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ERYTHROPOIESIS IN THE RAT: THE EFFECT OF ERYTHROPOIETIN ON RNA SYNTHESIS BY NORMAL AND LEUKEMIC HEMATOPOIETIC TISSUES, *IN VITRO*

by

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

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:

RNA synthesis has been studied in normal and leukemic hematopoietic tissues. The rat bearing the Shay chloroleukemia was the animal model system used in this study. This leukemia is an acute myelogenous leukemia that closely parallels the acute leukemia in humans. The course of the disease is monitored by an increase in the percent myeloblasts and a decrease in the percent of benzidine positive cells in the bone marrow. Heme synthesis shuts off very early in the course of the disease; an earlier event is probably responsible for this breakdown. Stimulation of RNA synthesis has been established to be the earliest response to erythropoietin in hematopoietic cell cultures and is therefore a reasonable candidate for a critical role in the malfunction of erythropoiesis in the leukemic state. Comparative studies measured the influence of erythropoietin on bone marrow and spleen cell suspensions.

In early stages of the disease when the percent of myeloblasts in the bone marrow is low, erythropoietin stimulated RNA synthesis, but the level of stimulation was below normal. The loss of responsivity to erythropoietin correlated with both the percent myeloblasts in the bone marrow and the length of time after seeding of these elements in the hematopoietic compartment. In late stages of the disease, when the

percent of myeloblasts in the bone marrow was over 50% there was no longer any response to the hormone.

Pure populations of leukemic myeloblasts in culture exhibited no stimulation of RNA synthesis in response to the hormone. These cell populations, however, did display a small but consistent depression of RNA synthesis in the presence of erythropoietin. Possible reasons for this are discussed.

Compensatory erythropoiesis occurs in the leukemic rat spleen. RNA synthesis in spleen cell suspensions from both normal and leukemic animals did not show a response to erythropoietin. It is conceivable that these cells do not operate via the same triggering mechanism as bone marrow cell suspensions.

When RNA was isolated from normal and leukemic bone marrow, differences appeared only when the average specific activity ($\Sigma cpm/\Sigma OD_{260nm} X10^3$) was calculated for each region of the sucrose density gradient. RNA from normal bone marrow displayed an increase in average specific activity predominantly in the 45S and 8-10S regions. Average specific activity patterns of RNA isolated from leukemic bone marrow was different from normal but no predictable pattern emerged. The normal bone marrow tissue seems to operate as a coordinately controlled system and the leukemic state perturbs this condition.

INTRODUCTION

Cell proliferation is perhaps one of the least understood areas of biology. Neoplasia usually represents a lesion in the normal pattern of differentiation and its study helps in an appreciation of normal control mechanisms. Various model systems exist to study these regulatory mechanisms. The one used in this study is a rat acute myelogenous leukemia, known as the Shay chloroleukemia. Leukemia is a severe malfunction at some step in the complex sequence of differentiation in blood forming tissues. It is through the comparative study of specific regulatory events in normal and leukemic tissues that the mechanisms involved in hemic cell differentiation can be further elucidated.

I. PATHOGENESIS OF THE SHAY CHLOROLEUKEMIA:

The pathology accompanying acute myelogenous leukemia (AML) in the rat is an ideal model system for the study of hematopoiesis. The Shay chloroleukemia, pathologically similar to human AML, was initially induced in rats by gastric instillation of 2-methylcholanthrene (Shay *et al.*, 1951; 1952). Intravenous inoculation of Shay chloroleukemic cells into the Long-Evans rat results in a predictable alteration of medullary histology (Handler and Handler, 1970). Leukemic myeloblasts can be detected in the bone marrow

from 3 to 5 days after the initial injection of leukemic myeloblasts. The bone marrow pathology is characterized by a progressive decrease in cellularity (Varsa et al., 1965), increase in percent myeloblasts (Handler et al., 1968) and decrease in normal myeloid and erythroid elements (Handler et al., 1968). Hepatosplenomegaly occurs in late stages of the disease (Handler and Handler, 1970). The progressive increase in percent bone marrow myeloblasts remain the most constant parameter of this leukemia and thus has been used in staging the course of the leukemia (Varsa et al., 1965; Handler and Handler, 1970). Infiltration and/or proliferation of leukemic myeloblasts in the spleen follows a less predictable pattern.

II. THE ROLE OF ERYTHROPOIETIN:

- PHYSIOLOGY OF RED CELL PRODUCTION

Erythropoietin is believed to be the triggering hormone in red cell development. Early in the century a humoral factor was postulated by Carnot and Deflandre (1906) to control red blood cell production. Since then erythropoietin (EPO) has been found to be a glycoprotein hormone (Goldwasser and Kung, 1968) which is produced primarily in the kidney (Jacobson et al., 1957) and induces differentiation of an EPO responsive stem cell (ERC) in the bone marrow (Till and McCulloch,

1961). The hormone produces a rapid release of reticulocytes from marrow into the peripheral blood (Gordon _et al._, 1962) and increases the red cell mass after prolonged injections (Schooley, 1965). EPO also acts to increase the number of erythroid precursor cells in bone marrow resulting in increase in iron incorporation into heme in newly formed red blood cells (Gordon and Zanjani, 1970; Hodgson, 1970).

The hormone also stimulates the proliferation rate of erythroblasts, the result of which is to shorten the mean transit time of the proliferating erythroblast compartment and has a direct reticulocyte releasing effect measurable in perfused limb preparations (Hodgson, 1970).

- CELL DIFFERENTIATION

At the level of cell differentiation, EPO is thought to act on an erythropoietin responsive cell. The ERC was originally proposed by Gurney (1965) and believed to be derived from a multipotential stem cell. A model of the postulated events occuring amongst the stem cell pools has been proposed by Stohlman (1970). The differentiated erythroid cell is not self sustaining and must be replenished from a precursor or pluripotential stem cell compartment which is responsive to population size. Intermediate stem cell compartments committed to granulopoiesis, erythropoiesis and megakaryocytopoiesis are interposed between the pluripotential stem cell

compartment and the differentiated cell compartment. These committed stem cell compartments are self-sustaining. Influx from the pluripotential stem cell compartment is important in maintaining the committed stem cell pool in the normal state as well as during physiological stress (Stohlman, 1970).

III. THE EFFECT OF ERYTHROPOIETIN ON MACROMOLECULAR SYNTHESIS:

- PROTEIN SYNTHESIS

Hemoglobin synthesis has been found to be the most reliable and convenient measurement of a response to EPO. ^{59}Fe incorporation into heme has been shown to be an index of hemoglobin production with over 90% of the assessed heme produced being attributable to hemoglobin synthesis (Gallien-Lartigue and Goldwasser, 1964). ^{59}Fe incorporation has been studied in mammalian bone marrow systems including rat (Krantz *et al.*, 1963; Hrinda and Goldwasser, 1969), dog (Ward, 1967), human (Krantz, 1965), in mouse fetal liver (Cole and Paul, 1966) and in mouse yolk sac (Cole and Paul, 1965). In all cases studied incorporation of ^{59}Fe has been demonstrated to increase in response to the hormone. Chui *et al.* (1971) using mouse fetal liver have shown that the EPO stimulated increase in hemoglobinized cells is due to an increase in the number of cells synthesizing hemoglobin. The hormone

acts to maintain the immature proerythroblast and basophilic erythroblast and to increase the immature population of erythroid cell presursors *in vitro*.

EPO has been shown to increase the synthesis of proteins other than hemoglobin. Dukes and Goldwasser (1965) demonstrated an increase of ^{14}C-glucosamine into stroma of rat bone marrow cells. The synthesis of delta-aminolevulinic acid synthetase, possibly the rate limiting enzyme in heme formation, is enhanced by the presence of the hormone in rabbit bone marrow culture (Bottomley and Smithee, 1969).

- DNA SYNTHESIS

During what phase of the cell cycle EPO exerts its effect is still an open question. Paul and Hunter (1968; 1969) based on observations obtained with a mouse fetal liver system, reported that DNA synthesis was a necessary prerequisite for increase in hemoglobin synthesis following EPO. Their conclusion was based on the use of 5-fluorouracildeoxyriboside (FUDR) which completely inhibited the effect of EPO stimulated heme synthesis. Gross and Goldwasser (1970) demonstrated that early in the incubation period, rat bone marrow cells do not require DNA synthesis or cell division in order to express short term EPO stimulated ^{59}Fe uptake or hemoglobin synthesis. Using erythropoietically repressed spleens from polycythemic mice, Orlic and Gordon (1972) measured the mitotic indices and suggested

that the period of DNA synthesis might also be the period of EPO sensitivity.

- RNA SYNTHESIS

RNA synthesis has been demonstrated to be an early response, possibly the initial response, to the hormone in rat bone marrow (Krantz and Goldwasser, 1965) and in mouse fetal liver (Djaldetti *et al.*, 1972). It was postulated that the hormone might act to induce mRNA synthesis and thereby initiate new protein synthesis. Gross and Goldwasser (1969; 1971) reported the appearance of a 150S peak on sucrose density gradient analysis unique to EPO stimulation of rat bone marrow cultures. This same group also reported enhancement of synthesis of 9S, 55-65S, 45S and 4-6S RNA. Terada *et al.* (1972) with a preparation of purified populations of EPO responsive precursor cells from mouse fetal liver demonstrated on sucrose density gradients that within one hour in culture the increase in RNA synthesis involves HnRNA, 45S and 4S, and subsequently 28S and 18S. No detectable stimulation in the synthesis of a 10S RNA occurs at this time. In addition, no biologically active mRNA for globin was recoverable from these cells prior to incubation with EPO.

- POSSIBLE SURFACE PHENOMENON

There is some implication that EPO has its primary effect on the cytoplasmic membrance of marrow cells. Chang *et al.*, (1974) found that when rat marrow cells

were exposed to trypsin, and incubated in the presence of cycloheximide, increased uridine incorporation observed with EPO was completely lost. When protein synthesis was permitted to occur, EPO stimulation approached normal levels. This suggested a protein receptor on the external surface of the ERC.

IV. ANEMIA ACCOMPANYING LEUKEMIA:

Anemias accompany human leukemias (Dameshek and Gunz, 1964) as well as a variety of neoplasms (Krantz and Jacobson, 1970).

The changing hematological pattern associated with the development of the Shay chloroleukemia in rats is an apparent elevation in circulating levels of EPO (Handler and Handler, unpublished observations) and is suggestive of a functioning EPO generating system. This parallels findings in human leukemias where high levels of circulating EPO have been reported (Krantz and Jacobson, 1970).

In cases of human chromic lymphocytic leukemia (CLL) and acute lymphocytic (ALL) and myeloblastic leukemia (AML) a reciprocal relation was found between the serum EPO level and erythropoiesis. These leukemias appeared to be associated with anemia because of a primary failure of the marrow to respond to the hormone (Thorling, 1965).

The stem cell has been implicated as the site of the erythropoietic lesion in leukemia, in rat (Hoelzer and Harriss, 1973), mouse (Lajtha, 1973) and human (Killman, 1968). Recently Chiyoda et al. (1974) reported that in bone marrow from patients with acute lymphocytic leukemia (ALL) there was a response to EPO as expressed by a measure of heme synthesis. Some degree of synthesis was maintained even though the number of erythroblasts was low compared to normal values thus indicating that the marrow cells retain the capacity to respond to the hormone. Similar studies on patients with AML failed to show a response to the hormone in vitro.

