
STD ERR OF EST -	10.97743	INTERCEPT (A) -	23.68109	SLOPE (B) -	-0.41767
PLOTTED VALUES -	49	EXCLUDED VALUES-	0	MISSING VALUES-	1

Figure 3A

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Per Cent Splenic Myeloblasts in Leukemic Rats: Subfiles 1-4.



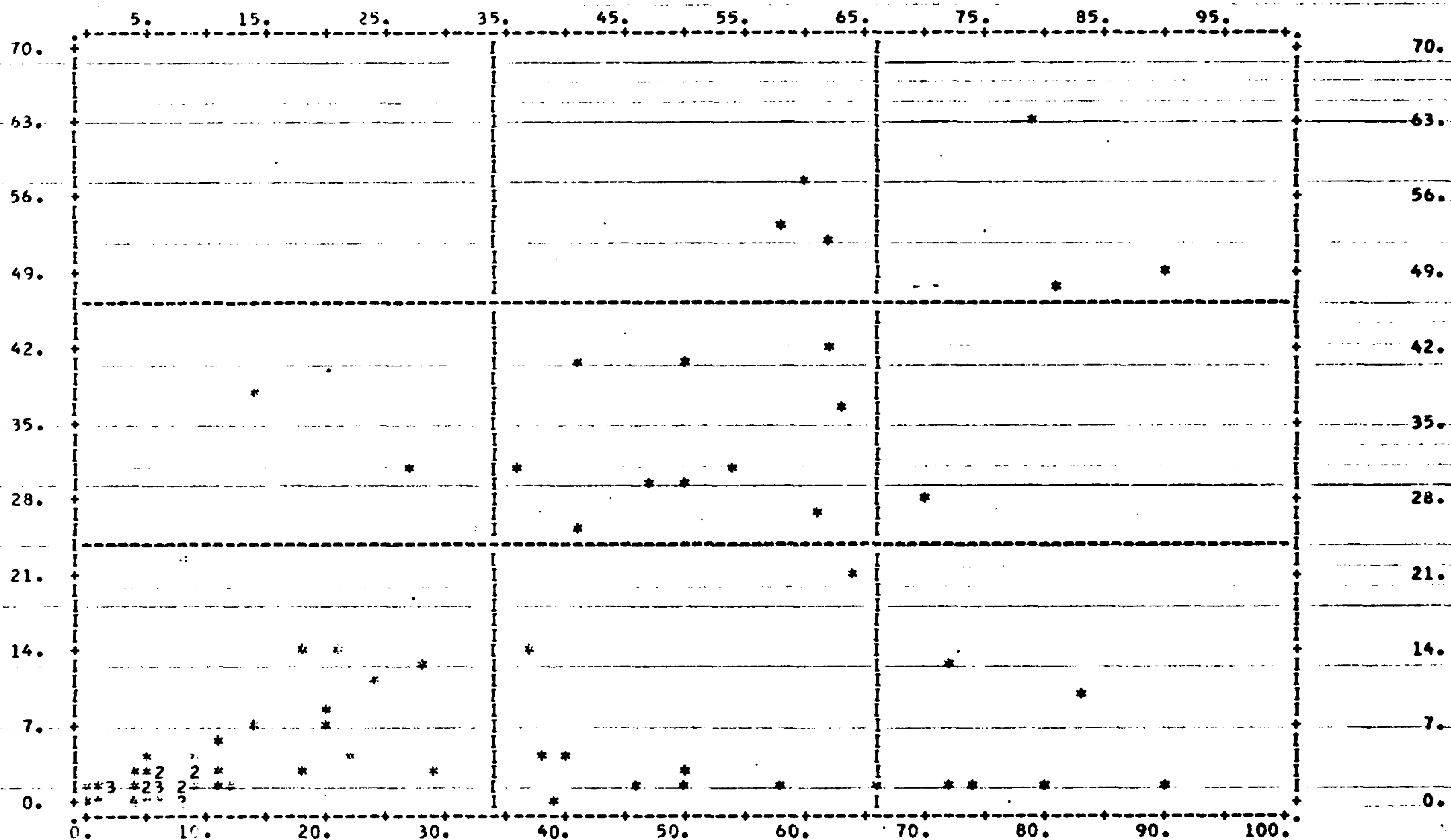
-109A-

STATISTICS..

CORRELATION (R)-	-0.29329	R SQUARED -	0.08602	SIGNIFICANCE -	0.00375
STD ERR OF EST -	12.57976	INTERCEPT (A) -	13.86612	SLOPE (B) -	-0.22931
PLOTTED VALUES -	82	EXCLUDED VALUES-	0	MISSING VALUES -	3

Figure 4

Relationship Between Per Cent Splenic Myeloblasts and Per Cent Bone Marrow Myeloblasts in Leukemic Rats: Subfiles 1-4.



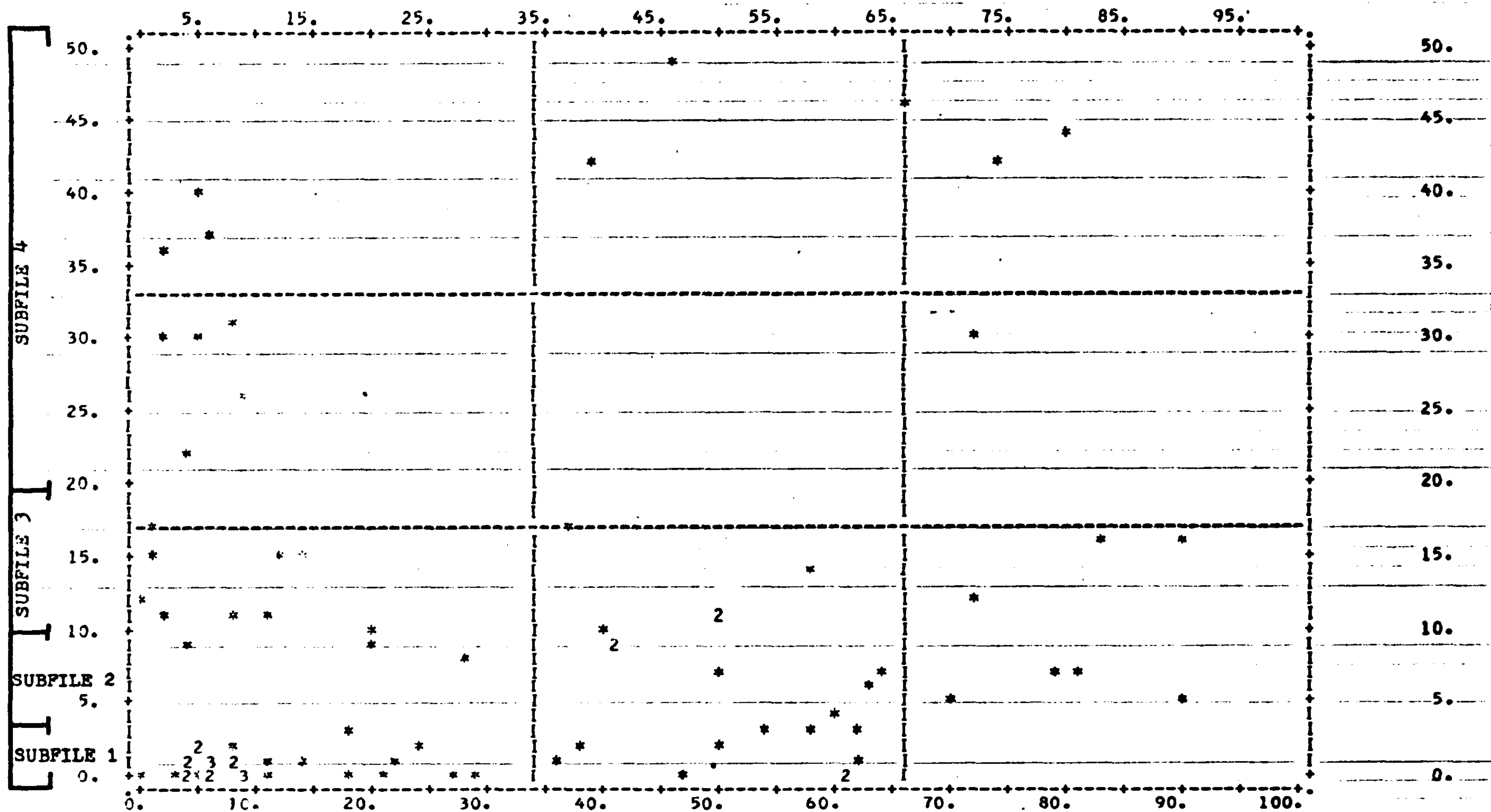
STATISTICS..

CORRELATION (R)-	0.56719	R SQUARED -	0.32171	SIGNIFICANCE -	0.00001
STD ERR OF EST -	13.86035	INTERCEPT (A) -	2.26941	SLOPE (B) -	0.34845

Figure 5

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Per Cent Bone Marrow Myeloblasts in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR002 SPLEEN BEN + (ACROSS) VAR007 BONE-MARROW MYELOBLAST

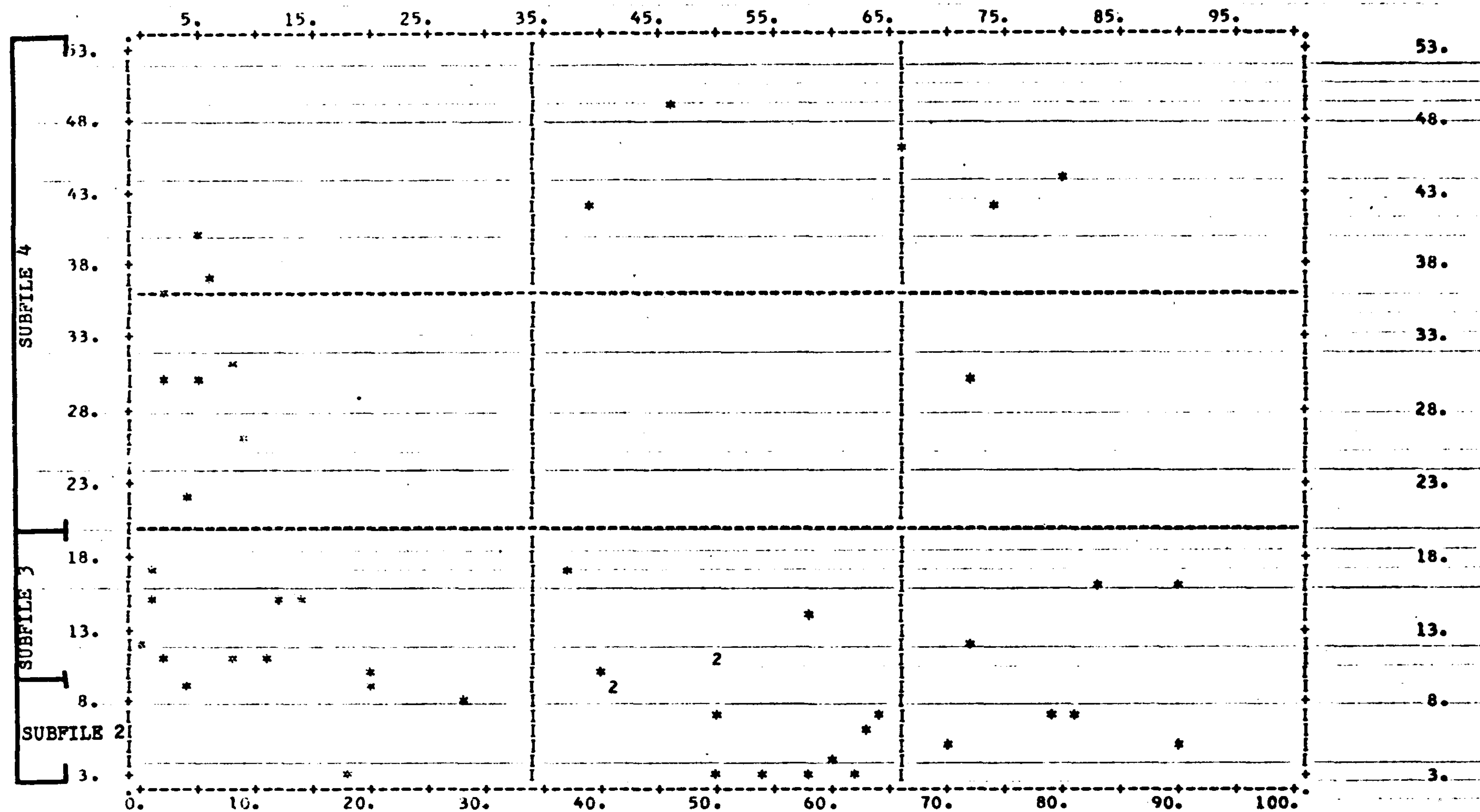


STATISTICS..

CORRELATION (R)-	0.15097	R SQUARED -	0.02279	SIGNIFICANCE -	0.08522
STD ERR OF EST -	12.94895	INTERCEPT (A) -	8.58830	SLOPE (B) -	0.07209

Figure 5A

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Per Cent Bone Marrow Myeloblasts in Leukemic Rats: Subfiles 2-4.

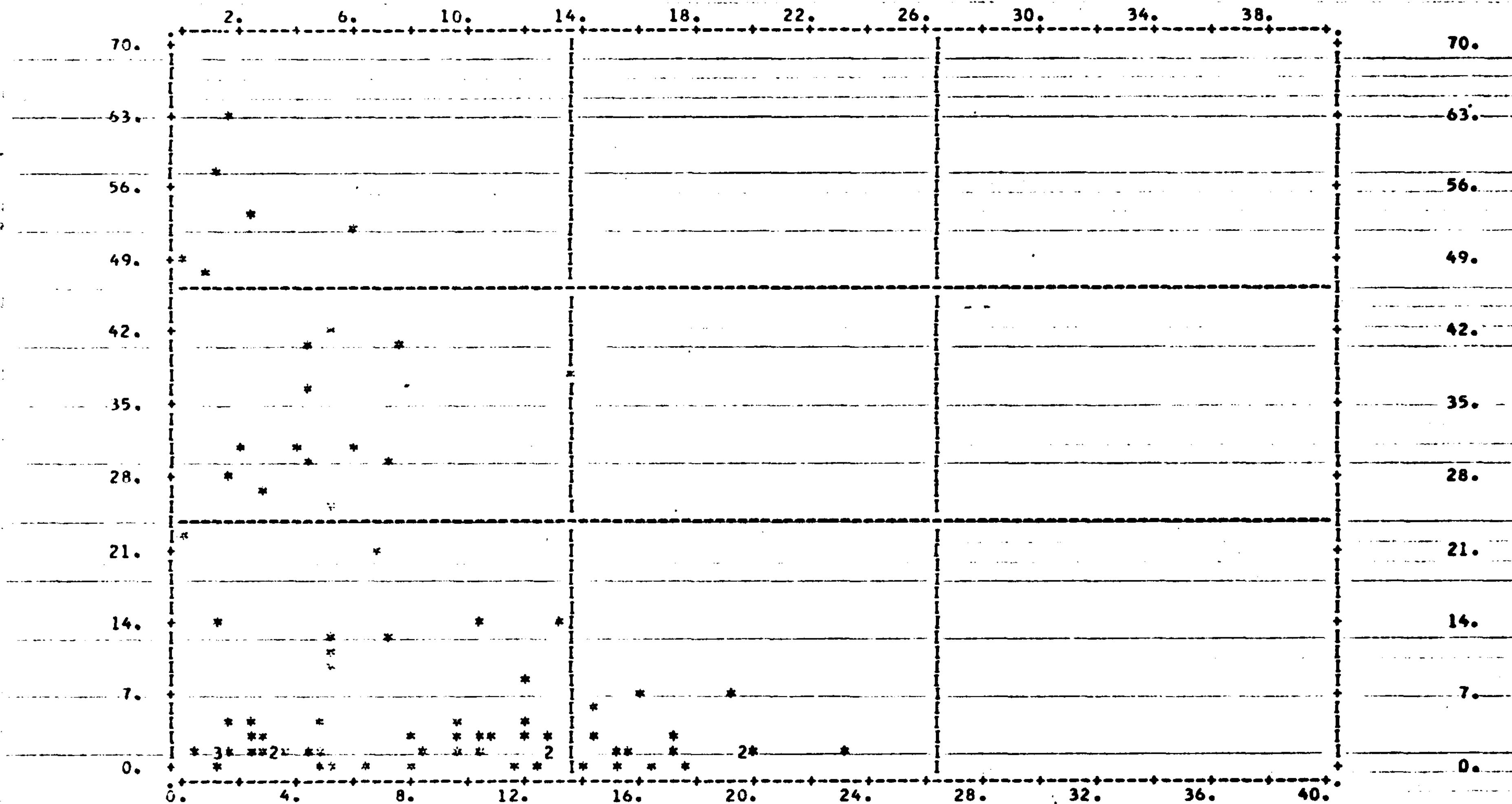


STATISTICS..

CORRELATION (R)-	-0.14916	R SQUARED -	0.02225	SIGNIFICANCE -	0.15317
STD ERR OF EST -	13.29681	INTERCEPT (A) -	20.20510	SLOPE (B) -	-0.06743
PLOTTED VALUES -	49	EXCLUDED VALUES-	0	MISSING VALUES -	1

Figure 6

Relationship Between Per Cent Splenic Myeloblasts and Per Cent Bone Marrow Benzidine-Positive Cells in Leukemic Rats: Subfiles 1-4.



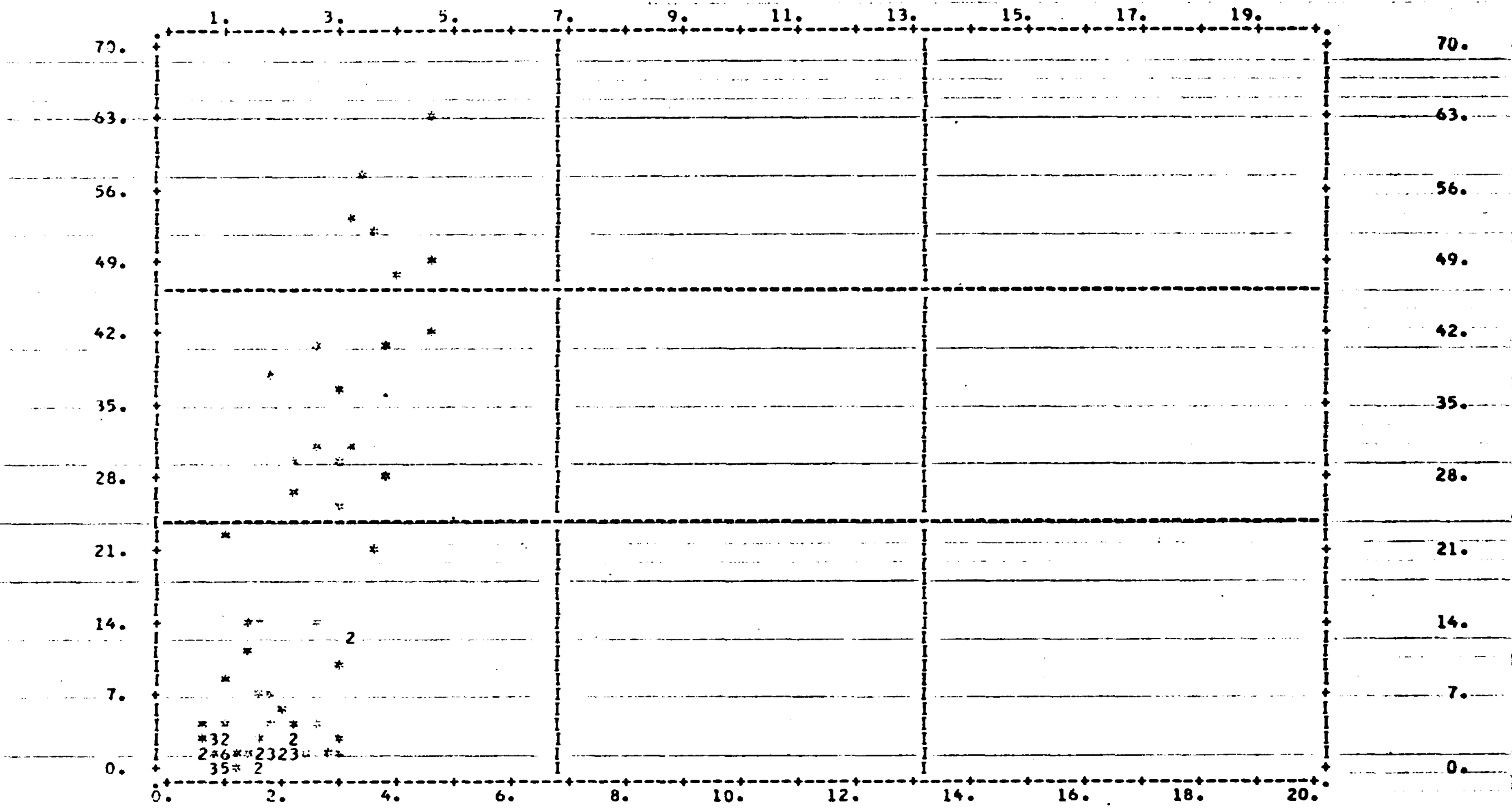
-113A-

STATISTICS..

CORRELATION (R)-	-0.38044	R SQUARED -	0.14474	SIGNIFICANCE -	0.00021
STD ERR OF EST -	15.56377	INTERCEPT (A) -	21.18893	SLOPE (B) -	-1.07663

Figure 7

Relationship Between Per Cent Splenic Myeloblasts and Weight of Spleen in Leukemic Rats: Subfiles 1-4.



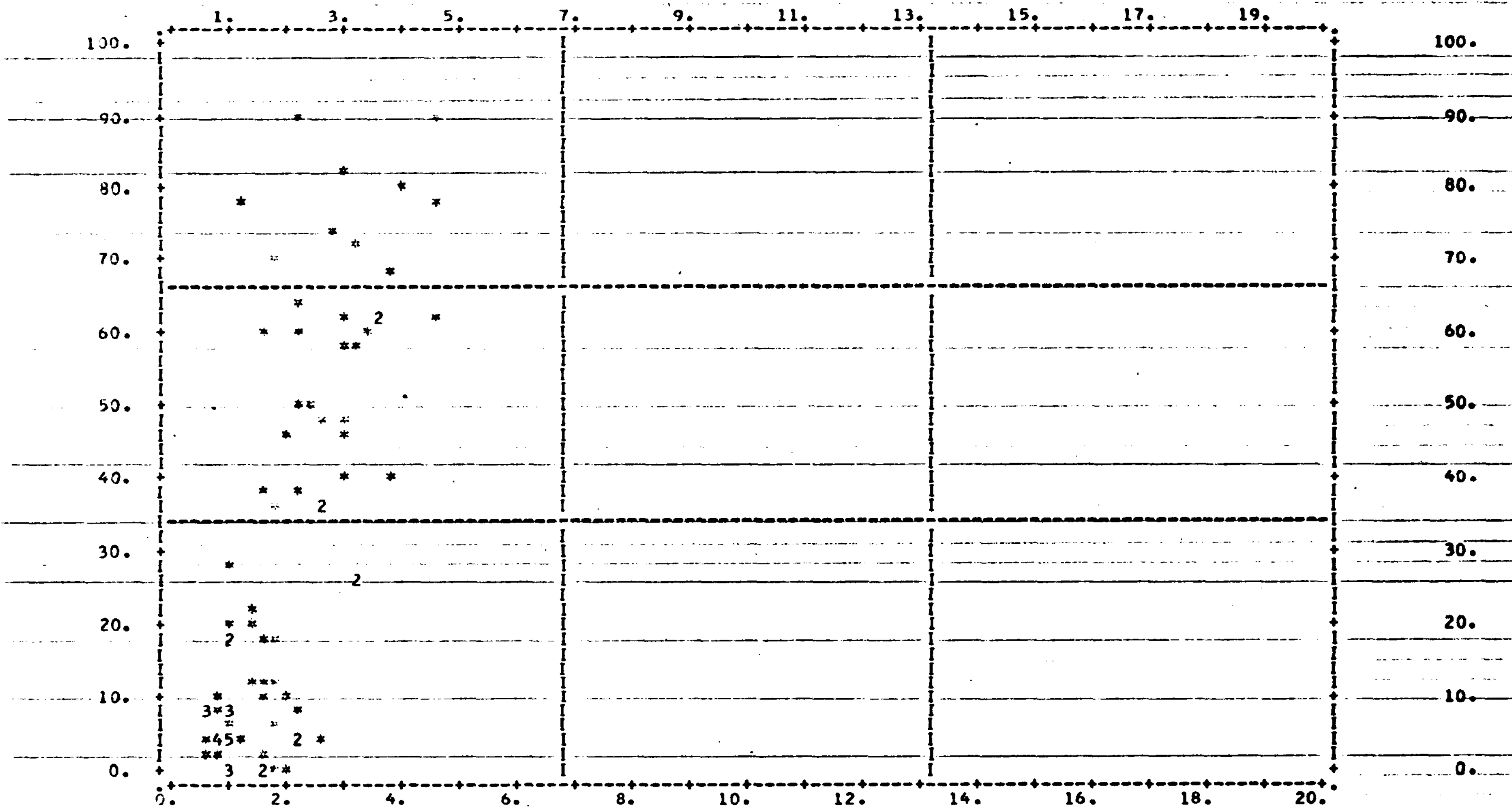
STATISTICS..

CORRELATION (R)-	0.73845	R SQUARED -	0.54531	SIGNIFICANCE -	0.00001
STD ERR OF EST -	11.33284	INTERCEPT (A) -	-10.17389	SLOPE (B) -	11.65636
PLOTTED VALUES -	81	EXCLUDED VALUES-	0	MISSING VALUES -	4

Figure 8

Relationship Between Per Cent Bone Marrow Myeloblasts and Weight of Spleen in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR007 BONE-MARROW MYELOBLAST (ACROSS) VAR005 WEIGHT OF SPLEEN



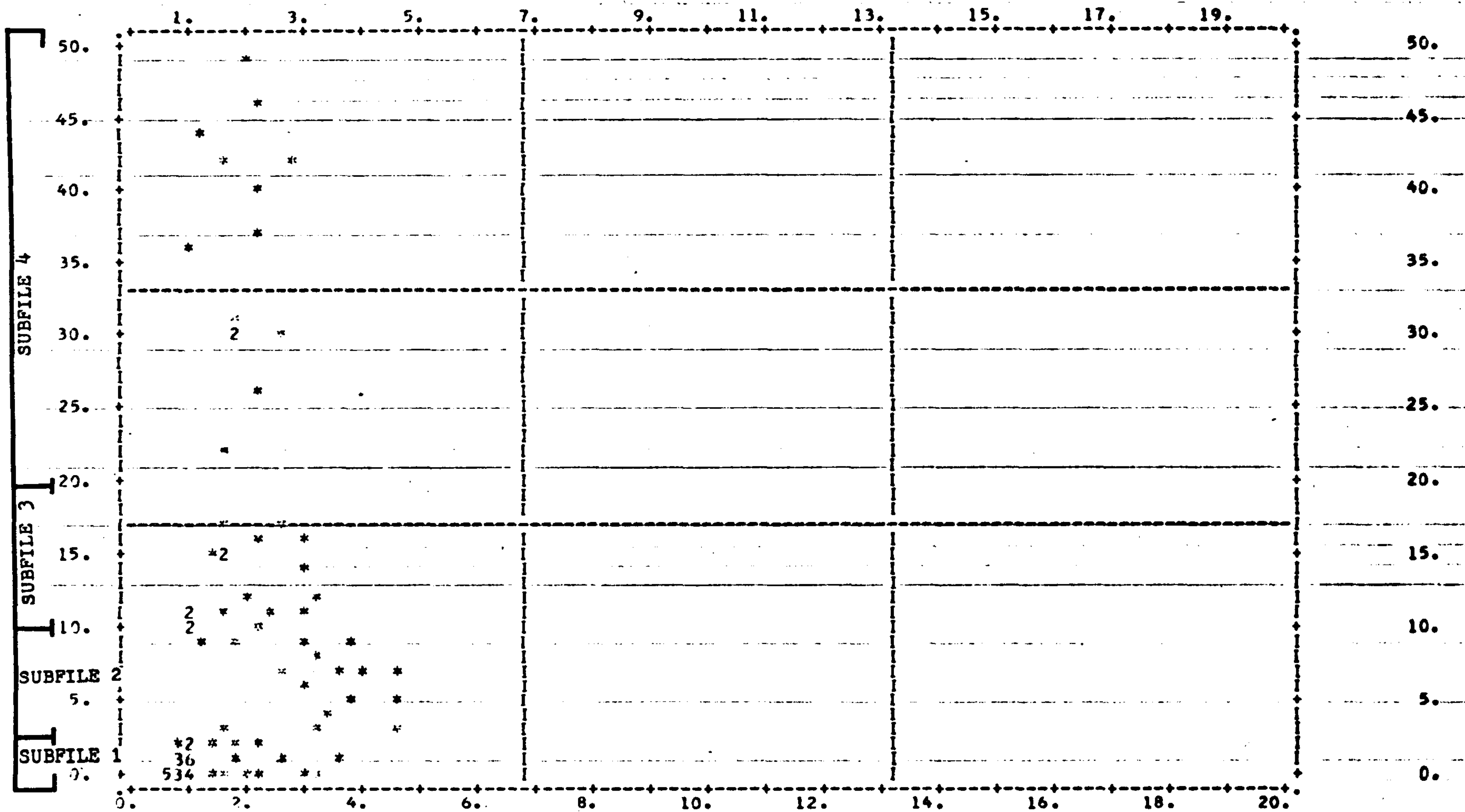
STATISTICS..

CORRELATION (R)-	0.73548	R SQUARED -	0.54094	SIGNIFICANCE -	0.00001
STD ERR OF EST -	18.60801	INTERCEPT (A) -	-6.96376	SLOPE (B) -	19.00625
PLOTTED VALUES -	83	EXCLUDED VALUES-	0	MISSING VALUES-	2

Figure 9

Relationship Between Per Cent Splenic Benzidine-Positive Cells and Weight of Spleen in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR002 SPLEEN BEN + (ACROSS) VAR005 WEIGHT OF SPLEEN



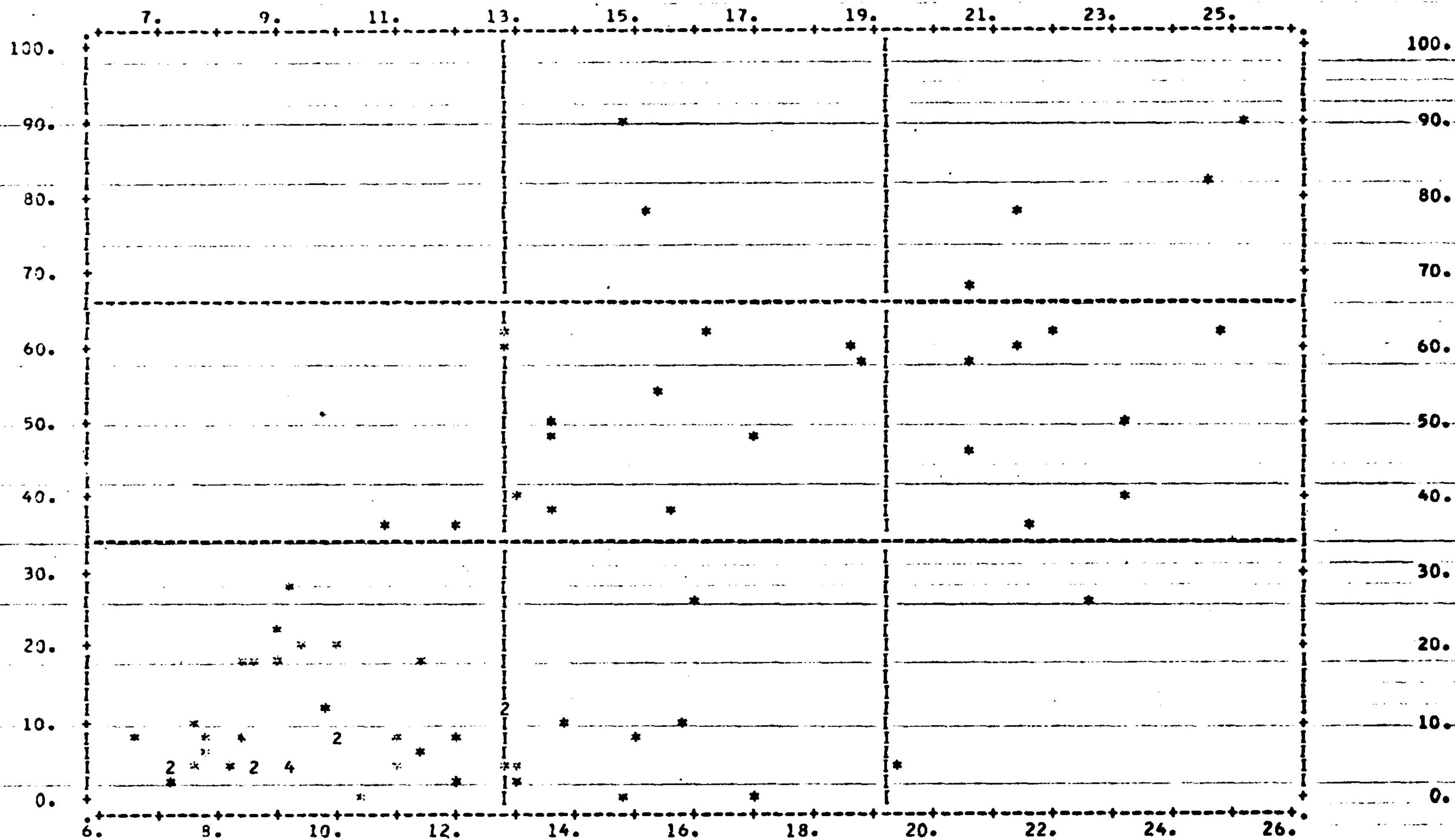
STATISTICS..