In some mouse leukemias EPO levels are depressed. Plasma levels of mice infected with Rauscher leukemia virus (RLV) and Friend leukemia virus (FLV) indicate a depression (Ebert et al., 1972) or a total absence of EPO (Mirand, 1967) in the blood. It has been suggested that these murine viruses induce erythropoiesis independent of normal EPO production (Mirand, 1967). Recent evidence indicates that in Rauscher leukemia both EPO production and clearance are accelerated (OKunewick and Erhard, 1974). Of interest is the recent observation that in vitro, Friend leukemia cells which have been induced to differentiate by dimethylsulfoxide (DMSO) will respond to EPO as evidenced by a further stimulation of heme synthesis (Preisler and Giladi, 1975).

V. RNA SYNTHESIS IN LEUKEMIA:

Much of the information concerning RNA synthesis of human leukemia derives from experiments with lymphocytes and lymphoblasts from peripheral blood. Wulff et al. (1972) studied RNA from lymphocytic cells of patients with ALL, infectious mononucleosis and normal healthy donors. Although the overall optical density profiles of the isolated RNA appeared similar, some quantitative differences in percentage distribution of RNA fractions were evident, namely a lower yield of higher molecular weight RNA and a higher yield of low molecular weight RNA was observed for leukemic cells when compared to normal cells.

Defective rRNA synthesis has been detected in leukemic blast cells from patients with AML and CLL. An impairment in the methylation of ribosomal precursor RNA (45S and 32S) has been shown in blast cells of human AML (Torelli et al., 1971). This group (Torelli and Torelli, 1973) also reported an increase in the number of polyA containing molecules in human AML blasts. Seeber et al. (1974) showed a failure of rRNA synthesis in leukemia cells from human AML by the differences in the ratios of specific activities, as well as the demonstration of very small amounts of label (^{32}P) in 28S rRNA of blast cells isolated on sucrose gradients. Billington and Itzhaki (1974) employed the technique of polyacrylamide gel electrophoresis to compare the kinetics of rRNA synthesis

in human CLL with normal lymphocytes and suggested a possible anomaly in rRNA synthesis. Rubin (1971) has demonstrated a sluggish growth response of CLL lymphocytes and associated this observation with a defect in mechanisms regulating ribosome assembly.

VI. RATIONALE:

Although the occurence of anemia accompanying leukemia is well documented, the fundamental mechanisms whereby leukemogenesis interrupts or interferes with erythropoiesis remains unclear. Reports exist of alterations at the end of the heme-hemoglobin biosynthetic pathway in leukemias of man as well as leukemias of rodents. However, the early biochemical events remain obscure. The Shay chloroleukemic rat presents the opportunity to compare the normal with the abnormal mechanisms in a system that closely parallels the disease in humans.

Altered sensitivity of EPO of erythroid tissue would appear to be associated with progress of the diseased state. Increased RNA synthesis is probably the earliest effect of EPO on normal rat bone marrow *in vitro*. In an attempt to elucidate the mechanisms of erythropoiesis and shed light on the possible lesions in erythropoiesis in the leukemic state, this early biochemical event, RNA synthesis, was chosen as a suitable candidate for the site of a possible malfunction in regulation of erythropoiesis.

Heme production has been found to decrease very early in the onset of the disease (Handler and Handler, 1972).

This suggested that a fruitful area of investigation would be an event that precludes the shutdown of heme synthesis. Prior studies of leukemia have examined RNA synthesis; the effect of EPO on normal erythropoiesis has been studied employing established cell lines and hematopoietic tissues from a variety of sources. To date, however, the ability of EPO to stimulate RNA synthesis in leukemic tissues has not been studied. These investigations were designed to elucidate the changing patterns of RNA synthesis in EPO stimulated rat bone marrow cultures during the progress of the disease.

MATERIALS AND METHODS

I. ANIMALS:

All animals used were male Long-Evans rats weighing between 180-200g maintained on a diet of Purina Lab Chow and tap water *ad libitum*. Rats were rendered leukemic by injection of $10X10^6$ leukemic cells via the jugular vein, and assays were performed 3-10 days post-inoculation.

The Shay chloroleukemia tumor was maintained by subcutaneous injection of $20X10^6$ Shay chloroleukemic cells into 40-60g male rats. This results in a localized tumor at the site of injection. The tumor is populated primarily by leukemic myeloblasts which are capable of inducing acute myelogenous leukemia in rats under the conditions described above.

II. STERILE PROCEDURES:

Surgical equipment and glassware were dry heat sterilized; glassware for RNA extractions was washed in 50% nitric acid, distilled water rinsed and dry heat sterilized. Media and sera were filtered through a 0.2u Nalgene Filter unit (Nalge, Sybron Corp., Rochester, N.Y.). All cell transfers were performed under a vertical laminar flow station (Baker Co.).

III. CELL SUSPENSIONS:

- BONE MARROW: The femurs of each test animal were asceptically excised. The femur head was removed and

the marrow flushed through with 2.0ml of incubation medium using a 21 gauge needle and hypodermic syringe. The marrow was then passed through a 44.0u nylon mesh (Nytex, Tobler and Traber Co., Elmsford, N.Y.) secured in a Swinny Hypodermic Adapter (Millipore Filter Corp., Bedford, Mass.). An aliquot of the resultant suspension was removed for determination of the number of nucleated cells by a viable cell count in a hemocytometer and preparation of slides for May-Grunwald and benzidine-hematoxylin stains.

- SPLEEN: The spleens were asceptically removed and transferred to a sterile petri dish where they were bisected. Each half was repeatedly flushed with 2.0ml of incubation medium until sufficient numbers of cells were harvested. Sample aliquots were removed for differential examination.

- CHLOROMA TUMOR: Subcutaneous tumors were asceptically excised from donor animals. Small chunks of tumor were homogenized gently with incubation medium in a sterile glass hand homogenizer equipped with a previously ground down, loosely fitting Teflon pestle. The suspension was passed through sterile cotton gauze and then through sterile glass wool. Sample aliquots were removed for differential examination.

IV. CELL CULTURE:

The medium consisted of 40% freshly heat in-

activated ($56^{o}C$, 30 min) fetal calf serum (BBL), 58% NCTC-109 (Microbiological Associates) and 2%, 7.5% sodium bicarbonate. Cells were cultured at $37^{o}C$ in a 5% carbon dioxide, 95% water saturated air atmosphere. Cells were incubated in Falcon plastic culture tubes (12mmX75mm) containing 350ul of cell suspension for analytical assays or in larger culture tubes (16mmX 100mm) containing 1000ul for preparative RNA extractions. Cell suspensions were incubated at $30X10^{6}$ cells/ml. Other conditions for either assay or preparative incubations for RNA extraction are described in the legends of the appropriate figures.

Erythropoietin* where used was added to cultures at the beginning of the incubation period at a concentration of 0.2U/ml.

V. CHARACTERIZATION OF RNA:

- CELL LABELING: RNA synthesis was monitored by labeling with radioactive uridine, 4-30 uCi/ml of ^{3}H-5-uridine (sp. act., 25 Ci/mmol) or with 0.2-1.0 uCi/ml of ^{14}C-2-uridine (sp. act., 50 Ci/mmol). Radiochemicals were purchased from New England Nuclear.
- TOTAL CELL RADIOACTIVITY: Total cell radioactivity was measured by uridine incorporation into trichloro-

*The erythropoietin was collected and concentrated by the Dept. of Physiology, University of Northeast, Corrientes, Argentina, and further processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under grant HE 10880 (National Heart and Lung Institute).

acetic acid (TCA) precipitable material. Analytical scale replicate cultures were either pulsed for 20 min. and/or labeled continuously. At the end of the incubation time the reaction was terminated by placing the culture tubes in an ice-water bath, then centrifuging in a clinical centrifuge at full speed (2000 rpm) for 3 min. at 4°C to remove the overlying medium. The cell pellet was washed once with 0.9% sodium chloride, centrifuged, suspended in 0.5 ml saline and precipitated with 0.1ml of 0.1M sodium pyrophosphate and 2.0 ml 7.5% TCA containing 0.001M ATP. The precipitates were collected on 0.01M ATP saturated glass fiber filters (Whatman GF/C), washed three times with ice cold 5% TCA and once with 5 ml of 95% ethanol, oven dried and counted in Liquifluor (New England Nuclear).

- DETERMINATION OF PERCENTAGE OF RADIOACTIVITY ATTRIBUTABLE TO RNA: Parallel sets of assays were incubated. One set was treated as above for measurement of the acid precipitable counts, the other was made 0.3M in potassium hydroxide incubated at 37°C for 18 hours, neutralized with hydrochloric acid and then precipitated with cold 5% TCA. The resulting precipitate was collected and treated as described above (Maniatis et al., 1973). Another method was also employed. Parallel sets of assays were incubated. Both sets were processed as described in measurement of total cell radioactivity. One group of filters was then placed in 5% TCA for 20 min. at 90°C

(Yu and Feigelson, 1971). Discs were then washed with cold 5% TCA, ethanol, dried and counted. Both these procedures indicated that 95%-99% of the precipitable counts could be considered counts due to RNA. To determine if ^{3}H-uridine was incorporated into RNA the method of Hausen _et al._ (1969) was used. TCA-insoluble material from triplicate assays were dissolved in 0.3M potassium hydroxide and hydrolyed for 18 hrs. at 37°C. A predetermined amount of 1.2 N hydrochloric acid was added to neutralize the mixture which was then adjusted to 0.25 N perchloric acid ($HClO_4$) with 70% $HClO_4$. The material rendered soluble contained practically all of the radioactivity and an absorbance ratio of 280nm/260nm of 0.5.

- RNA EXTRACTION: Cells were cultured in 1.0 ml replicate tubes. Following the incubation period, the cell suspension were cooled on ice, centrifuged in a clinical centrifuge at 4°C, washed twice with NCTC-109, pooled and suspended in an appropriate volume of saline to be at a concentration of 50-100X10^6 cells/ml. The suspension was dripped into a mixture of a equal volume of buffer A (46mM NaCl, 20mM NaAc, 2mM EDTA, 2% SDS: pH5.1) (Natta _et al._, 1973) and two volumes of water saturated freshly distilled phenol to which the equivalent of 2mg/ml Bentonite dry weight was added (Frankel-Conrat _et al._, 1961). The mixture was agitated for 3 minutes, either by a mechanical stirrer or with a Vortex mixer, depending on the total volume. The layers were separated by

centrifuging in a Sorvall centrifuge (12,000X g) for 10 minutes. The aqueous layer was removed and the interphase was discarded. The phenol layer was reextracted with half the volume of buffer B (100mM NaCl, 10mM NaAc, 1mM EDTA, 1% SDS; pH5.1). The centrifugation and layer separation procedure was repeated and the combined aqueous phase was reextracted with half the volume of water saturated phenol. The bentonite was removed by spinning in a Sorvall centrifuge at 31,000X g for 10 minutes. The aqueous phase was precipitated in 0.3M NaCl by addition of two volumes of 95% ethanol and stored at -20°C. The salt-ethanol precipitation was repeated twice.

● SUCROSE DENSITY GRADIENT ANALYSIS: The ethanol precipitate was dissolved in 5mM TRIS, pH 7.4 with 0.5% SDS. Between 1-5 OD_{260} units were layered on a 5ml, 5%-20% sucrose gradient in 5mM TRIS-HCl, pH 7.4. The samples were centrifuged in a SW50.1 rotor (Beckman Instrument Co.) at 45,000X g for 3 hours at 4°C. Following the centrifugation, the tubes were punctured and 13 drop samples were collected. The samples were diluted with 5mMTRIS-HCl, pH 7.4 for measurement of absorbance at 260nm. Carrier yeast RNA (50ug/tube) was added to each sample and precipitated with one ml ice-cold 15% TCA. The precipitates were collected on glass fiber filters and processed as described above.