CORRELATION (R)-	0.12964	R SQUARED -	0.01681	SIGNIFICANCE -	0.11994
STD ERR OF EST -	12.96546	INTERCEPT (A) -	7.77831	SLOPE (B) -	1.59600

Figure 10

Relationship Between Per Cent Bone Marrow Myeloblasts and Weight of Liver in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR007 BONE-MARROW MYELOBLAST (ACROSS) VAR011 WEIGHT OF LIVER



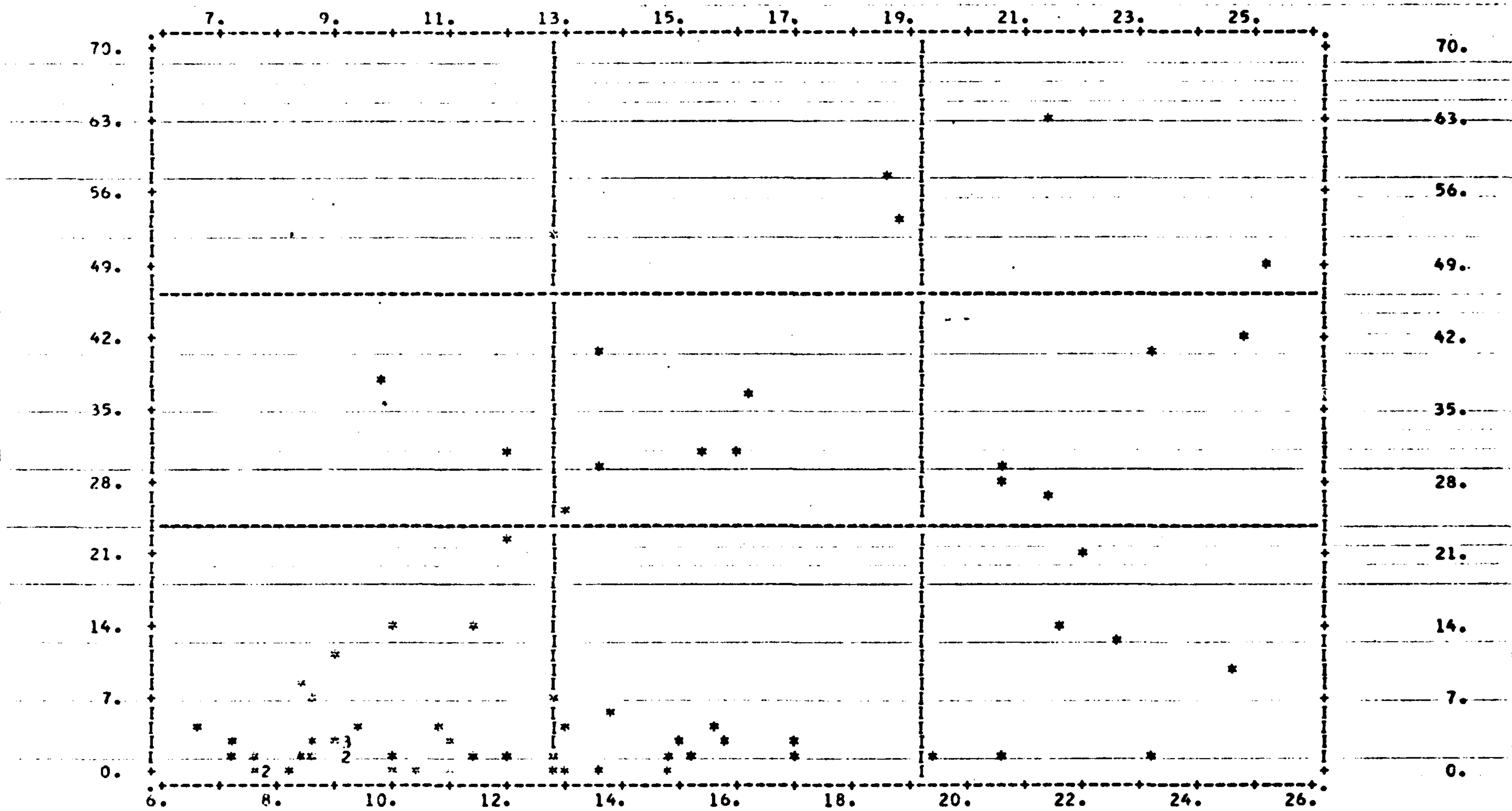
STATISTICS..

CORRELATION (R)-	0.69211	R SQUARED -	0.47902	SIGNIFICANCE -	0.00001
STD ERR OF EST -	18.72044	INTERCEPT (A) -	-18.91238	SLOPE (B) -	3.46755
PLOTTED VALUES -	74	EXCLUDED VALUES-	0	MISSING VALUES -	11

Figure 11

Relationship Between Per Cent Splenic Myeloblasts and Weight of Liver in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR006 SPLEEN MYELOBLAST (ACROSS) VAR011 WEIGHT OF LIVER



STATISTICS..

CORRELATION (R)-	0.50543	R SQUARED -	0.25546	SIGNIFICANCE -	0.00001
STD ERR OF EST -	14.70305	INTERCEPT (A) -	-9.31148	SLOPE (B) -	1.65791
PLOTTED VALUES -	72	EXCLUDED VALUES-	0	MISSING VALUES-	13

Figure 12

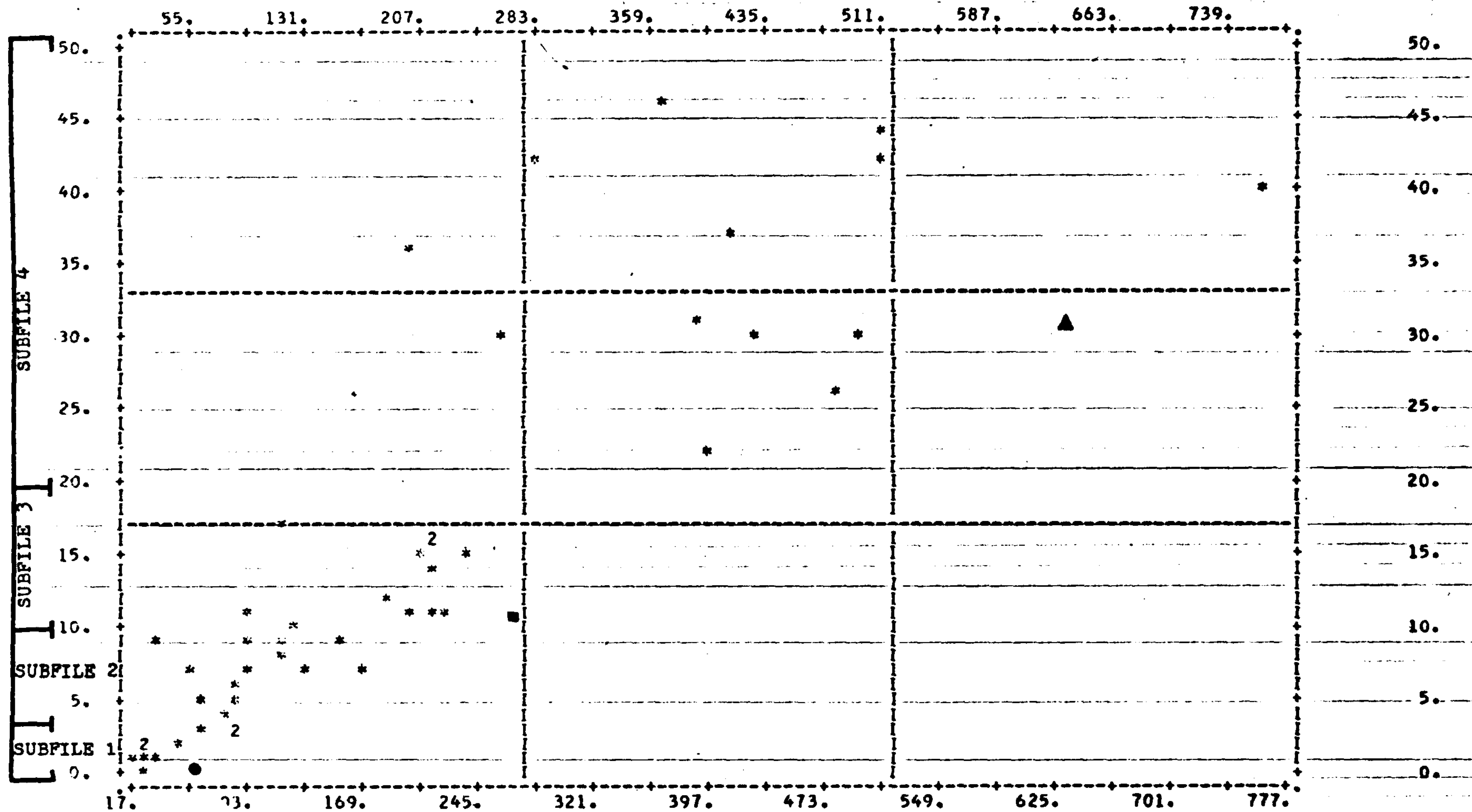
Relationship Between Per Cent Splenic Benzidine-Positive Cells and Specific Activity -Aminolevulinic Acid-Synthetase in Normal, Hemorrhagic and Hemolytic Anemic and Leukemic Rats: Subfiles 1-4.

● : Normal

■ : Hemorrhagic Anemic

▲ : Hemolytic Anemic

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR002 SPLEEN BEN + (ACROSS) VAR003 SPLEEN S.A.



STATISTICS..

CORRELATION (R) -	0.86430	R SQUARED -	0.74702	SIGNIFICANCE -	0.00001
STD ERR OF EST -	6.85467	INTERCEPT (A) -	1.98900	SLOPE (B) -	0.06961
		EXCLUDED VALUES -	0	MISSING VALUES -	37

Figure 13

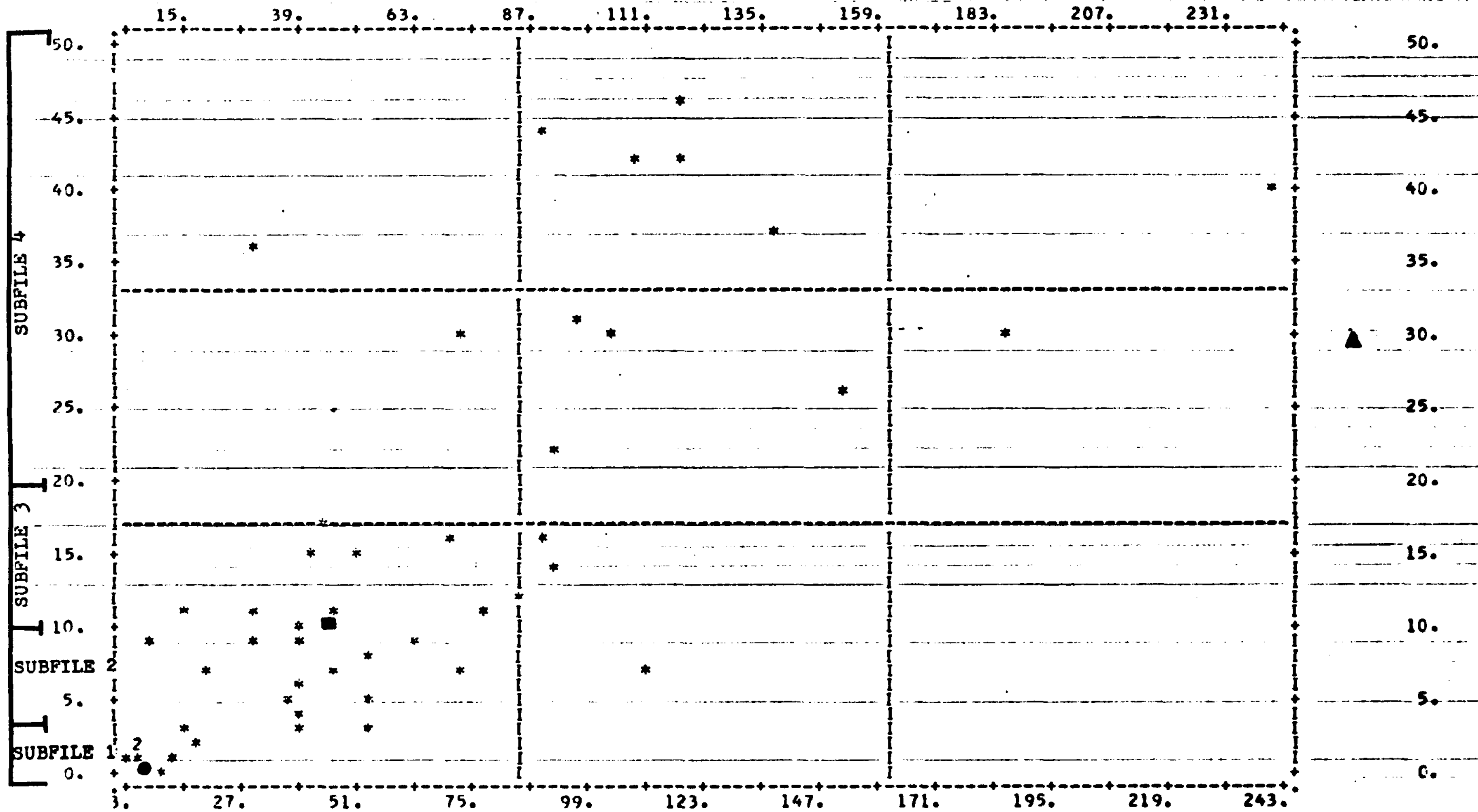
Relationship Between Per Cent Splenic Benzidine-Positive Cells and Net Activity δ-Aminolevulinic Acid-Synthetase in Normal, Hemorrhagic and Hemolytic Anemic and Leukemic Rats: Subfiles 1-4.

● : Normal

■ : Hemorrhagic Anemic

▲ : Hemolytic Anemic

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR002 SPLEEN BEN + (ACROSS) VAR004 SPLEEN N.A.



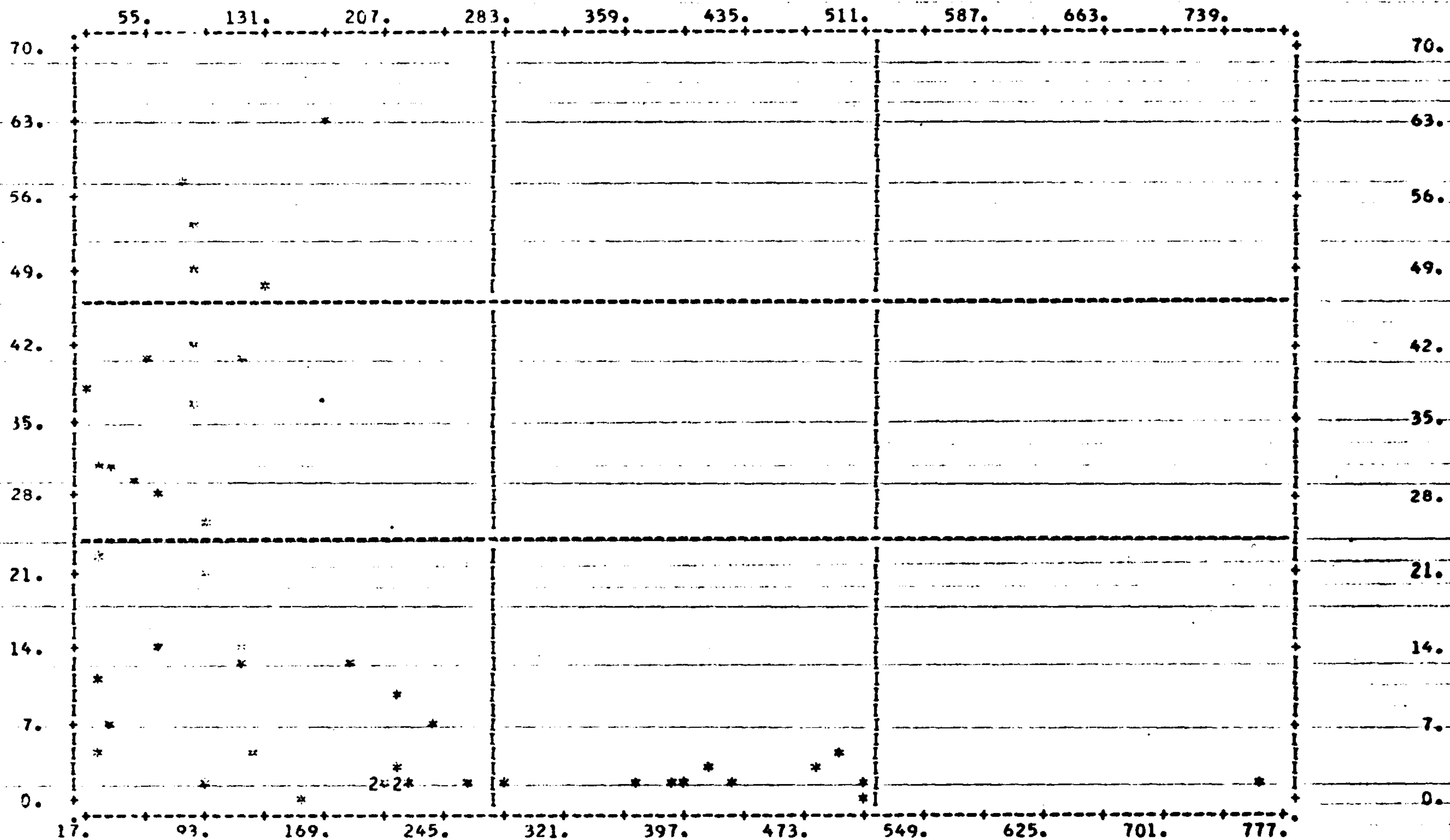
STATISTICS..

CORRELATION (R)-	0.72599	R SQUARED -	0.52706	SIGNIFICANCE -	0.00001
STD ERR OF EST -	9.37226	INTERCEPT (A) -	3.38245	SLOPE (B) -	0.19684
PLOTTED VALUES -	48	EXCLUDED VALUES -	0	MISSING VALUES -	37

Figure 14

Relationship Between Per Cent Splenic Myeloblasts and Specific Activity Splenic δ-Aminolevulinic Acid-Synthethase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR006 SPLEEN MYELOBLAST (ACROSS) VAR003 SPLEEN S.A.



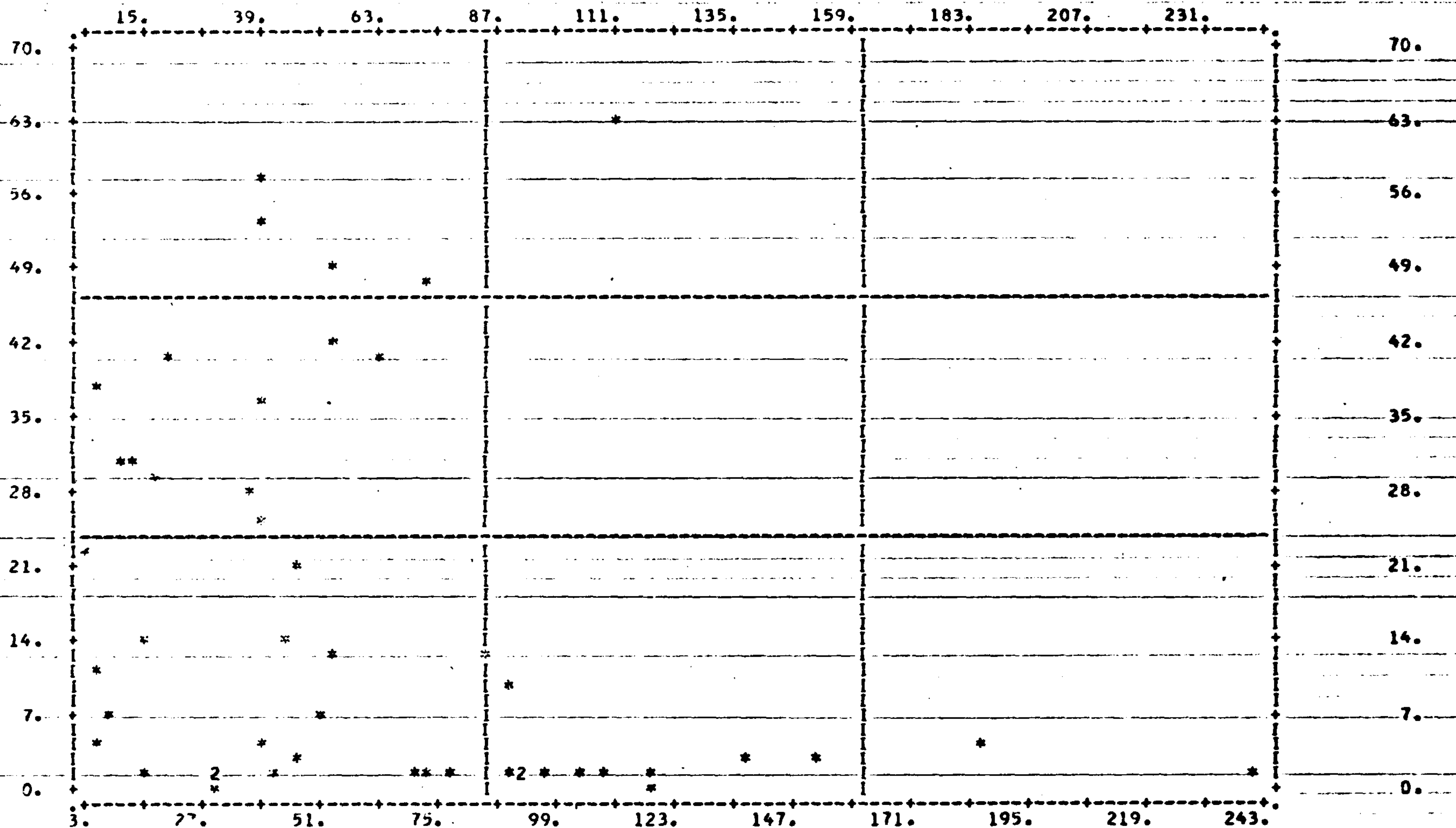
STATISTICS..

CORRELATION (R)-	-0.53592	R SQUARED -	0.28721	SIGNIFICANCE -	0.00004
STD ERR OF EST -	15.83380	INTERCEPT (A) -	28.46922	SLOPE (B) -	-0.05940
		EXCLUDED VALUES	0	MISSING VALUES	37

Figure 15

Relationship Between Per Cent Splenic Myeloblasts and Net Activity Splenic δ-Aminolevulinic Acid-Synthetase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR006 SPLEEN MYELOBLAST (ACROSS) VAR004 SPLEEN N.A.



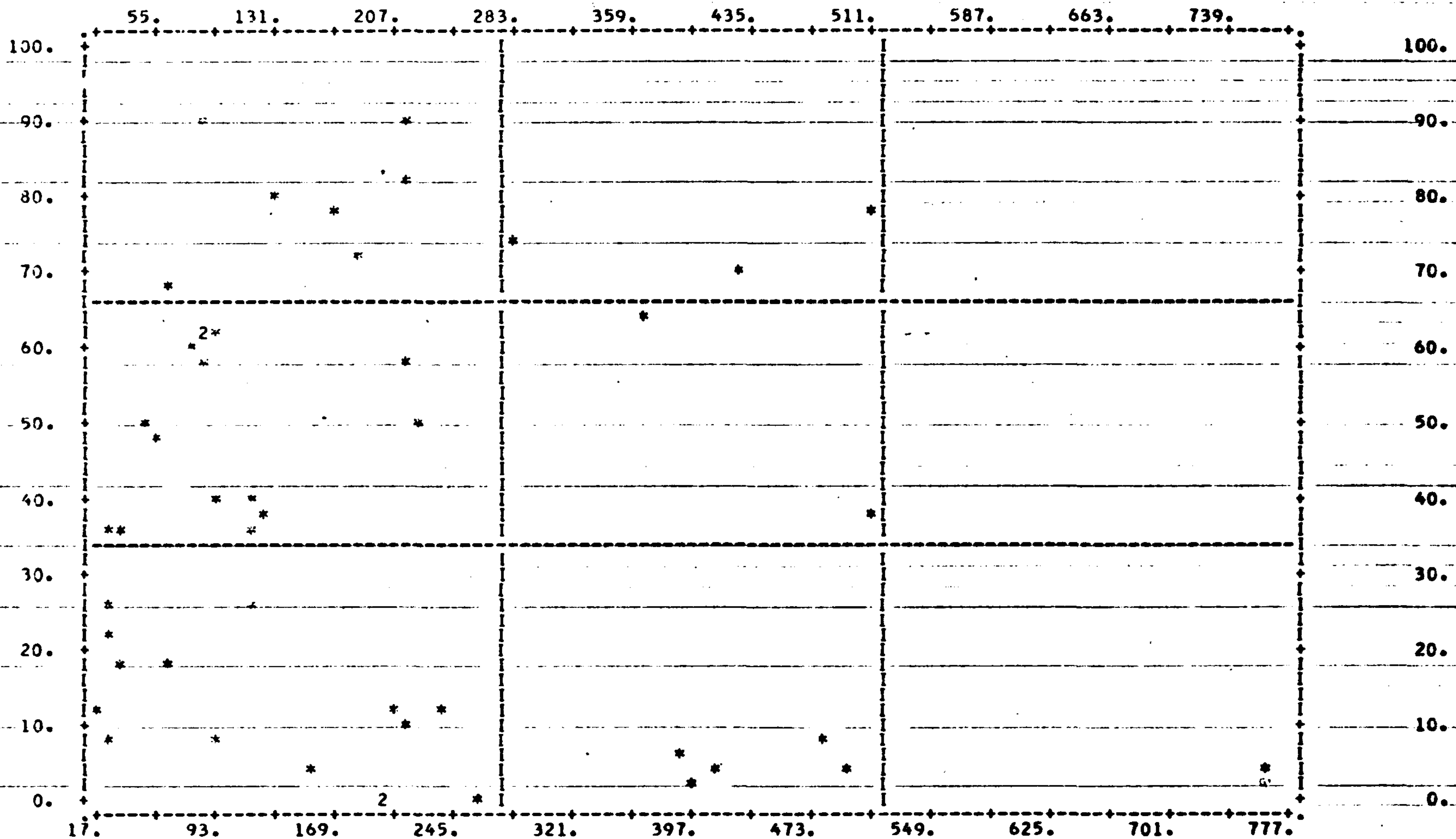
STATISTICS..

CORRELATION (R)-	-0.29481	R SQUARED -	0.08692	SIGNIFICANCE -	0.02097
STD ERR OF EST -	17.92096	INTERCEPT (A) -	23.67148	SLOPE (B) -	-0.11000

Figure 16

Relationship Between Per Cent Bone Marrow Myeloblasts and Specific Activity Splenic δ-Aminolevulinic Acid-Synthetase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR007 BONE-MARROW MYELOBLAST (ACROSS) VAR003 SPLEEN S.A.



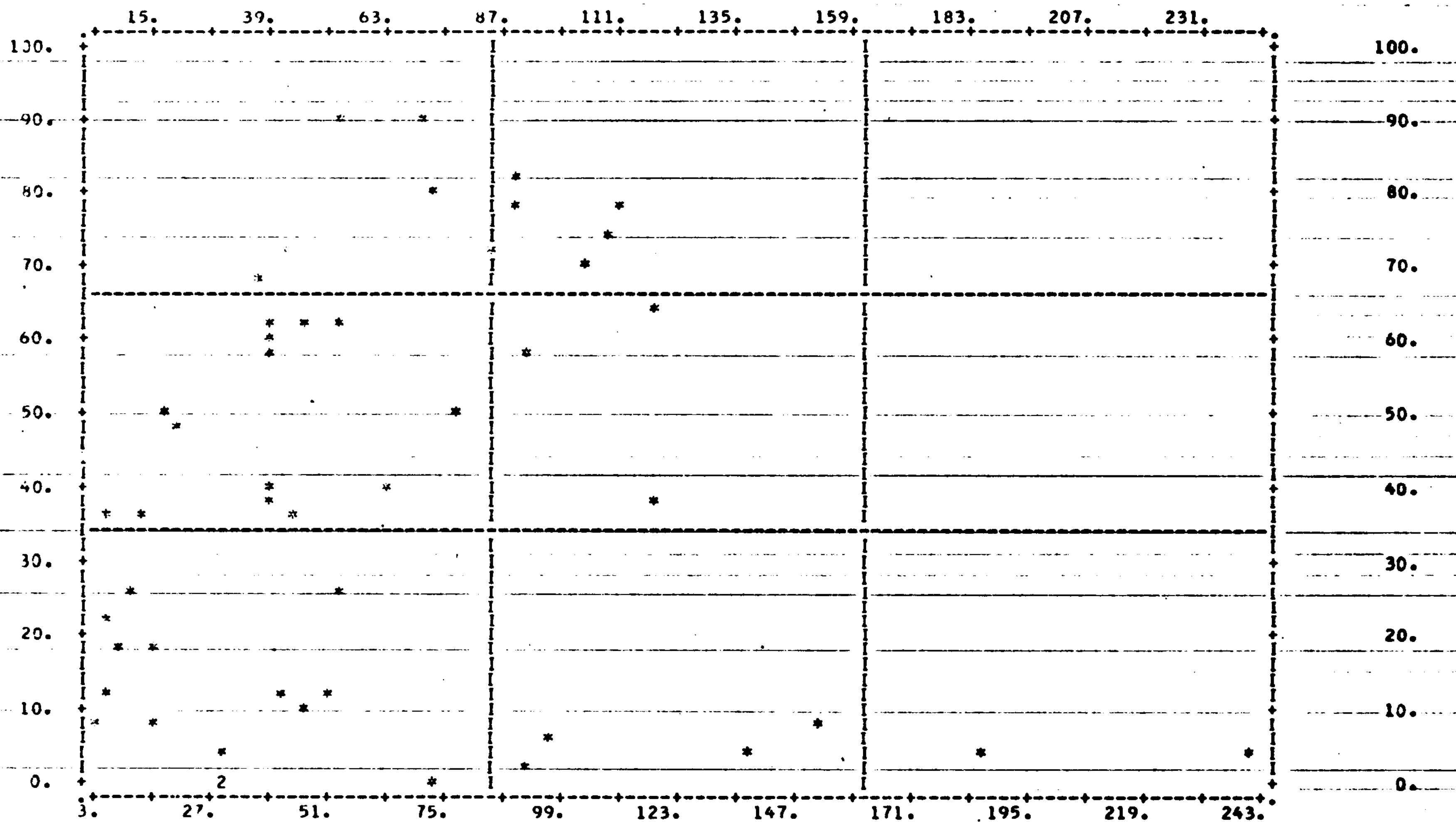
STATISTICS..

CORRELATION (R)-	-0.15716	R SQUARED -	0.02470	SIGNIFICANCE -	0.14304
STD ERR OF EST -	28.53814	INTERCEPT (A) -	44.22688	SLOPE (B) -	-0.02684
PLOTTED VALUES -	48	EXCLUDED VALUES-	0	MISSING VALUES -	37

Figure 17

Relationship Between Per Cent Bone Marrow Myeloblasts and Net Activity Splenic δ-Aminolevulinic Acid-Synthetase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR007 BONE-MARROW MYELOBLAST (ACROSS) VAR004 SPLEEN N.A.



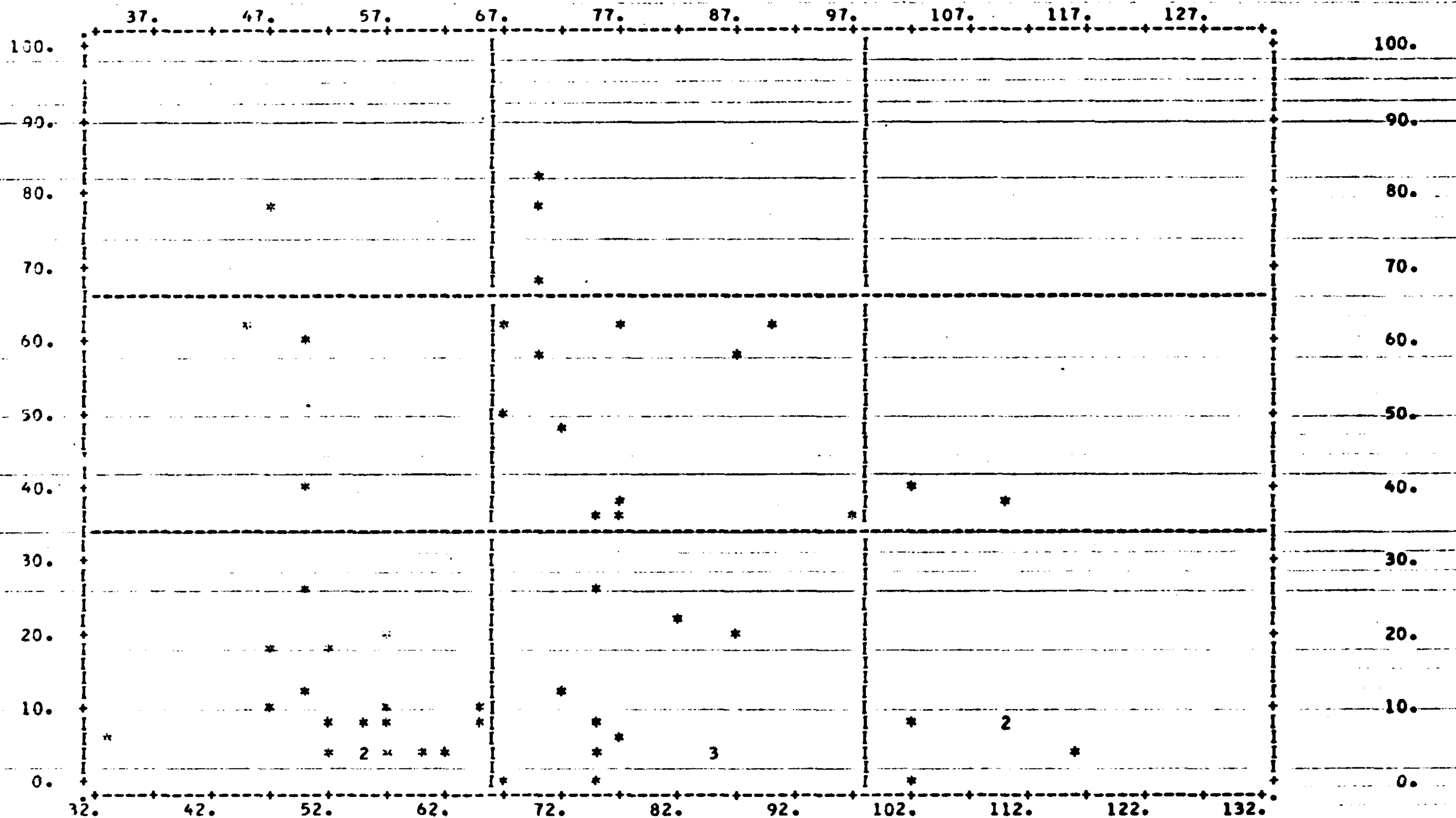
STATISTICS..