RESULTS

I. TOTAL RNA SYNTHESIS:

A. NORMAL BONE MARROW:

- Effect of Cell Concentration: The first consideration was to develop standardized optimal conditions for the measurement of total RNA synthesis in normal bone marrow cell suspensions. Concentration curves were initially performed for a 5-hour incubation period based on procedures established by Gross and Goldwasser (1969). Experiments indicated that the incorporation of ^{3}H-5-uridine during a 20 minute pulse at the end of the incubation period was linear over a wide range of cell concentrations (Fig. 1). Fluctuations were seasonally dependent, with greater incorporation during the summer months and lower levels apparent during the winter months. The slope of the curve ranged between 100-50 cpm incorporated per 10^6 cells.

- Effect of Length of Incubation Time: Choosing the middle of the linear portion of the cell concentration curve, the response to EPO with time was measured at $30X10^6$ cells/ml. Other concentrations were also measured with respect to EPO responsivity, and greatest sensitivity and reproducibility was found to be at $25-35X10^6$ cells/ml. Cumulative experiments covering a time period of 11 hours showed a maximal response to the hormone between 3 and 5 hours (Fig. 2).

Table I shows the values of the amount of isotope incorporated at the optimal times for assay at two cell concentrations. At $30X10^6$ cells/ml, in 10 separate experiments EPO stimulated isotope incorporation into RNA by a mean of 29.8% during a 3 hour incubation, and in 12 separate experiments the stimulation was a mean value of 42.4% during a 5 hour incubation. At $5X10^6$ cells/ml, in 6 separate experiments during 3 hour incubation the stimulation was a mean value of 27.8%, and in 5 separate experiments during a 5 hour incubation, 32.5%. Fig. 3 illustrates incubations performed at two different concentrations within the optimal time period, 1-5 hours. Fig. 3: A & C indicates that the amount of isotope incorporated during a 20 minute pulse was relatively linear within the time period and increased with cell concentration. Fig. 3: B & D shows that the amount of isotope incorporated per 10^6 cells was about the same throughout the time period and practically independent of the cell concentration.

- Effect of Continuous Versus Pulse Label: Fig. 3 also illustrates the incorporation of isotope into RNA during a continuously labeled incubation. There is a slight concentration dependent difference in the initial steep rise before reaching a plateau. When the slopes are calculated between the 1 and 3 hour points, m=2912 at $30X10^6$ cells/ml and m=2403 at $15X10^6$ cells/ml. The final level of incorporation was the same thereby

resulting in a final value of cpm per 10^6 cells greater in the more dilute population. Fig. 4 is a dual label time course study with EPO demonstrated that the increase due to the hormone was evident at all time points both for the pulse and the continuous label. The amount of label (^{14}C) incorporated during a 20 minute pulse remained virtually constant throughout the time period with increased incorporation in the presence of EPO. The continuous label (^{3}H) increases rapidly and then levels off. Both labels indicate the optimal time for observing the effect of EPO on RNA synthesis is between 3 and 5 hours.

- Relative Pool Size: For the purposes of our experiments cell suspensions were incubated between 3 and 5 hours, usually at 30×10^6 cells/ml. At this time interval uridine incorporation into TCA precipitable material can be equated with RNA synthesis as there is no appreciable change in the intracellular pool (Table II). Relative pool size was determined according to the method of Maniatis *et al.*, (1973) by incubating control and hormone treated cultures using three different levels of isotope. This procedure gives an indirect measurement of the relative pool size. Since gross changes do not occur it is inferred that the effective specific activity of the exogenous precursor entering the intracellular pool is the same for all cells being compared throughout the labeling period. Absolute quantitation would require an estimate of the contribution of endogenous synthesis to the effective input specific

activity of precursors (Cooper, 1973). These results are consistent with a constant intracellular pool.

B: LEUKEMIC BONE MARROW:

• 8-10 Days After the Onset of Leukemia: Tables III and IV present data for bone marrow suspensions from 25 animals sacrificed 8-10 days after intravenous administration of leukemic cells. The total cellularity of the bone marrow decreases as the disease progresses with a decrease in the percent benzidine positive cells and an increase in the percent myeloblasts. Assays were performed at $30X10^6$ cells/ml (Table III) or $5X10^6$ cells/ml (Table IV) when numbers of cells were at a premium. The qualitative results are similar and the conclusions drawn do not distinguish between the two groups. Animals within a group are listed according to increase in percent myeloblasts in the marrow.

EPO failed to stimulate RNA synthesis by bone marrow cells even when leukemic cells comprised a relatively small percentage of the marrow compartment. Animals L1-L5, L12, L13 had normal numbers of benzidine positive cells. Animals L6-L11, L14-L25 had reduced numbers of benzidine positive cells. Animals L1, L12-L15 exhibited an increase in RNA synthesis almost normal in magnitude following hormone treatment, however, note that L14 and L15 had low percent benzidine positive cells.

In several cases when bone marrow bells were labeled continuously (Table IV), there appears to be the suggestion

of a relationship between the response to EPO in a 20 minute pulse versus a continuous label in marrows of intermediate percent blast. If the pulse label shows a near normal increase in synthesis, the continuous label indicates a lesser percent stimulation, L15 approaches the normal pattern, L14 shows a decline in stimulation when labeled from the onset of incubation, even though the 20 minute pulse was in a normal range of incorporation. On the other hand at higher percent blast where the pulse label shows no response to the hormone, the continuous label exhibits some increase in RNA synthesis as seen in animals L16, L19 and L22.

- <u>3-5 Days After the Onset of Leukemia</u>: In order to determine whether the time after leukemic cell administration was important, bone marrow of animals 3-5 days post inoculation of leukemic cells was examined. Tables V and VI present data from 13 animals sacrificed 3-5 days after intravenous administration of leukemic cells. The data are grouped in the same manner as for the 8-10 day leukemic animals. Most of the animals exhibited EPO responsivity. However, except for L26, L28 and L38, the response was below normal levels. The response to EPO could not be correlated with the percent benzidine positive cells, L37 and L38 have low percent benzidine positive cells yet respond to the homone at levels approaching normal. As observed in the 8-10 day animals, there is also an elevation of control levels of RNA synthesis in the 3-5 day leukemic marrows.

Comparing the 8-10 day to the 3-5 day leukemic marrow data for low percent blast at 5 hours and $30X10^6$cells/ml (Tables III and V), the loss of response to EPO is more marked in the 8-10 day animals. At 3 hours and $5X10^6$cells/ml (Tables IV and VI) at low percent blast there is still responsivity, though reduced, to EPO in both groups of animals.

C: TUMOR CELLS:

- Effect of Cell Concentration: Pure populations of leukemic myeloblasts prepared from solid tumors exhibited linearity with a 20 minute pulse of ^{3}H-uridine at the end of a five hour incubation for a wide range of cell concentrations (Fig. 5). However, the amount of incorporation and the extent of linearity varied amongst tumor cell populations, seemingly an inherent property of the tumor. In all cases studied linearity was maintained up to $35\text{-}40X10^6$cells/ml. Cell concentrations above $45X10^6$cells/ml resulted in a decrease in isotope incorporation. Incorporation was greatest between $15\text{-}35X10^6$cells/ml within a 3-5 hour incubation period measured by the amount of incorporation of isotope during a 20 minute pulse.
- Effect of Length of Incubation Time: Unlike the situation in normal bone marrow cell suspensions, the rate of incorporation of ^{3}H-uridine declined with time in proportion to the cell concentration (Fig. 6).

When the cells were in the presence of isotope from the onset of incubation, accumulation of label varied inversely with cell concentration. At low concentration (10×10^6 cells/ml) even though the rate of incorporation was declining, the amount of accumulation during the time course, increased linearly whereas at the higher concentration (30×10^6 cells/ml) the decline in rate was followed by the decline in accumulation of radioactivity. This is more clearly seen when plotted as cpm/10^6cells.

The pure leukemic myeloblast cell suspensions exhibited no stimulatory response to EPO at either 3 or 5 hours (Table VII). If any difference is apparent, it is a slight reduction of isotope incorporation in the presence of the hormone.

- Relative Pool Size: Uridine incorporation can be equated with RNA synthesis since there is no appreciable change in the intracellular pool size (Table VIII).

D: 50:50 MIXTURES OF NORMAL MARROW CELL SUSPENSIONS WITH LEUKEMIC MYELOBLASTS

An attempt was made to duplicate the leukemic bone marrow *in vitro* by mixing normal tissue with leukemic myeloblasts. This led to results which are reported in Table IX. Normal bone marrow alone and tumor cells alone gave the expected results with EPO. However the 50:50 mixture of the two types of cell suspensions decreased the overall response to the hormone.

The overall level of incorporation in the 50:50 mixture reflected the contribution of the tumor cell moiety in most cases, being half that observed in the pure population at $15X10^6$cells/ml. Experiment C showed some deviation from this pattern. The 50:50 mixture without EPO is equal to the sum of half the contributions from each type cell suspension. However, the amount of incorporation by normal bone marrow cells alone in C is slightly higher than usual and the incorporation of the pure tumor cell suspension is lower than that in A, B and D.

E: NORMAL SPLEEN:

The adult rat spleen is not normally erythropoietic; it may become erythropoietic in the leukemic state. It was therefore of interest to examine spleen cell suspensions along with bone marrow suspensions. Spleen and bone marrow cell suspensions were assayed for RNA synthesis, as measured by ^{3}H-uridine incorporation, under the same conditions.

Table X summarizes the results of 8 separate experiments, 4 at 3 hours and 4 at 5 hours. Assays were performed at concentration of $30X10^6$cells/ml. Neither group showed any significant stimulation by EPO.

F: <u>LEUKEMIC SPLEEN</u>:

- <u>8-10 Days After the Onset of Leukemia</u>: Table XI presents data for spleen suspensions from 22 animals sacrificed 8-10 days after intravenous administration of leukemic cells. The numbers refer to the same animals reported in Tables IV-VI and are arranged in order of increase in the percent blast in the bone marrow. Isotope incorporation into RNA was performed at $30X10^6$cells/ml and, except for isolated cases (L1, L12, L20) was unaffected by exogenous EPO despite the routine observation of hemoglobinized cells in these samples. In general the level of incorporation appeared to increase with the percent blasts in the bone marrow rather than with the blast content of the spleen.

- <u>3-5 Days After the Onset of Leukemia</u>: Table XII presents data from 13 animals sacrificed 3-5 days after inoculation with leukemic cells. As in the 8-10 day leukemic spleens, very few spleen cell suspensions responded to EPO. L32 and L33 showed a response. Spleens of animals L33 and L34 showed an elevated number of erythroid cells and a modest response to the hormone even though the slides of the corresponding bone marrow appeared normal. Animal L33 showed no response in the marrow to EPO and L34 did show a response, -6.7% and +21.8% respectively.

II. CHARACTERIZATION OF RNA BY SUCROSE DENSITY GRADIENT ANALYSIS:

A: NORMAL BONE MARROW:

• Pulse Label: Three hour incubations were used routinely for the extraction of RNA. This was done because by the end of a five hour incubation the RNA extracted exhibited unacceptable levels of degradation as evidenced by a 28S to 18S ratio of less than 1.1. This has been observed in other erythropoietic systems (Spivak *et al.*, 1972). The optical density patterns reported and referred to have 28S and 18S ratios of 1.2-1.5 (mean=1.3$\pm$0.12 S.D.), (Table XIII and XIV).