CORRELATION (R)-	0.03555	R SQUARED -	0.00126	SIGNIFICANCE -	0.40521
STD ERR OF EST -	28.87897	INTERCEPT (A) -	37.69210	SLOPE (B) -	0.02044
PLOTTED VALUES -	48	EXCLUDED VALUES-	0	MISSING VALUES -	37

Figure 18

Relationship Between Per Cent Bone Marrow Myeloblasts and Specific Activity Hepatic δ-Aminolevulinic Acid-Synthetase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR007 BONE-MARROW MYELOBLAST (ACROSS) VAR009 LIVER S.A.

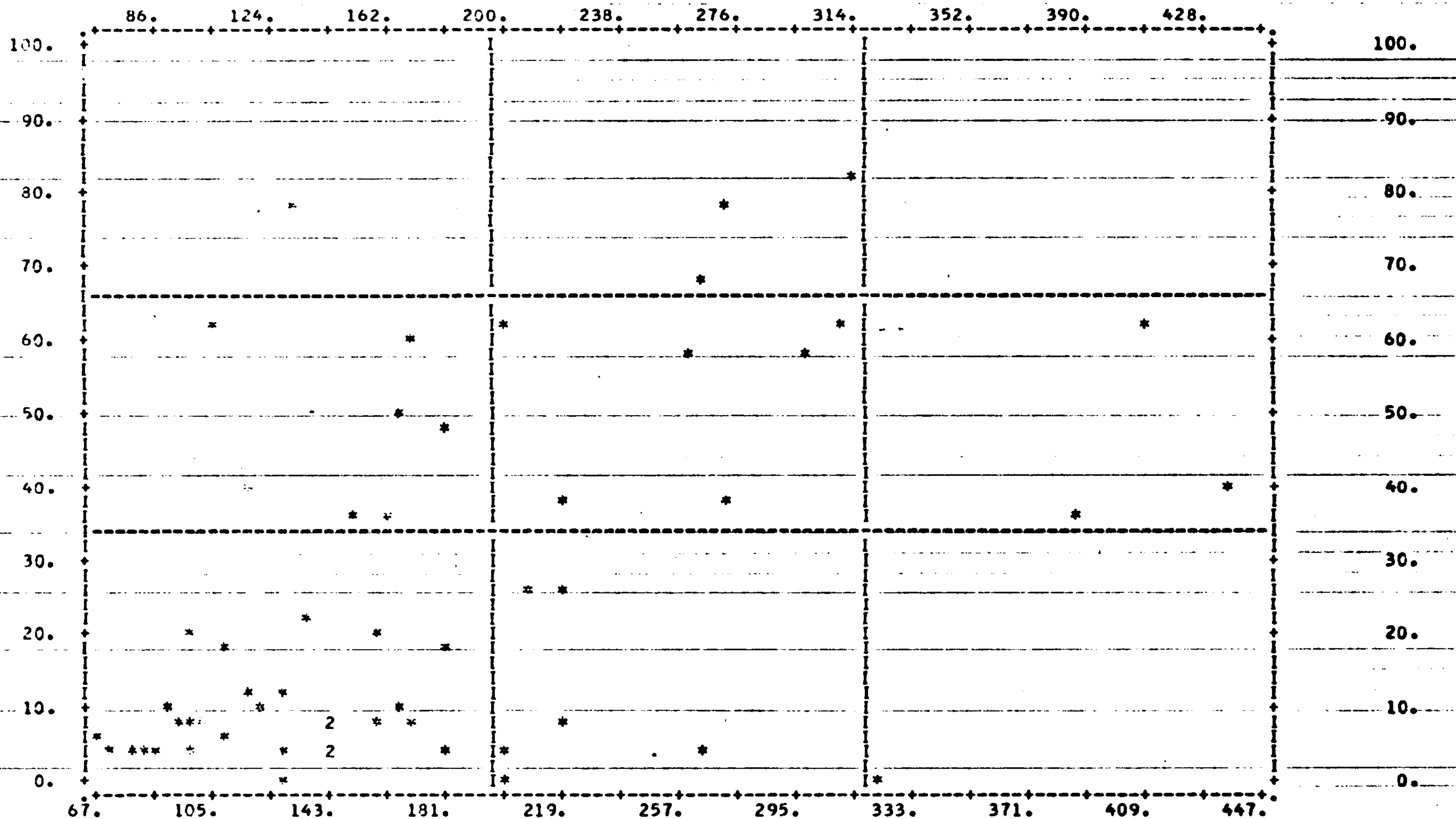


STATISTICS..

CORRELATION (R)-	-0.03416	R SQUARED -	0.00117	SIGNIFICANCE -	0.40130
STD ERR OF EST -	24.56217	INTERCEPT (A) -	29.32368	SLOPE (B) -	-0.04312
PLOTTED VALUES -	56	EXCLUDED VALUES-	0	MISSING VALUES-	29

Figure 19

Relationship Between Per Cent Bone Marrow Myeloblasts and Net Activity Hepatic δ-Aminolevulinic-Synthetase in Leukemic Rats: Subfiles 1-4.



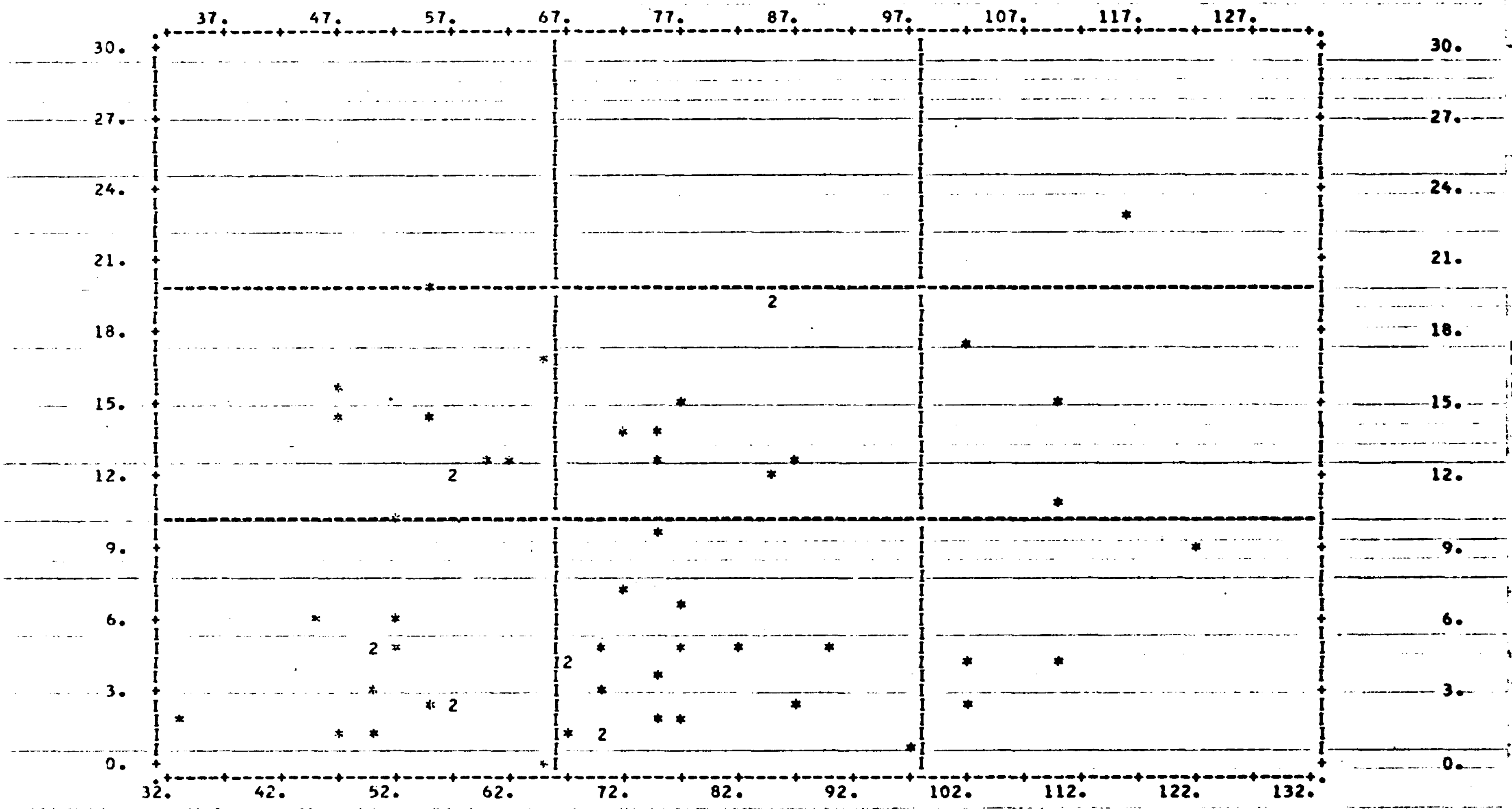
STATISTICS..

CORRELATION (R)-	0.50562	R SQUARED -	0.25565	SIGNIFICANCE -	0.00004
STD ERR OF EST -	21.20357	INTERCEPT (A) -	1.04118	SLOPE (B) -	0.14148
PLOTTED VALUES -	56	EXCLUDED VALUES-	0	MISSING VALUES-	29

Figure 20

Relationship Between Per Cent Bone Marrow Benzidine-positive Cells and Specific Activity Hepatic δ-Aminolevulinic Acid-Synthetase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR008 BONE-MARROW BEN + (ACROSS) VAR009 LIVER S.A.



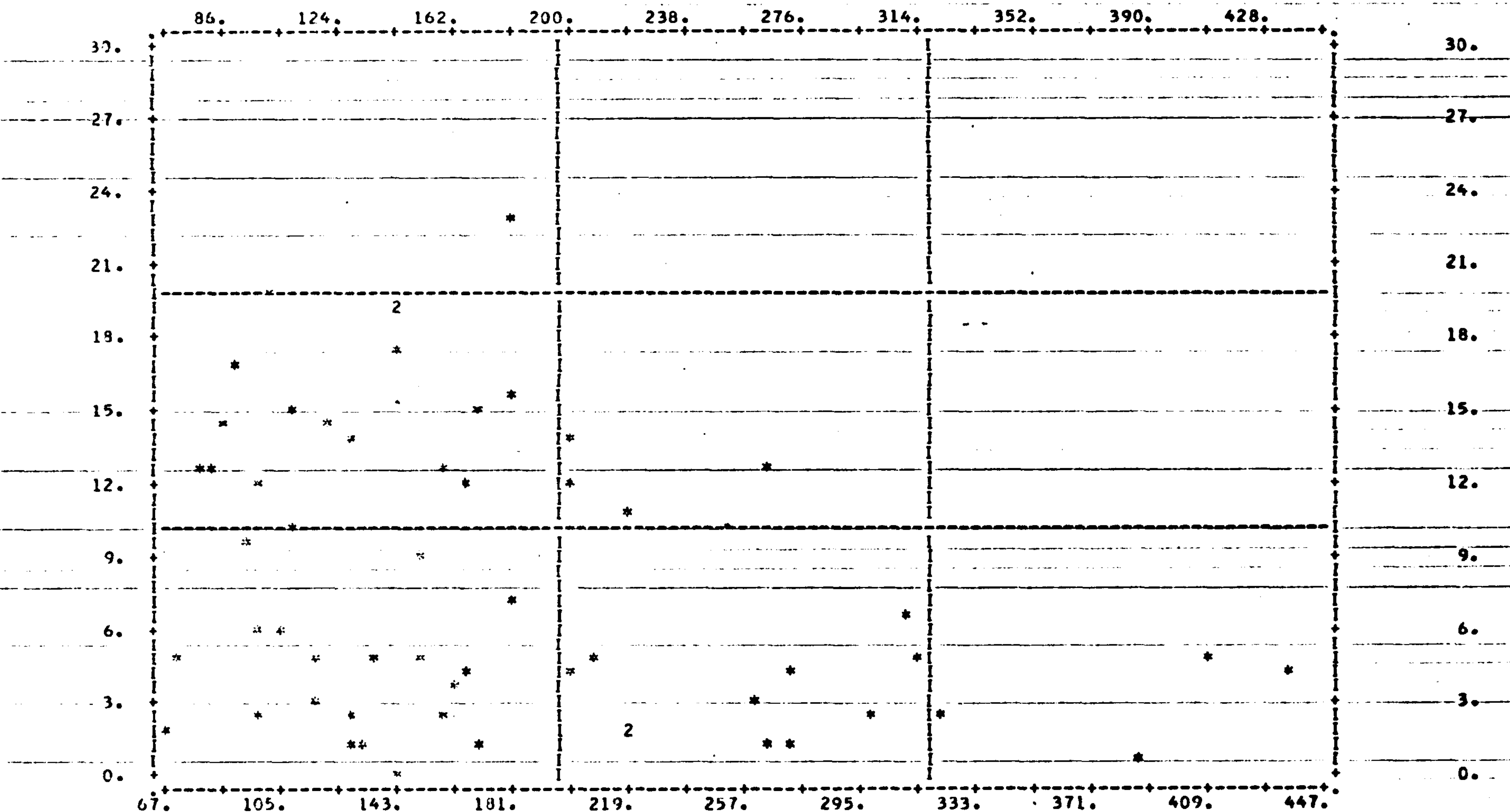
STATISTICS..

CORRELATION (R)-	0.20225	R SQUARED -	0.04091	SIGNIFICANCE -	0.06568
STD ERR OF EST -	6.00184	INTERCEPT (A) -	3.83421	SLOPE (B) -	0.06054

Figure 21

Relationship Between Per Cent Bone Marrow Benzidine-Positive Cells and Net Activity Hepatic δ-Aminolevulinic Acid-Synthetase in Leukemic Rats: Subfiles 1-4.

SUBFILE SUB1 SUB2 SUB3 SUB4
SCATTERGRAM OF (DOWN) VAR008 BONE-MARROW BEN + (ACROSS) VAR010 LIVER N.A.

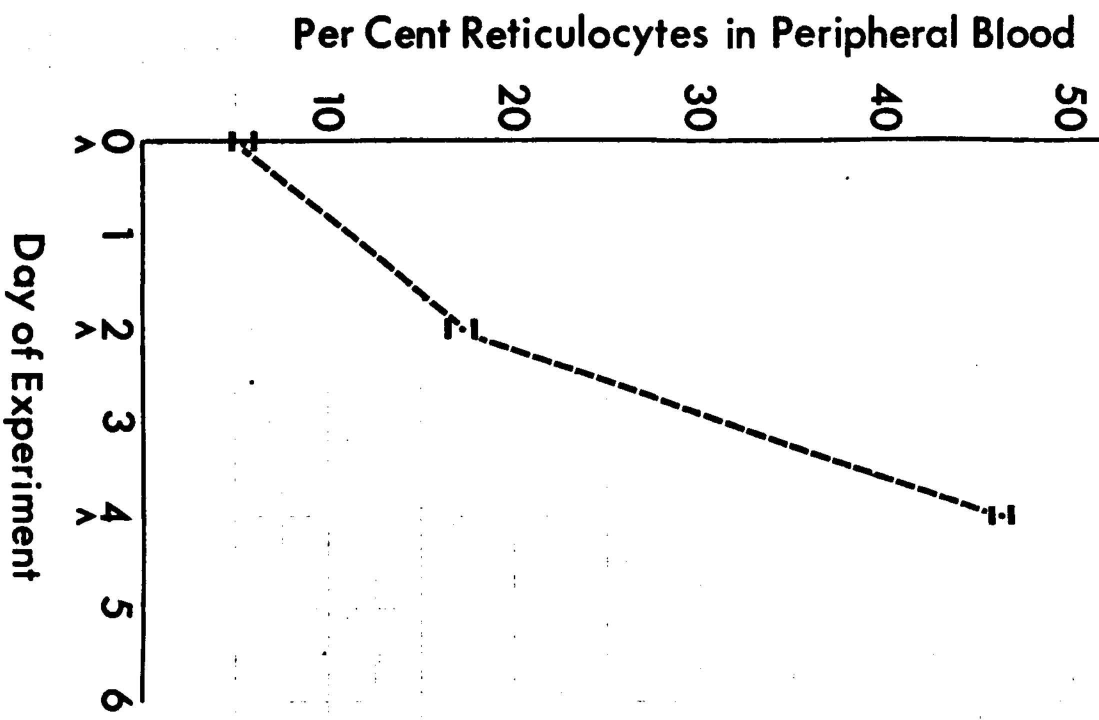


STATISTICS..