Fig. 7A and B represent one typical sucrose density gradient analysis of RNA with a 3 hour incubation with and without EPO. The cultures were pulse labeled, and the resulting distribution of TCA precipitable counts in each fraction of the gradient versus the corresponding optical density at 260nm is illustrated. The pattern for RNA extracted from cultures with EPO does not appear to differ appreciably from RNA isolated from cultures without EPO. The difference between RNA isolated from stimulated and unstimulated cultures becomes evident when the specific activity (SA: $cpm/OD_{260nm}X10^3$) is calculated and plotted for each fraction (Fig. 7C). The overall pattern displays an increase in SA of RNA from EPO treated cultures in the heavy and light regions of the gradient. Low SA values are found over

the 28S and 18s regions and a markedly higher level of SA between 18S and 4-5S, reaching a maximum difference in the region corresponding to 6-12S and 45S. These regions would be that of heterogeneous RNA and RNA with a high turnover rate, and possibly mRNA. Differences due to the response to EPO are more clearly seen in Fig. 7D. Each bar is the total of the specific activity of a region divided by the number of fractions in that region to give a value termed average specific activity (AvSA).

Fig. 8 illustrates two additional normal bone marrow average specific activity distributions. This demonstrates the slight variations in the state of bone marrow in different organisms. The general pattern is similar to that in Fig. 7 with increases in AvSA in the 45S and 8-10S regions. All normal bone marrow RNA studied exhibited a mean AvSA of 8 ± 3 S.D.

<u>Continuous Label</u>: Fig. 9A and 9B represent the optical density pattern and corresponding amount of TCA precipitable radioactivity of RNA isolated from cultures with and without EPO in the presence of isotope from the onset of incubation. The radioactivity follows the optical density trace. Again the difference between control and stimulated RNA is more apparent when the average specific activity is calculated. Fig. 9C is the plot of SA of each fraction of the gradient of RNA from treated and untreated cultures.

A general increase in SA is seen for EPO stimulated RNA. The bar diagram (Fig. 9D) shows this increase with EPO in all classes of RNA. Fig. 10 further illustrates this increase in all classes of RNA from EPO stimulated cultures. The mean AvSA for all extractions of RNA measured was 27 ± 8 S.D. A general increase in the level of counts throughout the entire gradient corroborates Goldwasser's earlier observations on the effect of EPO on RNA synthesis (Gross and Goldwasser, 1969).

B: LEUKEMIC BONE MARROW:

RNA profiles of leukemic bone marrow with and without EPO were indistinguishable from each other and from normal bone marrow. Not all RNA isolated from leukemic bone marrow is averaged. Since there are great individual differences in the time course of the disease amongst animals, each animal is recorded separately. Difficulty was experienced in isolating acceptable RNA based on 28S to 18S ratios of at least 1.1. Since the total cellularity decreases as the disease progresses it was often difficult to obtain enough cells for extraction. Frequently the actual amount of RNA extracted was so scant that calculation across the gradient of specific activity was not meaningful. Carrier RNA addition during the course of extraction was not practical because differences

between EPO stimulated RNA and control RNA show up in the specific activities and not always in the pattern of net amount of radioactivity. Despite these obstacles several valid observations can be made.

- 8-10 Days After the Onset of Leukemia:

Pulse Label: The average specific activities of different size classes of RNA isolated from animals 8-10 days post inoculation of leukemic cells are shown in Fig. 11. Animals L23 and L21 contain approximately the same high percent blast and the same percent benzidine positive cells, but did respond with a slight difference to EPO, +9.6% and +2.1% respectively (Table IV). This difference appears in the AvSA of the isolated RNA where L23 shows an increase with EPO at 45S and 8-10S, whereas L21 shows a depression in all classes. L20 did not show a response to the hormone either in the assay or in the extracted RNA. L13, a low percent blast animal, gave almost a normal level response to the hormone when assayed for total RNA and exhibited an increase in AvSA in the 45S and 8-10S region of the sucrose gradient.

Continuous Label: Animals L14 and L18 when assayed for total RNA (Table IV) with a pulse label showed a response to the hormone of +27.0% and +6.4% respectively. The assay of a continuous label was -4.9% and +1.2%. As noted in the normal animals with regard to the relationship between a pulse and continuous label,

the stimulation exhibited by the pulse labeled assay is higher than in the continuously labeled assay. The extracted RNA from marrow preparations displayed an increase in AvSA of RNA from EPO treated cultures (Fig. 12). The RNA from animal L18 showed an overall AvSA within the normal range. This animal is in the middle stages of the disease, and as stated previously there is an apparent depressed response to EPO when the pulse label is compared to the continuous label. Animal L14 exhibited an elevated AvSA of all classes of RNA in the absence of EPO and a further stimulation in EPO treated cultures.

- 3-5 Days After the Onset of Leukemia:

Fig. 13 shows the average specific activity of different size classes of RNA isolated from cell suspensions prepared from animals 3-5 days post inoculation of leukemic cells. RNA was isolated from pulse labeled 3 hour cultures. The most obvious difference between these animals and the 8-10 day leukemic and normal animals is the dramatic increase in levels of AvSA with a large contribution in the 45S region. The 3 animals studied (L35, L37, L38) all displayed an increase in AvSA in response to EPO. However, as opposed to the pattern observed from normal rat bone marrow, this increase was more evenly distributed amongst all RNA classes.

C: LEUKEMIC MYELOBLASTS:

Isolation and characterization of RNA from homogeneous populations of pure leukemic myeloblasts gave a ratio of 28S to 18S between 1.2 and 1.8 (mean = 1.5 ± 0.2 S.D.). These ratios were routinely closer to the expected ratios of the ribosomal RNA moietites than were obtainable from either normal or leukemic bone marrow cell suspensions. In all cases the pattern of radioactive distribution over the optical density profile appeared similar whether or not the cells had been exposed to EPO.

● Pulse Label: Fig. 14 shows RNA isolated from cells grown at two concentrations, 30 and 15×10^6 cells/ml. Fig. 14A illustrates the optical density pattern at 260nm and the corresponding amount of radioactivity found in each fraction of the gradient for RNA isolated from cell suspension incubated at 30×10^6 cells/ml without EPO. Label distributed mainly in 18S to 4-5S regions with some incorporation into 45S and 28S regions. The effect of concentration on the average specific activity is evident in Fig. 14B and C: Fig. 14B, AvSA of RNA from cultures at 30×10^6 cells/ml and Fig. 14C from cultures at 15×10^6 cells/ml. The most obvious difference is the pronounced increase in AvSA with decrease in cell concentration. The RNA from cells incubated at 15×10^6 cells/ml has over a 5 fold greater AvSA than the RNA from cells incubated at the higher concentration. This agrees with the data discussed earlier (Fig. 6).

The second effect noted is the repressive action of EPO in all classes of RNA independent of concentration. This reflects observations made in the assay of total RNA synthesis where a negative percent change was noted with EPO (Table VII).

• Continuous Label: Fig. 15 is the sucrose density pattern of RNA isolated from cell suspensions of tumor cells in the presence of isotope from the onset of incubation. Fig. 15A shows that the distribution of radioactivity follows the optical density trace. Fig. 15B and C are the levels of AvSA of the size classes of RNA isolated from cells incubated at 30 and $15X10^6$cells/ml respectively. The most marked difference in AvSA is the cell concentration dependence. RNA isolated from cell suspensions at $15X10^6$cells/ml have approximately 10 fold greater AvSA than that isolated at the higher concentration. The mild repressive effect of EPO is apparently concentration independent and class nonspecific. All classes show this slight repression, and as to be expected the effect is less pronounced in the continuously labeled isolated RNA than in the pulse labeled.

D: 50:50 MIXTURE OF NORMAL BONE MARROW CELL SUSPENSIONS WITH LEUKEMIC MYELOBLASTS

The RNA isolated from 50:50 mixtures of normal bone marrow cells and tumor cell suspensions showed no

distinguishing characteristics when analyzed for optical density and radioactivity distribution. Both the pulse labeled and the continuously labeled culture with or without EPO were indistinguishable from each other and from either of the components of the mixture. Fig. 16 displays the average specific activity pattern for two concentrations of 50:50 cell suspensions incubated for 3 hours.

When RNA was isolated from mixtures of total cellularity of $30X10^6$ cells/ml, the distribution of AvSA and the effect of EPO reflect the contribution of the tumor cells. However, the AvSA is higher than normal bone marrow alone and less than expected for tumor cells alone at $15X10^6$ cells/ml (see Fig. 14 and 15) for both the pulse and the continuous label. Also the repressive effect of EPO is evident in the pulse labeled culture in all classes save 45S and in the 4-5S region when cells were labeled from the onset of incubation.

When RNA was isolated from mixtures at $15X10^6$ cells/ml, the AvSA pattern appears to be midway between the two components. The pulse label shows a AvSA pattern closer to that observed with normal bone marrow than with tumor cells in magnitude of AvSA and in increase in the 8-10S and 4-5S regions. RNA from continuously labeled cultures shows an increase in all classes with EPO except in the 8-10S region, however the magnitude

of AvSA in all classes reflects the contribution of the tumor cells.

It would appear that the effect is essentially additive and *in vitro* with a short assay time there is little effect of one cell population on the other. The EPO effect is unmasked as the amount of RNA from the tumor cell is decreased.

DISCUSSION

I. RNA SYNTHESIS IN NORMAL HEMATOPOIETIC TISSUES:

Erythropoietin stimulation of RNA synthesis has been reported to be the earliest observable effect in erythropoiesis (Krantz and Jacobson, 1970). This early event is seen in the present study of normal rat bone marrow at 30 minutes after the start of incubation. Krantz and Goldwasser (1965) observed EPO stimulated RNA synthesis as early as 15 minutes after the start of incubation. The present reported increase in RNA synthesis over control levels of approximately 30% agreed with 35% obtained by Gross and Goldwasser (1969) in their assay system, which consisted of $25X10^6$cells/ml cultured in petri dishes containing a total volume of 1.0 ml and incubated for 5 hours. The cultures in the study presented here were capable of remaining viable for 7-11 hours, after that time there was a severe decline in overall RNA synthesis as well as response to EPO. Goldwasser (personal communication) has maintained his cultures for 72 hours.