CORRELATION (R)-	-0.32026	R SQUARED -	0.10257	SIGNIFICANCE -	0.00758
STD ERR OF EST -	5.80570	INTERCEPT (A) -	12.20227	SLOPE (B) -	-0.02254

Figure 22

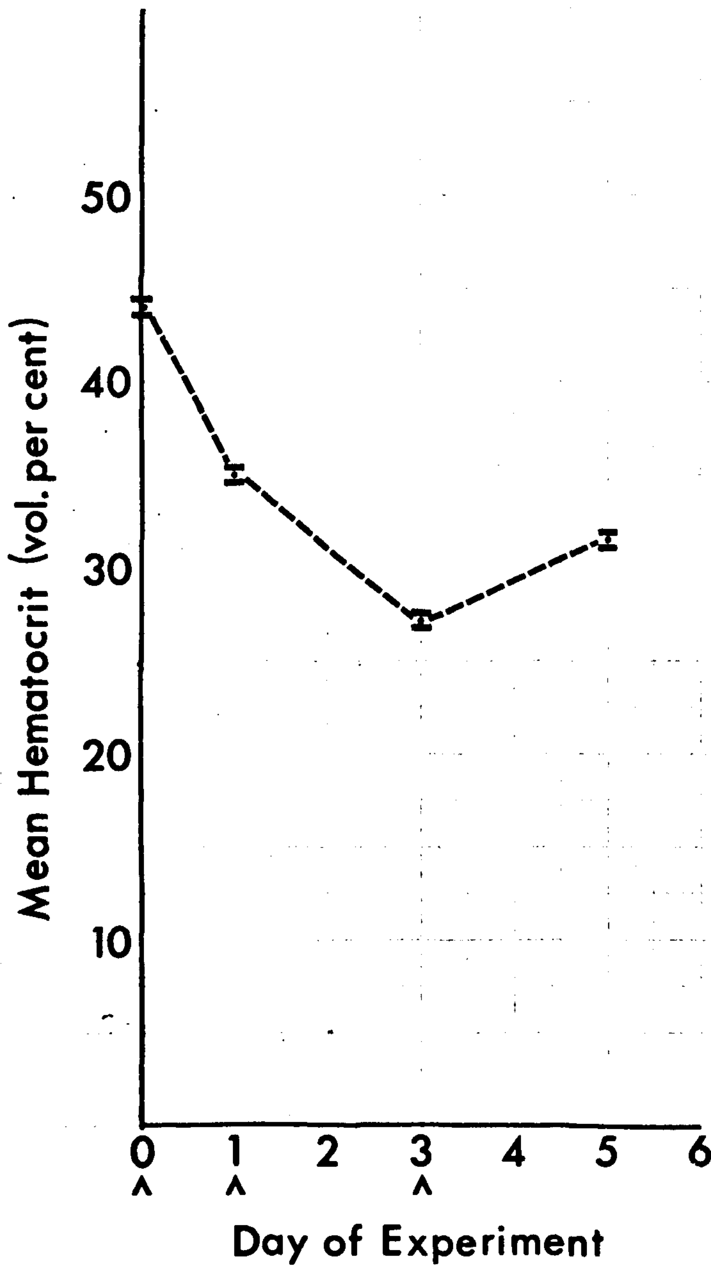
Effect of Three Intraperitoneal Injections of Phenylhydra-zine on Per Cent Reticulocytes in Peripheral Blood Over a Six Day Experimental Period. Arrowheads indicate days of injection.



Per Cent Reticulocytes in Peripheral Blood
10
20
30
40
50
Day of Experiment
0
1
2
3
4
5
6

Figure 23

Effect of Three Intraperitoneal Injections of Phenylhydrazine on Hematocrit Over a Five Day Experimental Period. Arrowheads indicate days of injection.



Mean Hematocrit (vol. per cent)
50
40
30
20
10
0
1
2
3
4
5
6
Day of Experiment

Figure 24

Effect of Three Intraperitoneal Injections of Phenylhydrazine on Peripheral Red Blood Cell Count Over a Six Day Experimental Period. Arrowheads indicate days of injection.

10 X 10 TO ½ INCH 46 1331
KEUFFEL & ESSER CO.

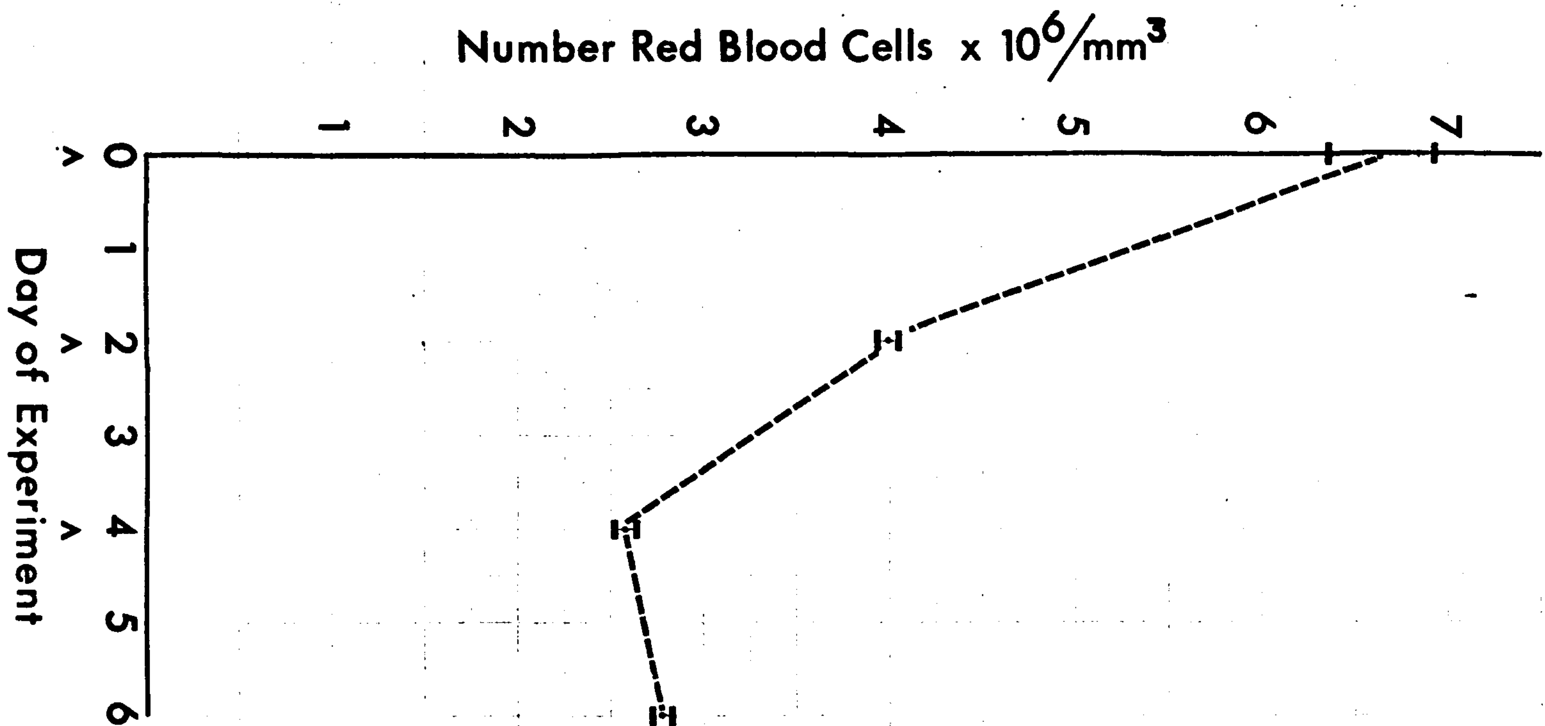
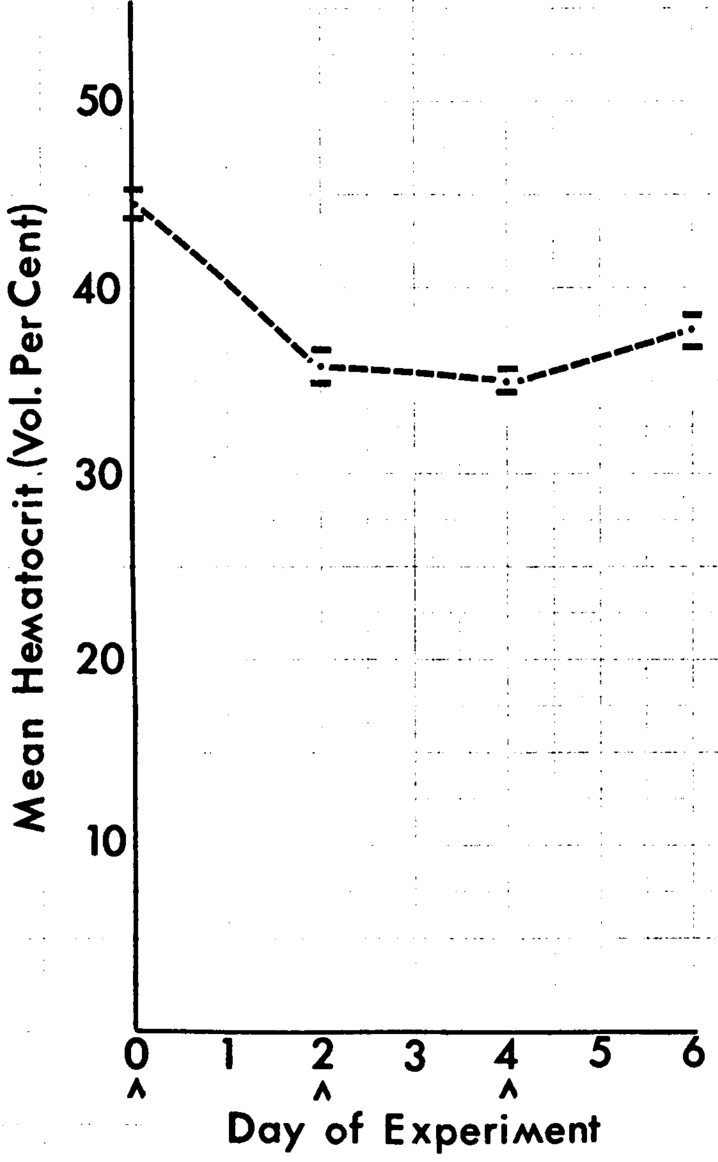


Figure 25

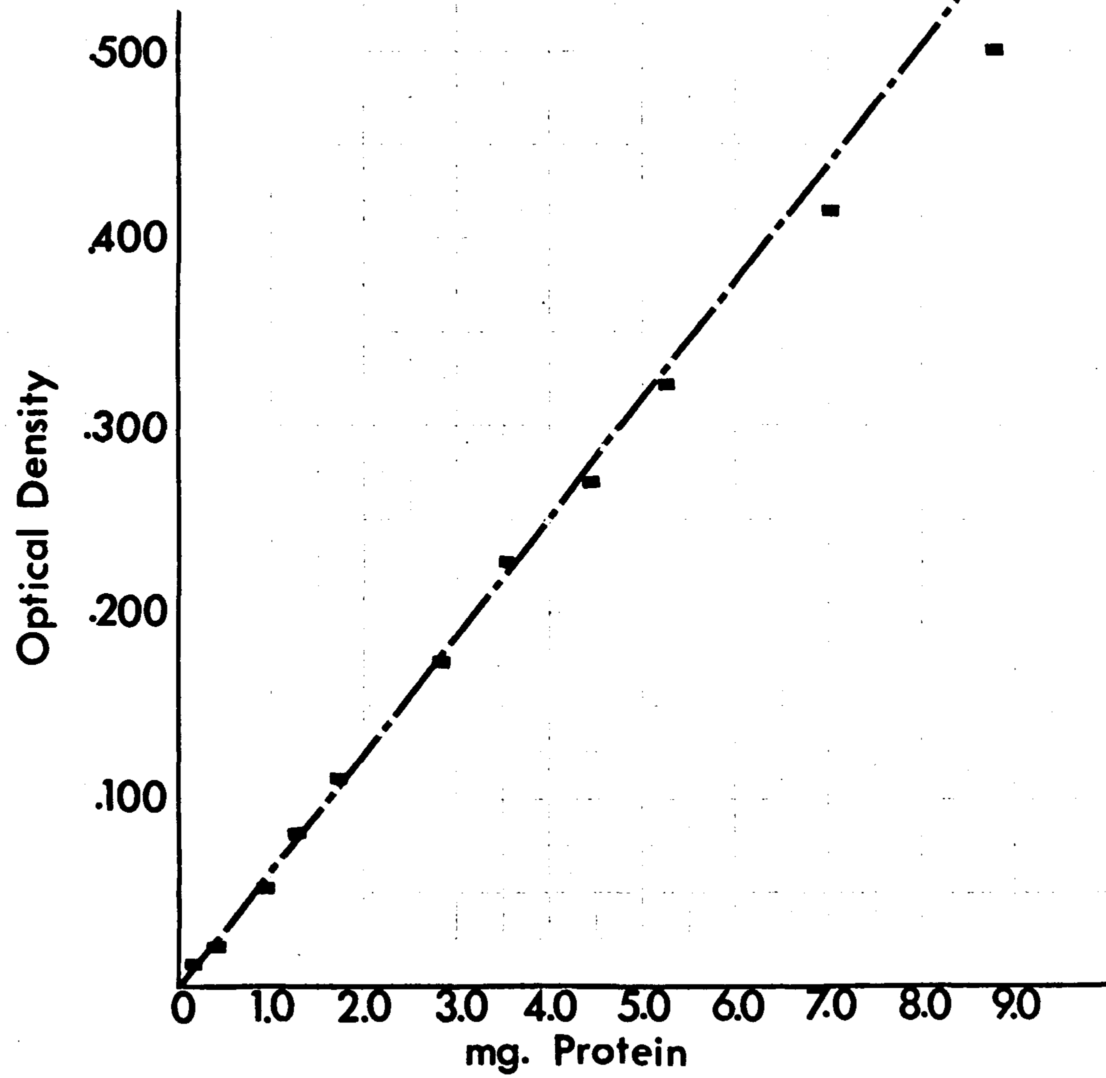
Effect of Three Hemorrhage Episodes on Hematocrit Over Six Day Experimental Period. Arrowheads indicate days of bleeding.



10 X 10 TO ½ INCH 46 1331
7 X 10 INCHES MADE IN U.S.A.
KEUFFEL & ESSER CO.

Figure 26

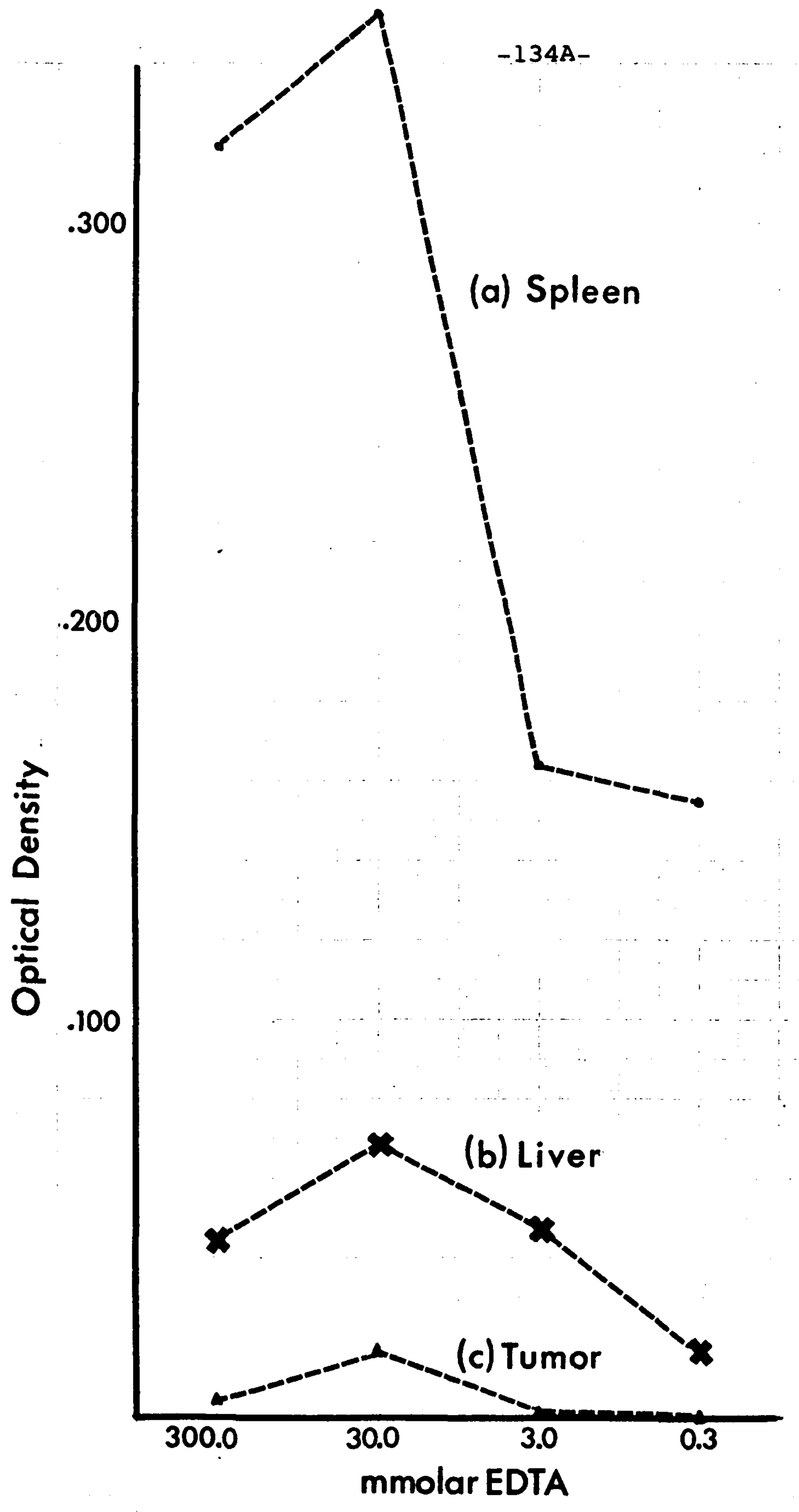
Biuret Standard Curve (BSA). λ = 550nm.