When a short exposure to label is given, the most obvious increase in average specific activity (AvSA) is seen in the 45S and 8-10S region of the sucrose gradient. Gross and Goldwasser (1969; 1971) have equated this increase in RNA synthesis with a rise in

mRNA synthesis, probably globin mRNA. This group has also reported the appearance of a 150S peak on sucrose density gradients, unique to EPO stimulated RNA synthesis in rat bone marrow cultures. In mouse fetal liver, the 8-10S region has been isolated and found to code for globin in a Krebs ascites tumor cell-free protein synthesizing system (Terada et al., 1972). This same region of the gradient of RNA isolated from chick embryos synthesized protein of only one size which coelectrophoresed with globin marker (Chan et al., 1974). Because of the highly reproducible optical density pattern of RNAs between 12S and 18S size range, these RNAs have also been interpreted to be the result of a specific type of breakdown that gives rise to smaller RNAs of certain sizes (Chan et al., 1974). HnRNA sedimenting at 45S can be construed as precursor to mRNA and/or to rRNA (Brawerman, 1974). Since some of the HnRNA comprises precursors of cytoplasmic mRNA it is not surprising that the two most prominent areas of change in AvSA are the 45S and 8-10S regions. Birnie et al., 1974) have reported that the area 8-10S comprises RNA with a rapid turnover. This region probably also contains mRNA that codes for globin (Marks et al., 1974). Other RNAs are probably also present. The bone marrow represents a heterogeneous population of cells. Stimulation by EPO involves both differentation and proliferation. EPO stimulated

differentiation not only induces the apparatus for hemoglobin synthesis but also promotes the development of material characteristic of the mature red blood cell. It is therefore highly likely that synthesis of proteins other than globin, and ipso facto mRNA, is stimulated. Increased synthesis has been demonstrated for uptake of glucosamine into stroma (Dukes et al., 1964; 1965), delta-aminolevulinic acid synthetase (Bottomley and Smithee, 1969), and specific antigens (Yunis and Ynis, 1963; Van den Engh and Golub, 1974). Increasing density of antibody reactive sites are a feature of erythroblast differentiation which parallels accumulation of hemoglobin (Minio et al., 1972). This property has been employed to isolate precursor erythroid cells from mouse fetal liver by immune hemolysis (Cantor et al., 1972). These precursor cells require EPO to be maintained in culture and proliferate and differentiate to erythroblasts with the initiation of hemoglobin synthesis.

Experiments with FUDR to inhibit DNA synthesis also suggest that mRNAs other than those for globin are stimulated. However, there is some controversy in the interpretation in the results of experiments with FUDR. Using mouse fetal liver Nicol et al. (1972) demonstrated that the analog inhibited stimulation of hemoglobin synthesis caused by EPO, but only partially inhibited stimulation

of RNA synthesis. Paul and Hunter (1969) reported the cessation of EPO stimulated RNA synthesis in mouse fetal liver whereas Gross and Goldwasser (1970) showed an inhibition of EPO stimulated RNA synthesis amounting to only six percent of control level of EPO stimulation.

In the present study, when bone marrow cell suspensions are exposed to isotope from the onset of incubation, all classes of RNA are labeled and the AvSA of RNA from stimulated cultures exhibits a uniform increase in all classes. Similar findings have been reported for rat bone marrow (Gross and Goldwasser, 1969) and rabbit bone marrow (Pavlov, 1969).

II. RNA SYNTHESIS IN LEUKEMIC HEMATOPOIETIC TISSUES:

The Shay chloroleukemia, pathologically similar to human acute myelogenous leukemia (AML), is characterized by a progressive decline in total cellularity in the bone marrow, together with a decrease in the percentage of benzidine positive cells and an increase in the percentage of myeloblasts (Handler and Handler, 1970). The observations presented in the present study corroborate these earlier observations. Erythropoiesis declines in the anemia accompanying leukemia as measured by ^{59}Fe incorporation studies in human (Thorling, 1965; Zucker *et al.*, 1974; Zaizov and Matoth, 1971) and in the Shay chloroleukemic rat (Handler and Handler, 1970). In the present study

bone marrow erythropoiesis declines as measured by EPO stimulated RNA synthesis.

The response to EPO decreases with increasing percent myeloblast in the bone marrow. This decrease in response to EPO is accompanied by an increase in the level of isotope incorporation in both EPO treated and untreated samples. This is probably due to the RNA synthetic activity of the leukemic myeloblast in the leukemic bone marrow cell suspension. Autoradiographic studies of bone marrow from patients with AML showed an increase in total percent cells labeled with uridine which was significantly greater than that in normals (Schumacher _et al._, 1971). However, in comparing normal blasts to leukemic blasts the labeling index appeared to be approximately equal.

The response to EPO appears to be independent of the number of benzidine positive cells. Although the bone marrow in acute leukemia is dominated by blast cells, there are a small number of differentiating red blood cells. These cells may represent the remnants of "normal" hemopoiesis, the progeny of precursors that passed through the EPO dependent stage prior to the "inhibitory" influence of the leukemic state. This might be a possible way to account for the stimulatory response to EPO seen under conditions of continuous label, which were not readily apparent in parallel cultures which received a pulse label.

This investigation also indicates that the loss of ability by bone marrow elements to respond to EPO depends not only on the presence of the leukemic cells in the marrow, but also on the length of time after seeding of these cells. Although there is a diminished response to EPO 3-5 days after the initiation of leukemia, the abolition of the response occurs only after further *in vivo* exposure.

An attempt was made to mimic the leukemic bone marrow *in vitro* by mixing normal bone marrow and tumor cell suspensions. This led to results that were both surprising and somewhat difficult to interpret. The response to EPO in a 50:50 mixture decreased by half the numerical value of the stimulation observed in normal bone marrow suspensions. This was unexpected, since it was assumed that the effect of tumor cells *in vitro* on normal bone marrow elements is minimal during a short incubation period. If the mixture were to behave as a simple addition of radioactivities, the following pattern might be observed:

	CPM -EPO	CPM +EPO	Percent Change
normal bone marrow	1500	2000	33
tumor	3000	3000	0
50:50	2250	2500	11

A possible explanation is that the response to EPO in the 50:50 mixture may be an artifact of the system *in vitro*. On the other hand it may reflect some conditioning of the medium to cause this apparent overstimulation. If one considers that in the 3-5 day leukemic animal there is an inordinately high increase in the specific activity of RNA isolated from marrow cell suspensions; it is feasible to conceive of the *in vitro* 50:50 mixture as representing day zero. Perhaps at this artificial day zero, *in vitro*, and possibly *in vivo*, the ERCs present in the bone marrow are "sensitized" and overcompensate in EPO stimulated RNA synthesis.

The RNA extracted from 50:50 mixtures reflected the numerical addition of the two types of cell suspensions. However, of interest is the observation that RNA extracted from a pulse labeled 50:50 mixed cell suspension at $30X10^6$ cells/ml exhibited stimulation due to EPO only in the 45S region of the gradient. The other regions of the gradient showed a depression of specific activity with EPO. At $15X10^6$ cells/ml an EPO effect is seen in the 8-10S region. At the present time no clear cut conclusions can be drawn about the synergistic actions between normal bone marrow elements and leukemic myeloblasts *in vitro*.

These data suggest that the sucrose density profiles obtained for RNA isolated from leukemic bone

marrow were similar to normal bone marrow and in each other in distribution of size classes and optical density profiles. Similar kinds of observations have been made in human leukemic cells. Wulff *et al.* (1972) compared RNA extracted from log phase human lymphocyte cells grown in large scale cultures with RNA isolated from lymphocytic cells derived from patients with acute lymphocytic leukemia and from normal donors. The overall patterns were similar and differences were only apparent as small variations in percent distribution of individual RNA fractions. Perhaps a fruitful area of investigation would be to explore differences in minor base sequences between normal and leukemic RNA.

When cultures were stimulated with EPO, again the sucrose density gradient profile was virtually indistinguishable from the RNA of unstimulated cultures. Differences appear only when the average specific activity (AvSA) of the various size classes are assessed. Neoplasia has frequently been defined as a defect in orderly cell growth due to alterations in the internal cellular control mechanisms. As discussed previously normal bone marrow displays a predictable AvSA and pattern of increase in RNA AvSA of different classes in response to EPO. The leukemic bone marrow on the other hand, displays a pattern different from normal and is not predictable. Each animal showed its own distribution of response to the

hormone and a wide range of AvSA.

Two generalizations that can be made based on the data of RNA isolated from 8-10 day animals: 1) when few myeloblasts were present in the marrow, and there was a response to EPO in the assay; the gradient profile reflects normal elements and the AvSA is similar to that observed for controls; 2) as the numbers of myeloblasts increase in the bone marrow, the control value of the AvSA increases and the AvSA gradient profiles vary considerably from controls and from each other.

The 3-5 day animals display a striking increase in AvSA of all classes of RNA. This increase is far above that observed in myeloblasts or 8-10 day leukemic bone marrow samples. One possible explanation of this behavior is that there is a marked increase in the permeability of the marrow cells (Schaefer _et al._, 1972). Another possibility is that there is "overcompensation" on the part of the normal elements of the bone marrow. The increase in AvSA in response to EPO is evident in all size classes during a 20 minute pulse. Since the extraction is of total RNA, the increase may reflect the huge amount of 45S or HnRNA which may have degraded and spread throughout the gradient. Or it may indicate a rate of synthesis rapid enough to incorporate isotope into rRNA in significant amounts. Another possibility is that the hyperactivity is due to a stimulation of the immune

system which disappears by day 8-10. The breakdown of the immune system has been implicated in AML and some remission of the disease following chemotherapy might be due to development of effective immunity to leukemic antigens (Harris, 1973).

Various interpretations are feasible for the observed characteristics of *in vitro* RNA synthesis of leukemic bone marrow. Sensitivity to EPO declines during the course of the disease with both time after seeding of the leukemic myeloblast and with increasing numbers of myeloblasts in the marrow. The observation that a 50:50 mixture gave a response to EPO different from that expected from the addition of the contributing moieties indicates that processes other than the presence of leukemic myeloblasts is implicated.

It has been postulated that at some point during the leukemic process medullary erythroid cells become refractory to high circulating levels of endogenous EPO (Thorling, 1965; Zaizov and Matoth, 1971; Chiyoda *et al.*, 1974). An alternate to decreased sensitivity is the possible depletion of the medullary erythron (Chiyoda *et al.*, 1974). These interpretations also hold for the Shay chloroleukemia. There seems to be no evidence for a malfunction of the EPO generating system. An elevation in plasma levels of EPO have been found and the observed anemia during the chloroleukemia cannot therefore be ascribed to lack of this regulatory

hormone (Handler and Handler, unpublished observations). The reduction in heme synthesis occurs at a time before extensive marrow infiltration by leukemic cells (Handler and Handler, 1972). When leukemic bone marrow suspensions are put into plasma clot cultures the number of erythroid colonies which develop are markedly reduced and EPO fails to stimulate the development of erythroid colonies (Handler and Handler, unpublished observations). This would indicate both a decline in the number of ERCs (CFU-E) and reduced sensitivity to the hormone.

The immediate chemical environment of a cell plays a central role in its proliferation and differentiation. By release of biologically active molecules the cell may directly influence its environment. It would appear that a change in the microenvironment might trigger changes in erythropoiesis. Curry and Trentin (1967) have brought forth evidence that microenvironments created by cytoplasmic function of stromal cells determines whether stem cells will differentiate along erythrocytic, granulocytic or megakaryocytic lines. Spleen and bone marrow hemopoietic studies in lethally irradiated mice transfused with limited numbers of bone marrow cells have revealed that the direction of differentiation of pluripotential stem cells is controlled by their interaction with hematopoietic inductive micro-

environments (HIM) of the spleen and bone marrow reticuloandothelial stroma (Curry and Trentin, 1967). Low doses of bone marrow cells injected into lethally irradiated mice give rise to colonies in both the bone marrow and spleen. Based on cytogenetic studies, these colonies have been shown to be derived from a single pluripotential stem cell (Becker *et al.*, 1963).

Experiments of Curry *et al.* (1967) indicated that the role of the erythroid HIM is to induce pluripotential stem cells to become EPO sensitive stem cells. This was based on experiments designed to test the role of EPO on spleen colony formations. If an irradiated host is made polycythemic, granulcyte colonies develop, but only presumptive erythroid colonies are seen. These undifferentiated spleen colonies are composed of EPO sensitive cells, since on administration of EPO they transform into differentiated erythroid colonies.

Further evidence has been provided by use of the genetically anemic Steel mouse (Sl/Sl^d) which has normal stem cells (McCulloch *et al.*, 1965) and normal or elevated levels of EPO (Bernstein *et al.*, 1968). Spleen and bone marrow colony assays in lethally irradiated Steel mice resulted in formation of normal numbers of granuloid colonies but no erythroid colonies. The low erythroid to granuloid colony ratios indicate a defect or absence of the erythroid HIM

(Gallagher *et al.*, 1971).

Another group of studies to test the HIM concept involves xenogeneic (rat to mouse) bone marrow transplantation. Isologous rat marrow transfused into lethally irradiated rats gave rise to erythroid and undifferentiated colonies (Rauchwerger *et al.*, 1973). Rat marrow transfused into lethally irradiated mice gave rise to both erythroid and granuloid colonies (Rauchwerger *et al.*, 1973; Trentin *et al.*, 1974). This indicated that HIM can induce rat stem cells to differentiate along lines seen in mouse spleen but not in rat spleen, and gives further credence to the role of microenvironments in the course of hematopoietic cell differentiation. Rates of colony growth also differed, rat colonies grew more rapidly in mice spleens than in rat spleens, suggesting that HIM also influences rate of proliferation (Rauchwerger *et al.*, 1973).

The microanatomy also contributes to the microenvironment. The function of marrow depends significantly on the microanatomy of the vascular network (Weiss, 1965). The arterial supply to the marrow ultimately breaks up into networks of intercommunicating sinuses that permeate parenchymal cords. Data is not available to support the possibility that physical segregations is a random phenomenon with no specific relationships between the cell. Indirect evidence

suggests that cell-to-cell inductive regulatory processes must be operative in bone marrow (Beck, 1974). Striking segregation is found in chickens, with granulocytopoietic cells lying in aggregates outside the sinusoids. In mammals however, both granulocytopoiesis and erythropoiesis seems to be extravascular (Beck, 1974). McCuskey *et al.* (1972) have suggested that stem cells committed to the erythroid line will complete their development only in a microenvironment that is highly vascularized, has a high rate of blood flow, and contains neutral mucopolysaccharides. During the course of the Shay chloroleukemia, progressive alteration in bone marrow architecture, including destruction of marrow sinuses, has been demonstrated (Chen *et al.*, 1972). "Hot pockets" have been observed in bone marrow smears from patients with aplastic anemia and may represent remaining hematopoietic tissue in intense proliferative activity (Erslev, 1974).

In the present study it is difficult to assess the role of the microenvironment. Intact marrow structure does not appear to be required for the effect of EPO *in vitro* of EPO on marrow cell RNA synthesis by bone marrow: the experimental procedures employed perturb the bone marrow architecture. However, it is conceivable that the residual conditioning effect started *in vivo* manifests itself *in vitro*. It is not

known whether inhibiting influences of the leukemic myeloblast on erythropoiesis requires direct cell-to-cell interaction and/or elaboration of substances from cells which specifically inhibit the responsiveness of the ERC. The recently developed erythroid colony assay (Axelrad, 1974) should prove to be a useful tool in the "dissection" of the microenvironment.

III. THE SPLEEN IN HEMATOPOIESIS:

In the normal adult mouse, splenic erythropoiesis contributes significantly to the total erythropoietic contribution of the animal (Fruhman, 1970). Erythropoiesis in the normal adult rat spleen is not considered to be a large contributor to the total erythropoietic output (Handler and Handler, 1970). Nevertheless, it is capable of substantial levels of compensatory red blood cell production during conditions of anemic stress as well as during the pathogenesis of AML, no doubt the result of anemia associated with leukemia (Handler and Handler, 1970).

In vivo, EPO stimulates erythropoiesis in mice and in rats (Fruhman, 1970). The RNA isolated from spleens of mice rendered anemic by phenylhydrazine treatment will direct the synthesis of globin (Cheng *et al.*, 1974). In leukemic rats the specific activity of splenic delta-aminolevulinic synthetase appears to be linear with the percent benzidine positive cells

in the spleen (Horowitz, 1975). The present study indicates that EPO did not effect RNA synthesis by normal rat spleen cell suspensions. *In vitro* RNA synthesis by leukemic spleen cells was not affected by EPO despite the presence of benzidine positive cells. It is conceivable that the early erythroid elements in the spleen may differentiate in response to a different triggering mechanism. Alternatively, it is possible that the *in vitro* conditions optimal for RNA synthesis by bone marrow cell suspensions are not favorable to spleen cell suspensions.

IV. RNA SYNTHESIS IN HOMOGENEOUS POPULATIONS OF LEUKEMIC MYELOBLASTS:

The tumor cell cohort when injected intravenously into the animal "seeds" in part in hemopoietic tissues. *In vitro*, the leukemic myeloblast exhibits some properties of cells in long term culture. Amongst these is the high dependency of incorporation of labeled precursors on cell concentration. Unlike the normal bone marrow cell suspensions, which appear to be relatively devoid of any marked concentration dependency, the tumor cells exhibit a rate of RNA synthesis directly related to cell concentration at low density, and inversely related at high density. This is readily observed in both the assay and average specific activity of isolated RNA.

The basis for "density-dependent" inhibition is unknown, but appears to be characteristic of untransformed cells in culture (Burger and Noonan, 1970). It has been postulated that dense cell populations do not receive an adequate supply of nutrients, one of the reasons being that serum factors raise the internal nutrient concentration by making the transport system more active (Holley, 1972). In chick fibroblasts a low rate of rRNA synthesis and accumulation could be reversed by the addition of fresh serum (Emerson, 1971). The stimulation by serum factors would be attributed to replenishment of nutrients. An earlier hypothesis suggests that the actual contact of a cell with its neighbors is inhibitory (Stoker, 1971; Dulbecco, 1971). The stimulation by serum factors would then be attributed to the ability to dimish the inhibitory contacts. In 3T3 cells the first recognizable event following the addition of serum is an increase in the rate of RNA labeling within the first 30 minutes. This rate is not maintained and starts to decline within one hour (Todaro *et al.*, 1965). This is not unlike the observation made with the Shay chloroleukemic cells in culture. Suspensions prepared from solid tumor mass represent a new environment for the leukemic myeloid cell (Handler *et al.*, 1969) and could be considered similar to the situation where fresh media is added

to density inhibited cells. Simple depletion of nutrients and/or anoxia being the cause of the progressive decline in the rate of RNA synthesis is unlikely during the relatively short incubation period of three to five hours. The normal bone marrow cells are maintained in the identical medium and culture conditions for periods as long as eleven hours with no appreciable decline in RNA synthesis. Tumor cells have also been maintained in this laboratory for seven days with a feeding on day four. Throughout this time the cells were viable and increased in number (Handler *et al.*, 1974).

Other laboratories have been successful in the longterm culture of these leukemic cells (Moloney, 1974; Yunis *et al.*, 1975) and in DMBA (dimethylbenz(a)anthracene) induced chloroleukemia *in vitro* (Ioachim *et al.*, 1971). It is still an open question whether or not chemically induced Shay chloroleukemia operates as a virally transformed cell, as the presence of a latent virus cannot be excluded. "C" type particles have been observed both in intercellular spaces of leukemic marrow (Weinstein and Moloney, 1965) and budding from leukemic myeloblasts (Chen *et al.*, 1972).

If an inhibitory substance is produced by the tumor cells, it is possibly a granulocytic chalone.

Chalones are a group of molecules that act as humoral "inhibitors" of cell proliferation. There is no rigorous definition of a chalone, but there is general agreement on four principal characteristics of these substances: 1) chalones inhibit mitosis *in vitro* and *in vivo*, 2) their action is reversible; they are not cytotoxic, 3) they are synthesized by mature cells of the tissue upon which they act, and 4) they are tissue specific, but not species specific (Maugh, 1972).

Chalones have been implicated in the control mechanisms of several normal tissues (Bullough, 1973) and in malignant tumors, epidermal carcinoma (Bullough and Laurence, 1968a), melanocytic and amelanocytic melanomata (Bullough and Laurence, 1968b) and granulocytic leukemia (Rytomaa and Kiviniemi, 1968a). The chalones produced by these malignant tissues appear to be similar to those produced from normal tissues. However, at this time little is known about the structure of the molecule(s). Preliminary biochemical studies indicate considerable diversity. The epidermal, fibroblast and lymphocyte chalones appear to be glycoproteins of molecular weight from 30,000 to 100,000, whereas the melanocyte, liver, granulocyte and erythrocyte chalones are of molecular weight 2,000 to 5,000 and are peptides or very small glycoproteins (Bullough, 1973). It would appear that chalones differ depending on the cell of origin.

Malignant cells seem to lose their chalone at a very high rate, yet do respond to exogenous chalone *in vitro* (Rytomaa and Kiviniemi, 1968b). In the culture system employed here, it may be that the chalone produced by the leukemic myeloblast is rapidly lost to the medium, where it accumulates in adequate amounts to inhibit the metabolism of the myeloblast, as evidenced by a decline in RNA synthesis.

Granulocytic chalone is undoubtedly present in the system employed for these studies. Its effect however, is not assessable. Rytomaa and Kiviniemi (1968b) using autoradiography in studying the proliferative activity of normal bone marrow and chloroleukemic cells, have observed that in the absence of exogenous chalone, the rate of cell proliferation is lower in chloroleukemic than in normal bone marrow cells. However, due to delayed maturation, a larger number of progenitor cells are present in chloroleukemia; hence the rate of cell production and growth is greater in the leukemic bone marrow.

It has been noted previously that the response to EPO is still evident in the mixed, 50:50, *in vitro* cell culture. Possibly a granulocytic chalone is inhibiting the overall synthesis of RNA of both marrow cells and myeloblasts, but not affecting the ERC with respect to EPO stimulated RNA synthesis.

The granulocytic chalone has been implicated in the suppression of myelopoiesis in culture when 10^5 normal marrow cells plus 10^6 mature granulocytes were implanted intraperitoneally in a diffusion chamber into mice (Stohlman _et al._, 1973). The presence of chalone is also implicated in colonies grown in methylcellulose from leukemic bone marrow where an initial high level of CSF (colony stimulating factor) causes large numbers of colonies to appear. Conditioned medium from these cells prevents colony growth in normal bone marrow cultures (Handler _et al._, 1974). Two opposing forces seem to be acting on the development of the CFC (colony forming cell) during this leukemia. First a CSF active within the animal before culturing and second an inhibitor derived from the leukemic cells also present within the animal with increasing effect due to increasing numbers as the disease progresses. These opposing forces act to elicit an increase in the numbers of CFC coupled with an inhibition of their subsequent development leading to large numbers of abortive colonies seen in culture (Handler _et al._, 1974).

The granulocytic chalone has been used successfully to induce tumor regressions (Rytomaa and Kiviniemi, 1969). This is noteworthy because of its ramifications in possible clinical role of chalones. Granulocytic chalone obtained partly from normal granulocytes and

partly from subcutaneous tumors of the Shay chloroleukemic rat was able to cause regression of large chloromas. Treatment of generalized leukemia with chalone was not as effective, but prolongation of the mean survival time was observed.

In the present study it has been observed that the cell suspensions of pure populations of leukemic myeloblasts exhibit a small but consistent depression of RNA synthesis in the presence of the hormone. This is seen in the assay of total RNA and in the isolated RNA as a decrease in average specific activity in all size classes. The mechanism which is responsible for these observations is open to speculation.