.500
.400
.300
.200
.100
Optical Density
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
mg. Protein

Figure 27

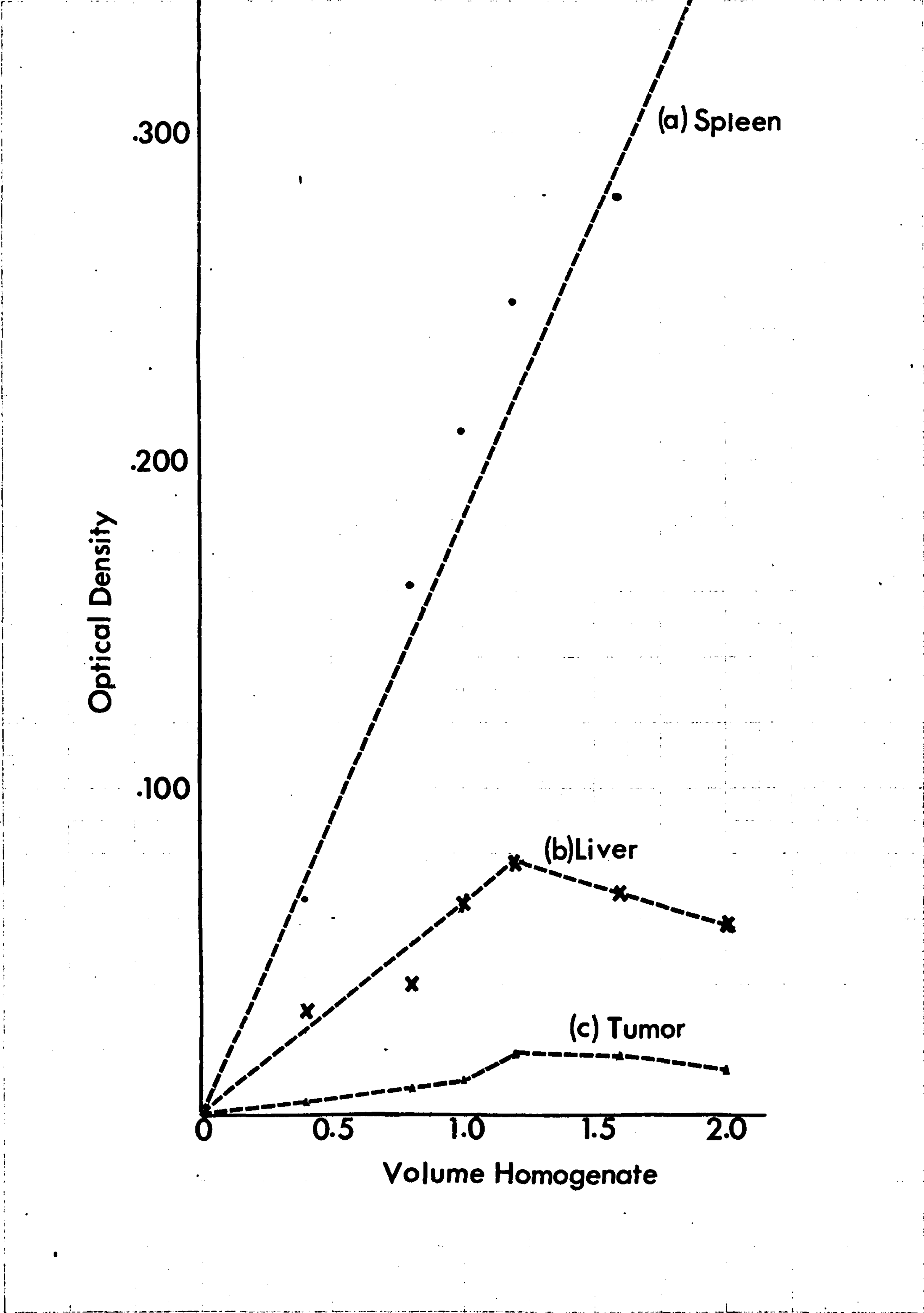
Optimalization of ALA-S Assay: EDTA Concentration.
(a) Spleen Homogenate
(b) Liver Homogenate
(c) Tumor Cell Homogenate



.300
.200
.100
Optical Density
(a) Spleen
(b) Liver
(c) Tumor
300.0
30.0
3.0
0.3
mmolar EDTA

Figure 28

Optimalization of ALA-S Assay: Volume Homogenate.
(a) Spleen Homogenate
(b) Liver Homogenate
(c) Tumor Cell Homogenate



.300
.200
.100
Optical Density
(a) Spleen
(b)Liver
(c) Tumor
0
0.5
1.0
1.5
2.0
Volume Homogenate

Figure 29

Optimalization of ALA-S Assay: Time Incubation.
(a) Spleen Homogenate
(b) Liver Homogenate
(c) Tumor Cell Homogenate

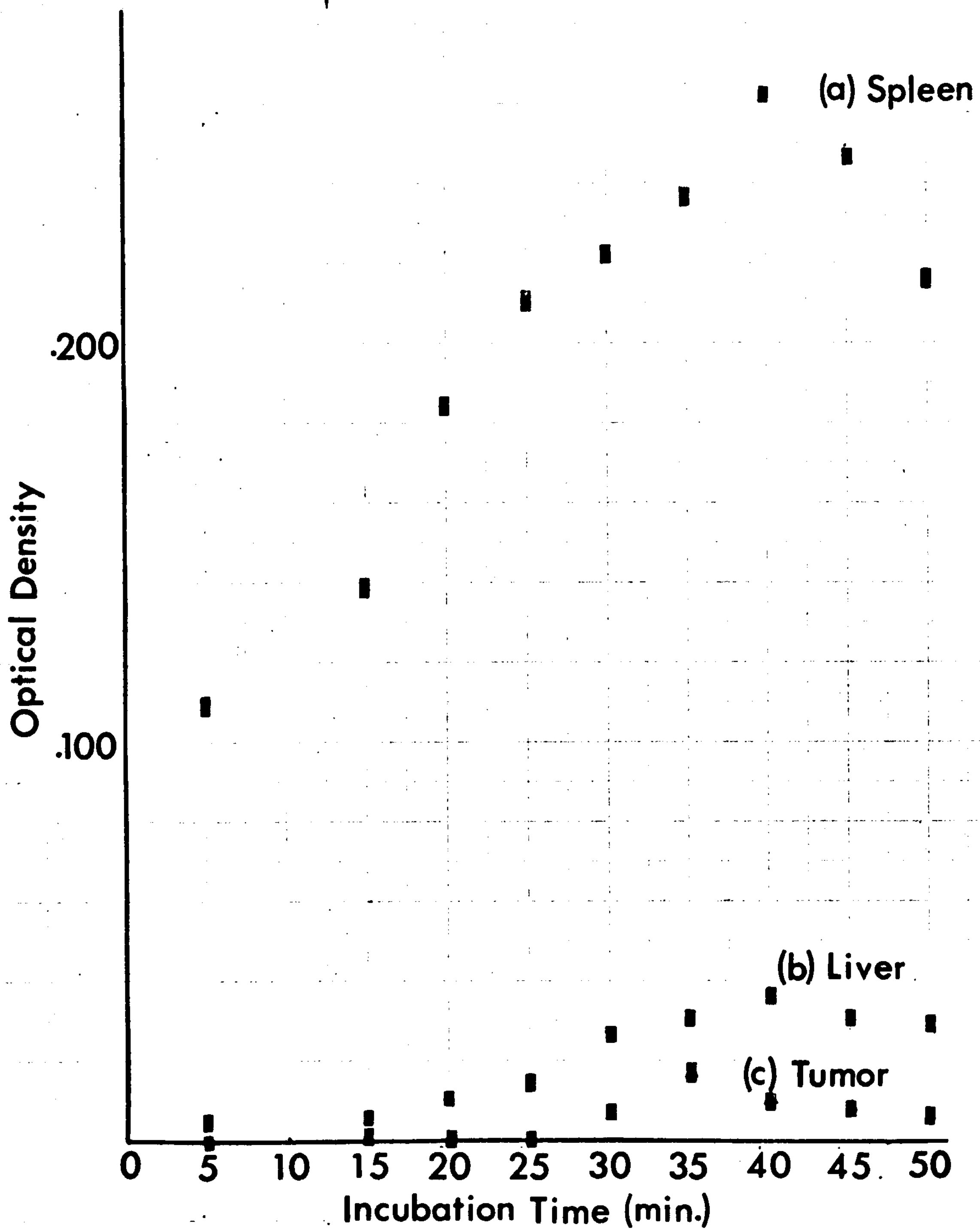

(a) Spleen
.200
Optical Density
.100
(b) Liver
(c) Tumor
0
5
10
15
20
25
30
35
40
45
50
Incubation Time (min.)

Figure 30

Effect of Miniaturization to Twenty-Five Per Cent Original Volumes on Optical Density Generated by Changing Volumes Homogenate in ALA-S Assay.

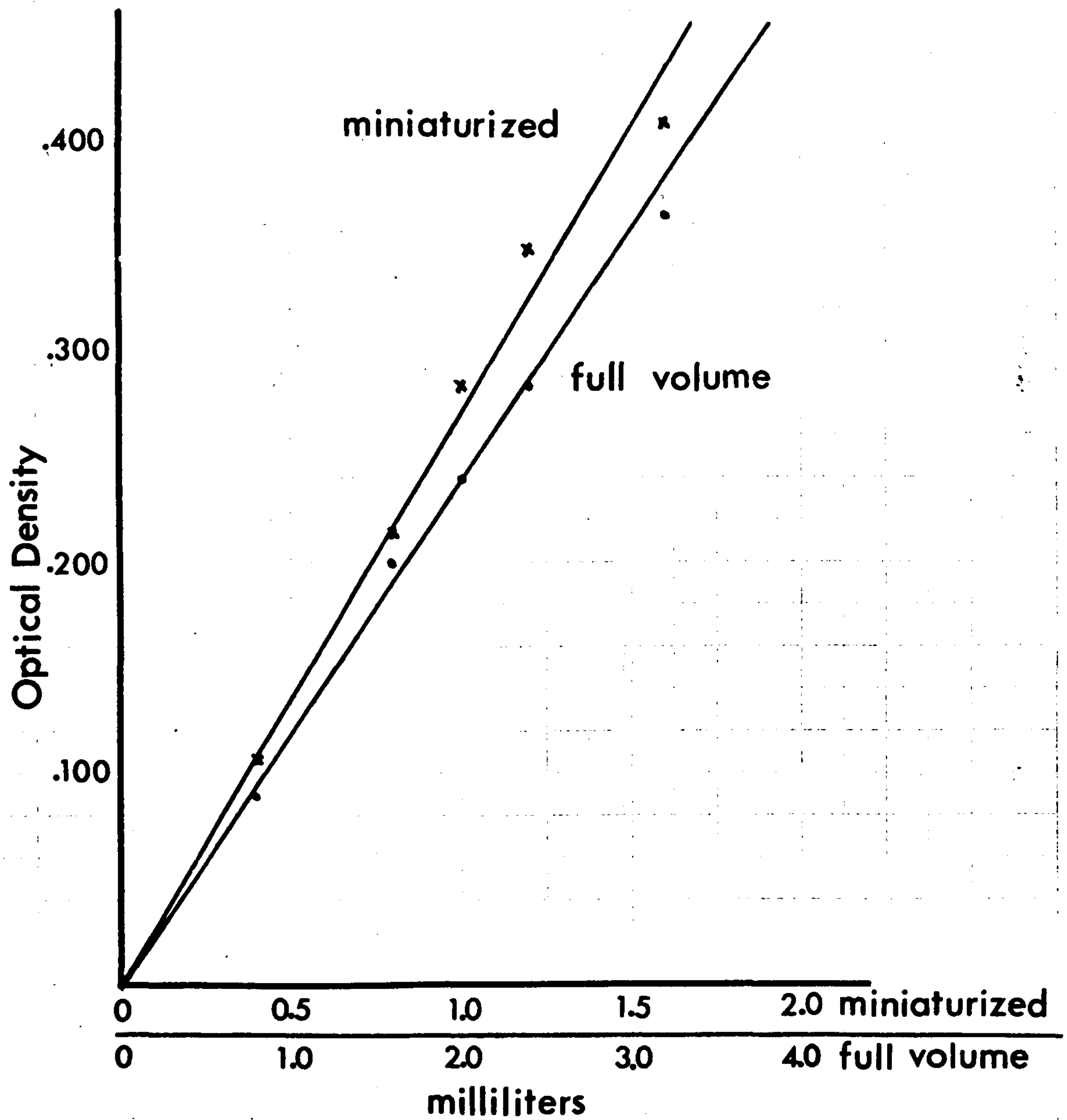

miniaturized
full volume
Optical Density
.400
.300
.200
.100
0
0.5
1.0
1.5
2.0 miniaturized
0
1.0
2.0
3.0
4.0 full volume
milliliters

Table 1

Protein Content of Normal, Leukemic Spleens and Liver and Tumor Cell Homogenates.

No. Samples	Type Cell Homogenate	Mean Per Cent Protein (±SE)*	Mean $\frac{\text{mg wet weight}}{\text{mg protein}}$
12	Normal Spleen	14.0 ± 0.2	7.14
12	Normal Liver	16.9 ± 0.6	5.92
39	Leukemic Spleen	14.4 ± 0.3	6.94
39	Leukemic Liver	17.6 ± 0.4	5.68
8	Tumor Cells	14.7 ± 0.7	6.80

*SE = Standard Error

Table 2

Effect of Per Cent Splenic Myeloblasts on Splenic Erythroid Compensation. SA = Specific Activity, pmoles ALA/mg prot./hr.; NA = Net Activity, nmoles ALA/mg prot./hr.

% SPLENIC MYELOBLASTS	SUBFILE 1 0-2.9 % Benz.-Pos. Cells			SUBFILE 2 3.0-9.9 % Benz.-Pos. Cells			SUBFILE 3 10.0-19.9 % Benz.-Pos. Cells			SUBFILE 4 20.0-50 % Benz.-Pos. Cells		
	No. Cases	SA*	NA*	No. Cases	SA*	NA*	No. Cases	SA*	NA*	No. Cases	SA*	NA*
0-10	1	20.9	5.1	1	28.5	7.4	11	190.1 (±15.8)	52.7 (±7.5)	13	422.0 (±42.3)	119.5 (±15.9)
11-19	0	-	-	2	88.4 (±27.5)	33.4 (±19.7)	2	150.0 (±32.0)	64.1 (±19.8)	0	-	-
20+	5	24.0 (±2.9)	7.2 (±1.8)	13	90.9 (±9.0)	48.3 (±5.2)	0	-	-	0	-	-

NORMAL SPLEEN:

SA*	NA*
57.3 (±5.2)	7.2 (±1.0)

*(± Standard Error)

Table 3

Effect of Hemolytic and Hemorrhagic Anemias on Hematocrit, Per Cent Benzidine-Positive Cells in the Bone Marrow and Spleen, and Spleen and Liver Weights. Values presented are from Day of Assay.