The cell surface is known to play an important role in cell growth control. Cell membrane proteins and carbohydrates seem to be responsible for recognition of signals, both local and distant, affecting growth control (Cook, 1968). Receptor sites have been localized on the cell membrane for insulin (Cuatrecasas, 1972) and concanavalin A (Chase and Miller, 1973) and inferred for EPO (Chang _et al._, 1974). Proteolytic activity has been found to be associated with the surface membrane of transformed cells (Reich, 1974). Ovomucoid, a glycoprotein, is a protease inhibitor of the enzyme associated with Py3T3 cells and when added to cultures of these cells will inhibit growth (Talmadge _et al._, 1974). These workers, in a series

of elegant experiments using polyacrylamide bead coupled ovomucoid to study the effect on growth of Py3T3 and 3T3 cells, suggested that growth inhibition of Py3T3 does not involve uptake of the ovomucoid, but may be related to a critical event in the cell membrane. It is highly speculative, but conceivable, that an analogous situation exists in the tumor cell studies presented here with respect to the effect of EPO on these cells in cultures. With the development of the understanding of biological regulatory mechanisms one cannot overlook the possibility that cyclic 3', 5'-adenosine monophosphate (cAMP) plays a role as a controlling component. This compound is now viewed as a key regulatory effector of the particular process peculiar to a given cell type or of the event of importance at a given point in time in the maturation of a cell (Goldberg et al., 1974). There is evidence that in a number of cell cultures adenylate cyclase activity and cAMP levels may be dependent on the growth rate and/or cell density. Hormone-stimulated adenylate cyclase activity may be enhanced by cell-cell interaction or contact as indicated by studies of cultured fibroblasts, HeLa cells, Chang's liver cells and rat hepatoma cells (Makman, 1971). Abell and Monahan (1973) have shown that addition of dibutyryl cyclic AMP and cAMP inhibit DNA synthesis in phytohemagglutinin (PHA)-stimulated lymphocytes from patients with CLL at concentrations (10^{-5}M) that have essentially no effect on normal lymphocytes.

The adenyl cyclase-cAMP system has been suggested to regulate the cell cycle of hematopoietic stem cells. There is also some suggestion that EPO mediated stimulation of differentiation might operate via this membrane bound system (Fisher, 1972). At the present time it is not possible to say what the effect of EPO is on the tumor cell. It may be a nonspecific binding to the cell, or a possible binding of EPO to serum proteins with subsequent inhibition of RNA synthesis, *in vitro*. It is of interest to note that stimulatory and inhibitory proteins associated with the hematopoietic system (EPO, CSF, chalones) are glycoproteins. This class of compounds when purified and their structure elucidated may prove to be a system of exquisitely fine control for cellular differentiation.

SUMMARY:

A short term culture system was developed in which cell suspensions of bone marrow, over a range of concentrations, could be handled with ease. It was possible, using this system to analyze replicate samples when numbers of cells were at a premium.

In the Shay chloroleukemia impaired erythropoiesis, as reflected by RNA synthesis, is evidenced by a decrease in response to EPO. The decline in responsivity to the hormone is dependent not only on the presence of myeloblasts in the bone marrow, but also on the length of time after seeding of these leukemic cells.

Normal bone marrow cell suspensions respond to EPO with a predictable increase in average specific activity of different classes of RNA. Average specific activity increases were observed predominently in the 45S and 8-10S regions of sucrose gradients for RNA isolated from pulse labeled cultures, and in all size classes for RNA isolated from continuously labeled cultures. The leukemic state disrupts this pattern. In all cases studied it could not be predicted from the percent of myeloblasts in the bone marrow which, if any, class of RNA would be stimulated in response to EPO. However, all samples were different from those of normal bone marrow.

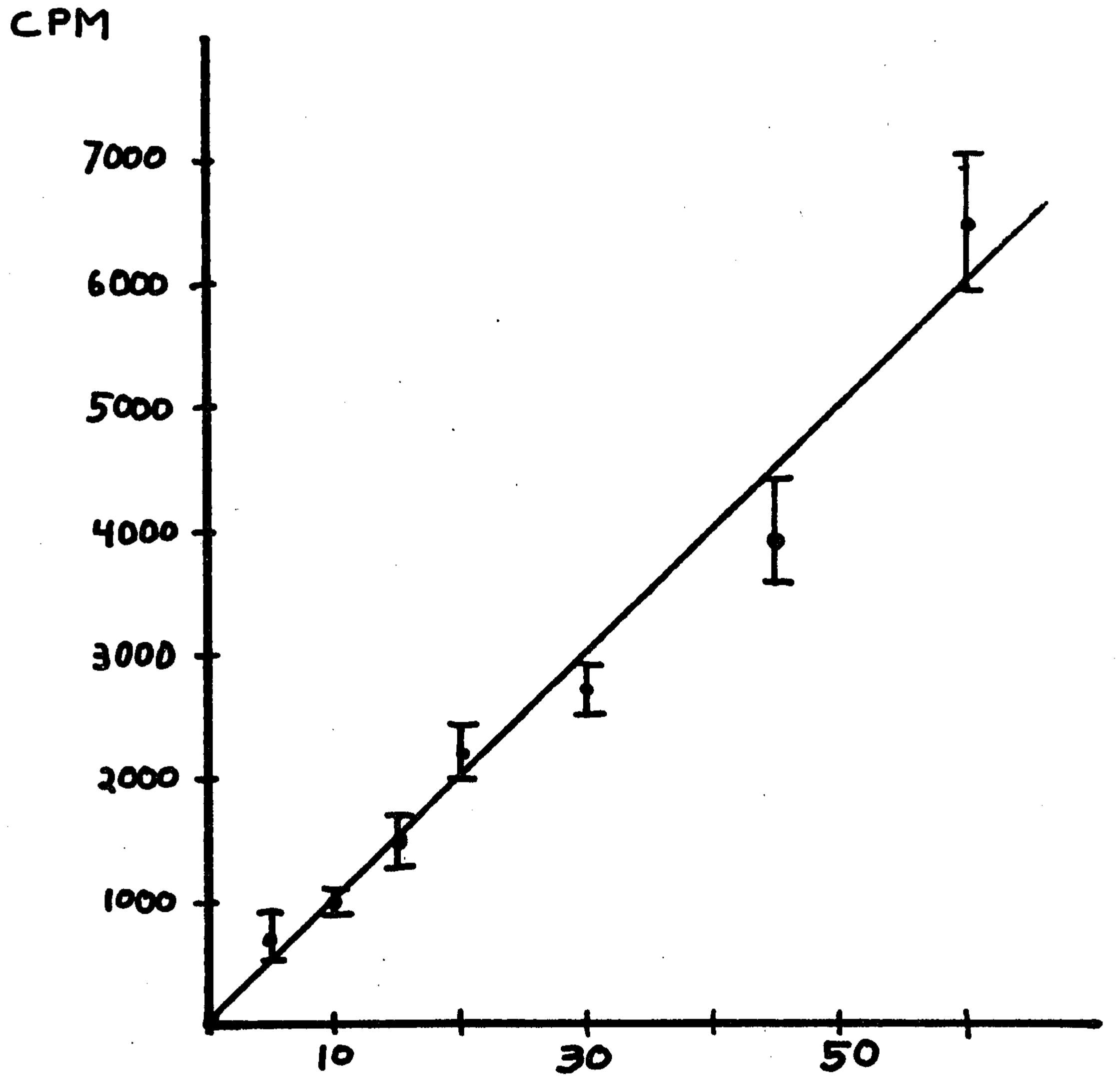
The bone marrow in early stages of leukemia i.e. a low percent myeloblast content in the bone marrow of a 3-5 day leukemic animal, showed a hyperactivity as evidenced by a markedly high level of average specific activity of all classes of RNA. The average specific activity of RNA isolated from these marrow was greater than that observed in normal, 8-10 day leukemic marrow or leukemic myeloblasts alone.

Spleen cell suspensions prepared from both normal and leukemic animals did not respond to EPO by an increase in RNA synthesis.

Leukemic myeloblast cell suspensions exhibit a small depression of RNA synthesis when incubated in the presence of EPO. This depression was evidenced as a decrease in the average specific activity in all classes of RNA isolated from these cell suspensions. The leukemic myeloblast also demonstrated a cell concentration dependent rate of RNA synthesis. Synthesis varied directly with cell concentration at low density and inversely at high cell density.

FIGURE 1:

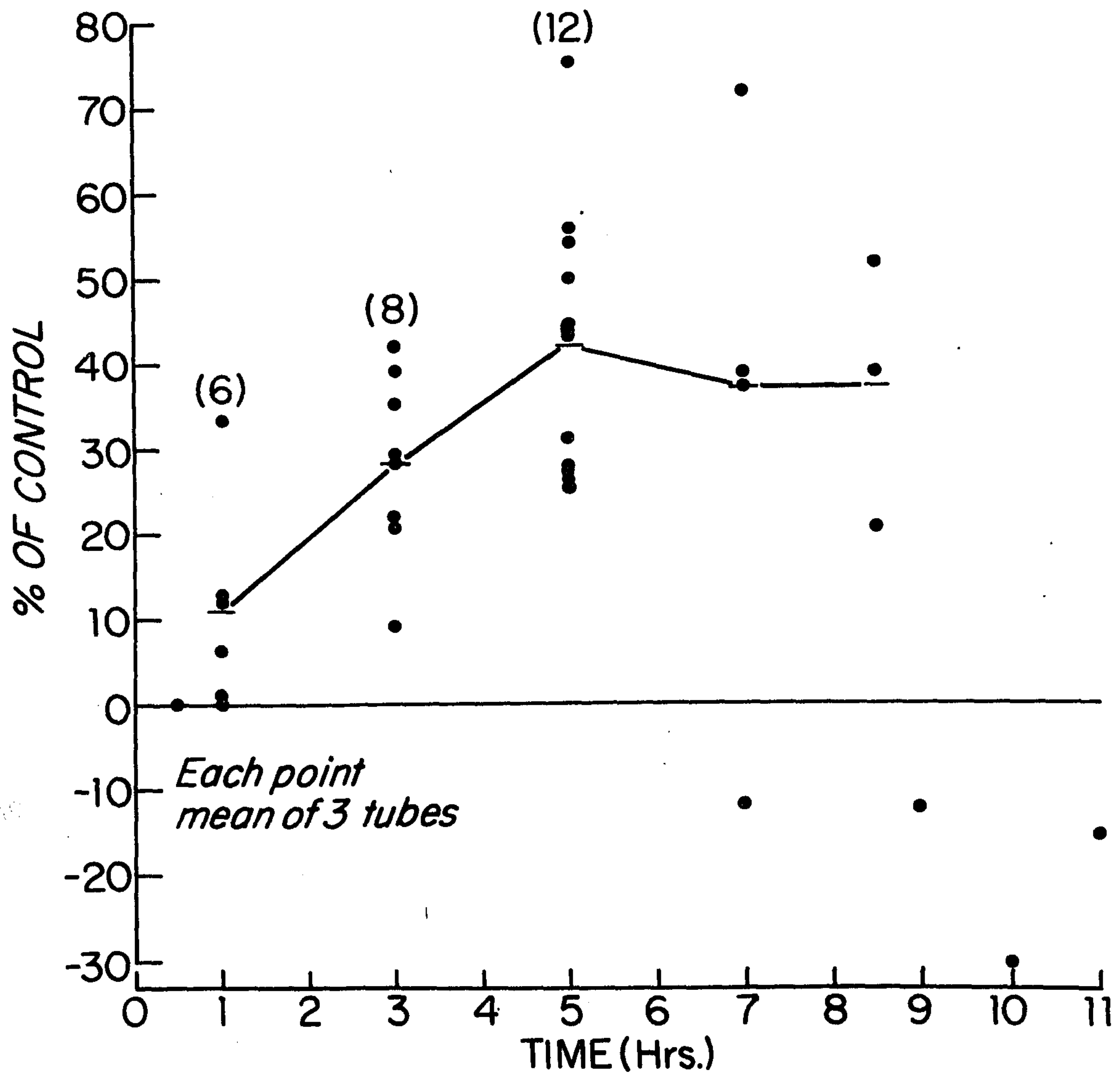
Incorporation of ^{3}H-uridine into RNA of normal rat bone marrow cell suspensions as a function of cell concentration. Cell suspensions were incubated for 5 hours and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-uridine at the end of the incubation period. Each point represents the mean of two separate experiments $\pm$S.E.M. $r=0.98$, $m=99$, $b=16$



CONCENTRATION (10^6 cells/ml)

FIGURE 2:

The effect of EPO on ^{3}H-uridine incorporation into RNA by normal rat bone marrow cells *in vitro*. Cells ($30X10^{6}$ cells/ml) were incubated in triplicate cultures for the time indicated and pulse labeled with 4uCi/ml of ^{3}H-uridine at the end of the incubation period. The curve represents the mean; numbers in parentheses indicate the number of experiments.