TYPE TREATMENT	BONE MARROW $\bar{m}$ % benz.-posit. cells (± SE)*	SPLEEN $\bar{m}$ % benz.-posit. cells (± SE)	Hematocrit	weight spleen (g)	weight liver (g)
Phenyl- hydrazine:	31.6 (± 1.2)	29.5 (± 1.0)	31.6 (± 0.3)	2.9 (±0.1)	8.8 (±0.2)
Hemorrhage:	29.9 (± 1.2)	10.5 (± 1.0)	37.7 (± 0.8)	1.2 (±0.1)	8.5 (±0.2)
Normal:	16.8 (± 0.9)	0.4 (± 0.2)	47.2 (± 0.7)	0.9 (±0.01)	9.2 (±0.3)

*SE = Standard Error

Table 4

Effect of Hemolytic and Hemorrhagic Anemia on Specific and Net Activities of ALA in Spleen and Liver.

TYPE OF TREATMENT	SPLEEN $\bar{m}$ Spec. Activ. ALA-S ($\pm$SE)* pmoles/mg prot/hr.	SPLEEN $\bar{m}$ Net Activ. ALA-S ($\pm$SE) mumoles/spleen/hr.	LIVER $\bar{m}$ Spec. Activ. ALA-S ($\pm$SE) pmoles/mg prot/hr.	LIVER $\bar{m}$ Net Activ. ALA-S ($\pm$SE) mumoles/liver/hr.
phenylhydrazine	627.2 ($\pm$39.4)	257.4 ($\pm$10.9)	91.1 ($\pm$7.1)	146.5 ($\pm$11.5)
hemorrhage	275.0 ($\pm$40.2)	46.3 ($\pm$9.6)	93.9 ($\pm$9.8)	145.2 ($\pm$17.4)
normal	57.3 ($\pm$5.2)	7.2 ($\pm$1.0)	81.4 ($\pm$9.0)	135.3 ($\pm$15.2)

*SE = Standard Error

Table 5

Spectrophotometric Analysis of BSA Stock Solution: Calculation of BSA Concentration.

Wavelength: (nm)	260	280	320	260-320	280 320	$\frac{280}{260}$
Optical Density:	0.364	0.605	0.016	0.348	0.589	1.69

$$x = (OD_{280}) - (OD_{320}) \times 20$$

and

$$y = \frac{x}{6.7} \times 10$$

where: y = actual BSA ;
6.7= extinction coefficient BSA, E^{550}_{icm}

$$x = 0.589 \times 20 = 11.78$$

$$y = 11.78 \times 10$$

$$= 17.58 \text{ mg BSA/ml}$$

Table 6

Optical Densities of Standard Solutions for Standard Curve.

	Volume BSA Stock (ml)	Volume H_2O (ml)	Volume Biuret Rgt. (ml)	Optical Density (A_{550})	mg. protein
1-	0.01	0.99	4.00	0.011	0.18
2-	0.02	0.98	4.00	0.021	0.35
3-	0.05	0.95	4.00	0.054	0.88
4-	0.07	0.93	4.00	0.082	1.23
5-	0.10	0.90	4.00	0.121	1.76
6-	0.15	0.85	4.00	0.175	2.84
7-	0.20	0.80	4.00	0.225	3.52
8-	0.25	0.75	4.00	0.268	4.40
9-	0.30	0.70	4.00	0.322	5.27
10-	0.40	0.60	4.00	0.415	7.03
11-	0.50	0.50	4.00	0.500	8.79
blank-	0.00	1.00	4.00	---	--

linear regression: O.D. v. mg prot./ml:

$r = +0.998$

$m = 17.347$

$b = -0.155$

Table 7

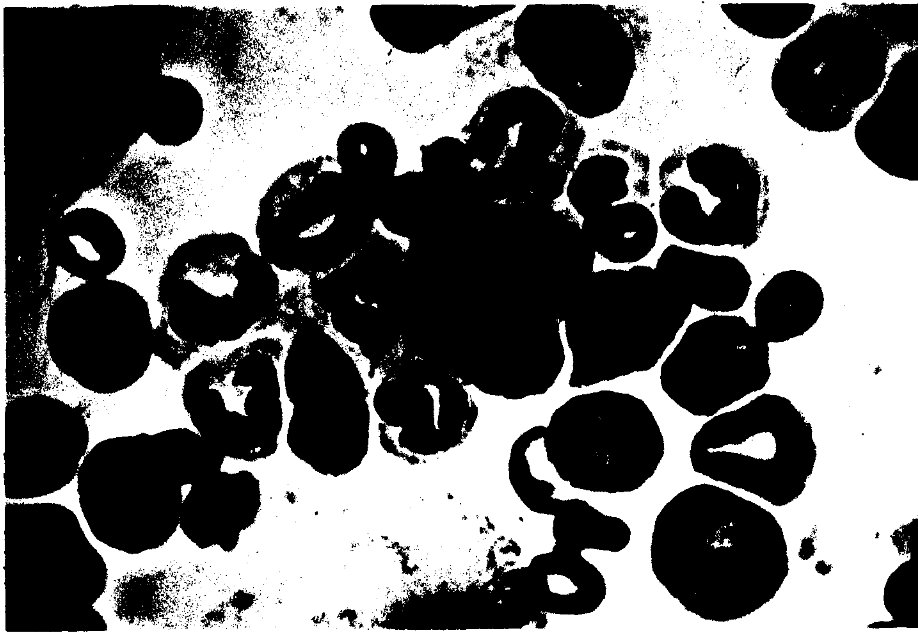
Comparison of R_f Values and Differential Staining of Standard and Experimental pyrroles.

	SOURCE OF MATERIAL SPOTTED:	Rf	Ninhydrin Reaction	E.R. Reaction	No. times spotted
I. STANDARD REFERENCE	(a) synthetic ALA-p	0.20	-	+	5-10
	*(b) synthetic AA-p	0.93	-	+	
II. EXPERIMENTAL	(a) phenylhydrazine-spl.	0.20	-	+	3-5
	(b) normal spleen	0.21	-	+	15-30
	**(c) leukemic spleen	0.29	-	+	15-30
	(d) phenylhydrazine-liver	0.20	-	+	15-30

*Literature value (Mauzerall and Granick, 1956)
**High per cent Benzidine-positive samples

PHOTO 1

Bone Marrow: (a) Normal Smear; (b) Leukemic Smear, Leukemic Myeloblasts predominate. Magnification: X 1600.

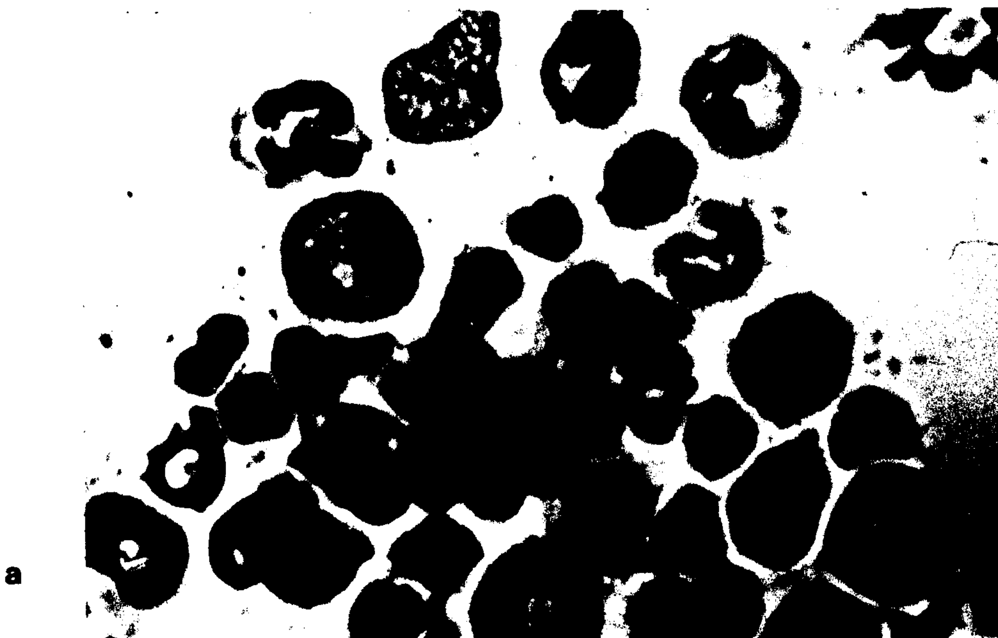


a

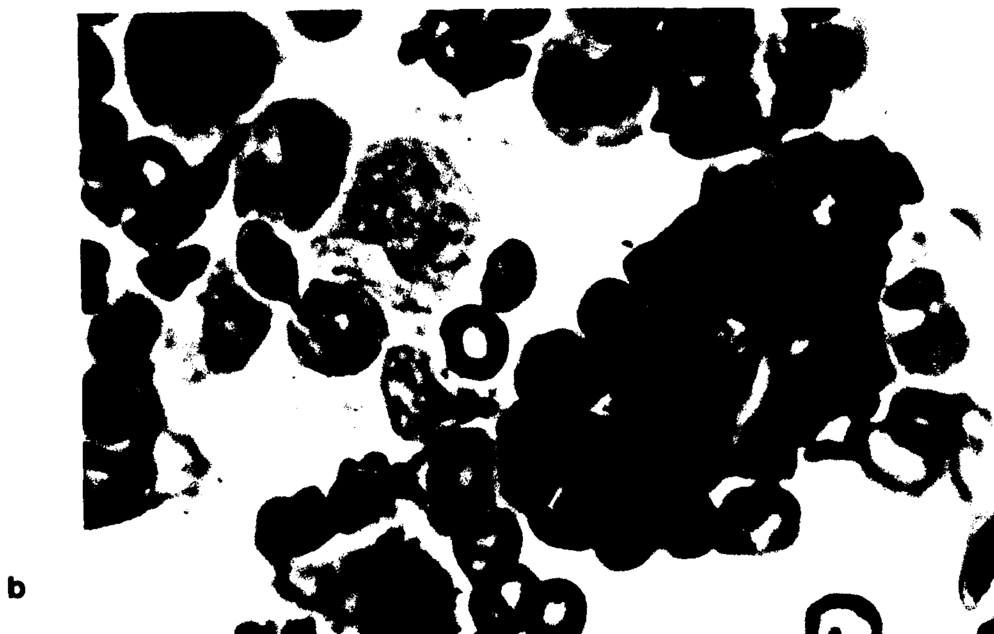
b

PHOTO 2

Bone Marrow: (a) Phenylhydrazine-treated Rat; (b) Bled Rat Erythroid nests can be seen in (a) and (b). Magnification: X 1600.



a



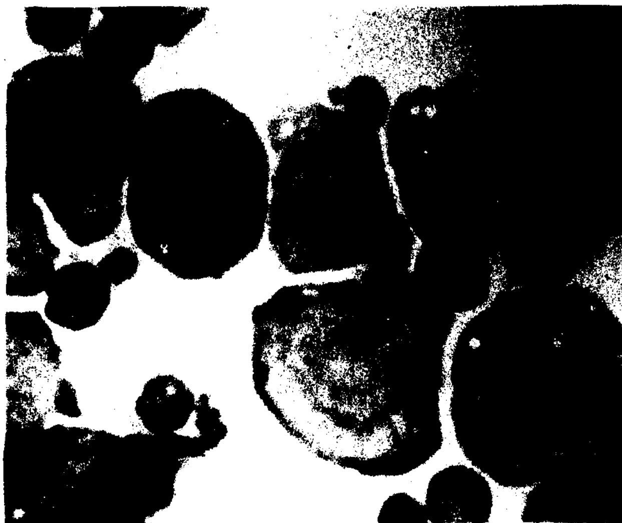
b

PHOTO 3

Spleen: (a) Normal Smear; (b) Leukemic Smear, Erythroid nest; (c) Leukemic Smear, Leukemic myeloblasts predominate. Magnification: X 1600.

a

b



c

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APPENDIX: FOOTNOTES

[1] Simenson Labs, Gilroy, Calif.

[2] Marland Breeding Farms, Hewett, N. J.

[3] Ralston Purina Co.

[4] SterilGARD Hood, Baker Co.

[5] Spencer Bright-Line

[6] Eastman Chemical

[7] Fisher

[8] Harleco

[9] Chase Instrument Co.

[10] Adams Autocrit Centrifuge

[11] Model B

[12] Thomas

[13] Labline, Inc.

[14] Precision Scientific

[15] International Clinical Centrifuge

[16] BioRad Labs., Richmond, Calif.

[17] Beckman, Model DUR

[18] CalBiochem., Grade A

[19] Sigma

[20] Schwartz-Mann

[21] National Appliance

APPENDIX A

BENZIDINE/WRIGHT'S-GIEMSA STAIN PROCEDURE:

1. Slides were placed in staining rack and immersed in staining jar containing 1% 3,3'-dimethoxybenzidine (aged 2-4 days) for 2 minutes.

2. Rack and slides were drained on toweling and quickly immersed in 7½% H_2O_2 for 1 minute.

3. Rack and slides were drained on toweling and rinsed in distilled water for 1 minute.

4. Slides were individually drained and placed in a horizontal position on rack.

5. Undiluted Wright's stain, sufficient to cover the slide uniformly, was added for 1 minute.

6. Approximately 2½ ml of phosphate buffer (Giordano) PH 6.4-6.5 was added to the slide containing Wright's stain and was mixed by blowing on slide. The diluted stain remained on the slide for 3 minutes.

7. While in a horizontal position, the diluted Wright's stain was rinsed off with tap water. The horizontal position prevented the precipitation of metallic elements on the smear.

8. Excess water was drained from the slide; slides were again placed in horizontal position and flooded with freshly prepared Giemsa stain (1/10 dilution of stock in distilled water) for 3¼ minutes.

9. Stain was rinsed as in step (7) above.

APPENDIX B

NEW METHYLENE BLUE STAINING PROCEDURE:

1. An equal volume of blood and stain is drawn into a plain capillary tube. The mixture is expelled onto a square of Parafilm and mixed thoroughly.

2. The mixture is drawn into the capillary tube and allowed to remain there for 10 minutes.

3. At this time, the sample is again mixed, and a drop is placed on a slide for smearing.

APPENDIX C

HOMOGENIZING SOLUTION: pH = 7.4

One liter contains:

9.0 g. NaCl
1.21 g. Trizma Base
0.15 g. $EDTA\text{-}Na_2$

made to volume with distilled H_2O

APPENDIX D

INCUBATION SOLUTION: pH = 7.2

Each 50 ml incubation flask contains to following:

1. In the spleen:

   0.4 ml 1M glycine (Fisher)
   1.2 ml solution containing 0.27 M Tris (Sigma) with
   0.3 M $EDTA\text{-}Na_2$ (Sigma)
   0.4 ml distilled water
   2.0 ml 25% spleen homogenate
   4.0=Total Volume

2. In the liver:

   0.4 ml 1M glycine
   1.2 ml solution containing 0.27 M Tris (Sigma) with
   0.3 M $EDTA\text{-}Na_2$ (Sigma)
   1.2 ml distilled water
   1.2 ml 25% liver homogenate
   4.0 = Total Volume

APPENDIX E

MODIFIED EHRLICH'S REAGENT:

Per 50 ml:

1 g diethylaminobenzaldehyde (Eastman)
10 ml 60% perchloric acid (Baker)
10 ml 1.5% $HgCl_2$ in glacial acetic acid (Fisher)
Bring to volume (50 ml) with glacial acetic acid.

APPENDIX F

BIURET REAGENT:

1.5 g. of cupric sulfate ($CuSO_4$-$5H_2O$) and 6.0 g. of sodium tartrate ($NaKC_4H_4O_6$-$4H_2O$) were dissolved in 500 ml water. With constant swirling, 300 ml of 10% sodium hydroxide (prepared from stock, carbonate-free, 65 to 75% sodium hydroxide solution) was added. The solution was diluted to 1 liter with water and stored in a polyethylene bottle.

(from: Layne, 1957, Methods in Enzymology, v. III, pp. 450-1.)

APPENDIX G

ORGANIZATION OF THE DATA:

Previous investigations on the SCL in this laboratory have been analyzed using per cent bone marrow myeloblast as an independent variable, with other histological paramenters regarded as secondary and dependent on it. This has been most essential in characterizing the progression of the leukemia and in correlating such parameters with the pathogenesis. In the present study, the data presented is organized according to increasing per cent splenic benzidine-positive cells. Justification for this arrangement, rests with the fact that the thrust of the present investigation focuses, primarily, on the phenomenon of splenic erythroid compensation, per se, and not directly on bone marrow myeloblast content, though clearly, it is a vital parameter in this study.

A cursory inspection of the data reveals the existence of 4 natural groups, or subfiles, each possessing a fairly characteristic histological pattern. Accordingly, the 4 subfiles with their general histological relationships are presented below:

SUBFILE	% spleen benz. +	% spleen myelobl.	% bone marrow benz. +	% bone marrow myelobl.
1*	0-2.9	(a) gener. low (b) high	(a) gener. normal (b) very low	(a) low (b) moderate
2	3.0-9.9	high	generally very low	generally high
3	10.0-19.9	low	moderate-very low	mixed but gener. low
4	20.0 +	low	very low	mixed

*Subfile 1 appears to be composed of 2 populations: (a) fairly normal, early or preleukemic animals; (b) animals exhibiting advanced pathology.