80
70
60
50
40
30
20
10
0
-10
-20
-30
% OF CONTROL
(6)
(8)
(12)
Each point
mean of 3 tubes
0
1
2
3
4
5
6
7
8
9
10
11
TIME (Hrs.)

FIGURE 3:

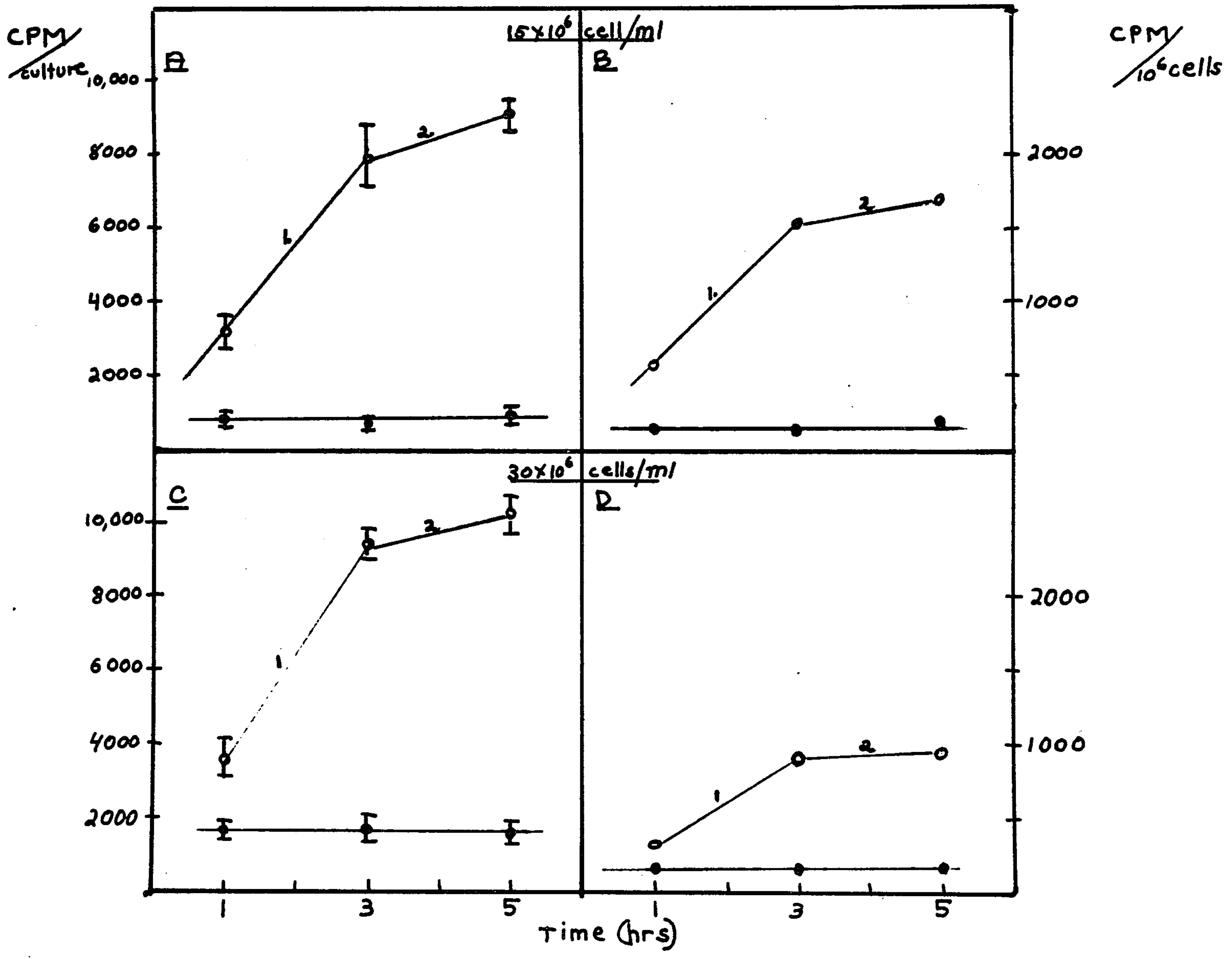
Incorporation of ^{3}H-uridine into RNA by normal rat bone marrow cell suspensions at two concentrations. Cell suspensions were prepared from pooled marrow of 3 rats. In A and C each point represents the mean of triplicate cultures $\pm$S.E.M. In B and D each point is the mean divided by the number of cells per culture.
(●-●-●) 20 min. pulse; (o-o-o) continuous label

20 min. pulse:

A: m=46, b=708
B: m=9, b=135
C: m=-15, b=1715
D: m=-1.5, b=164

continuous label:

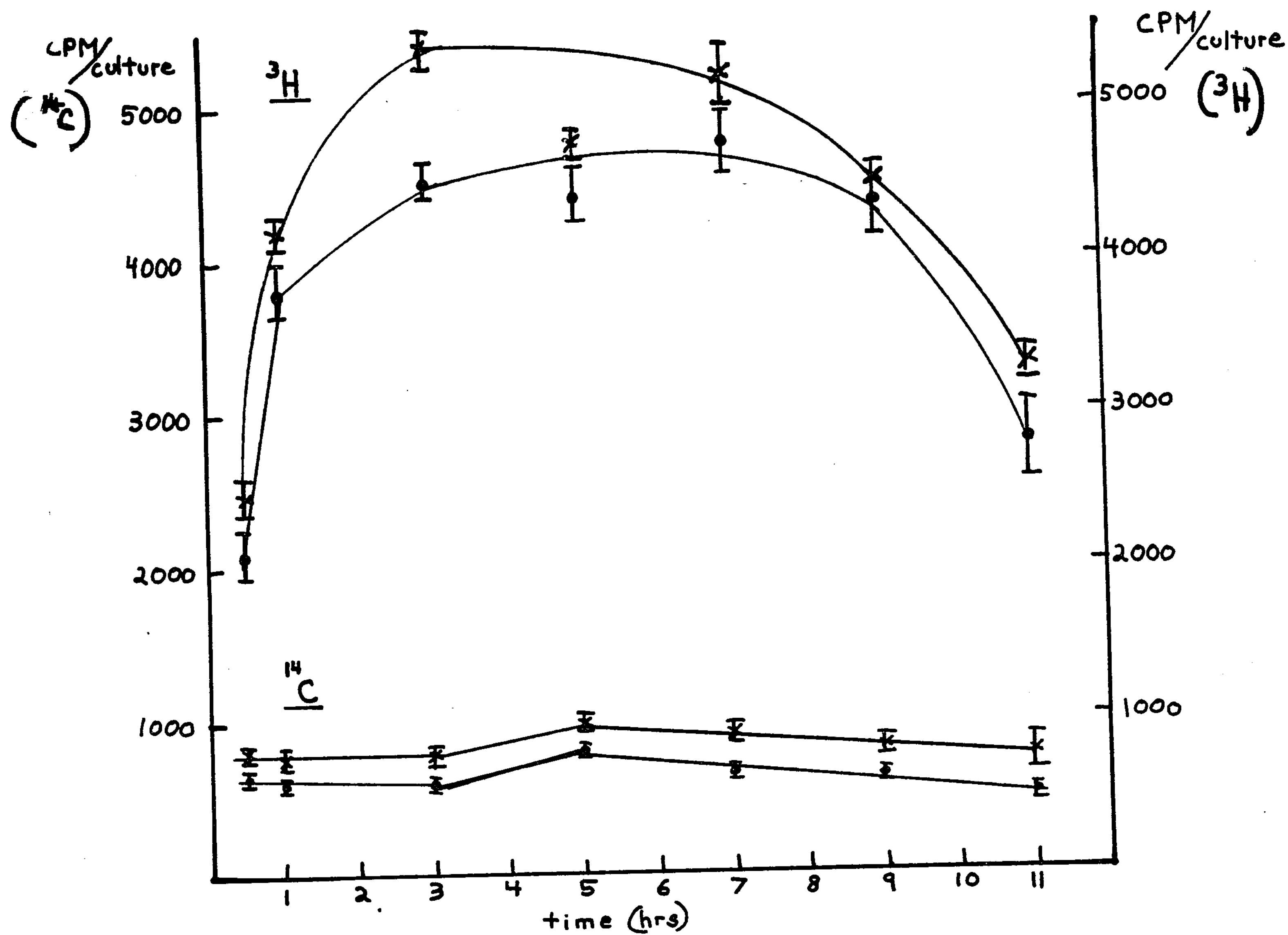
A: 1) m=2403, b=724; 2) m=572, b=6215
B: 1) m=458, b=138; 2) m=110, b=1181
C: 1) m=2912, b=684; 2) m=380, b=8279
D: 1) m=290, b=30; 2) m=35, b=795



CPM/culture
CPM/10^6 cells
15x10^6 cell/ml
30x10^6 cells/ml
A
B
C
D
10,000
8000
6000
4000
2000
1000
1
2
1
3
5
Time (hrs)

FIGURE 4:

The effect of EPO on uridine incorporation into RNA by normal rat bone marrow cells *in vitro*. Cell suspensions were prepared from pooled marrow of 6 rats and incubated at $30X10^6$ cells/ml for the times indicated. Cell suspensions were pulse labeled for 20 minutes with 0.2uCi/ml of ^{14}C-uridine at the end of the incubation period and labeled from the onset of incubation with 4uCi/ml of ^{3}H-uridine. (●-●-●) -EPO, (x-x-x) +EPO.



CPM/culture
(^{14}C)
5000
4000
3000
2000
1000
^{3}H
^{14}C
CPM/culture
(^{3}H)
5000
4000
3000
2000
1000
1
2
3
4
5
6
7
8
9
10
11
time (hrs)

FIGURE 5:

Incorporation of ^{3}H-uridine into RNA by homogeneous suspensions of leukemic myeloblasts prepared from one chloroma. Cell suspensions were incubated for 5 hours and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-uridine at the end of the incubation period. Each point represents the mean of triplicate cultures $\pm$S.E.M. r=0.99, m=-41, b=430

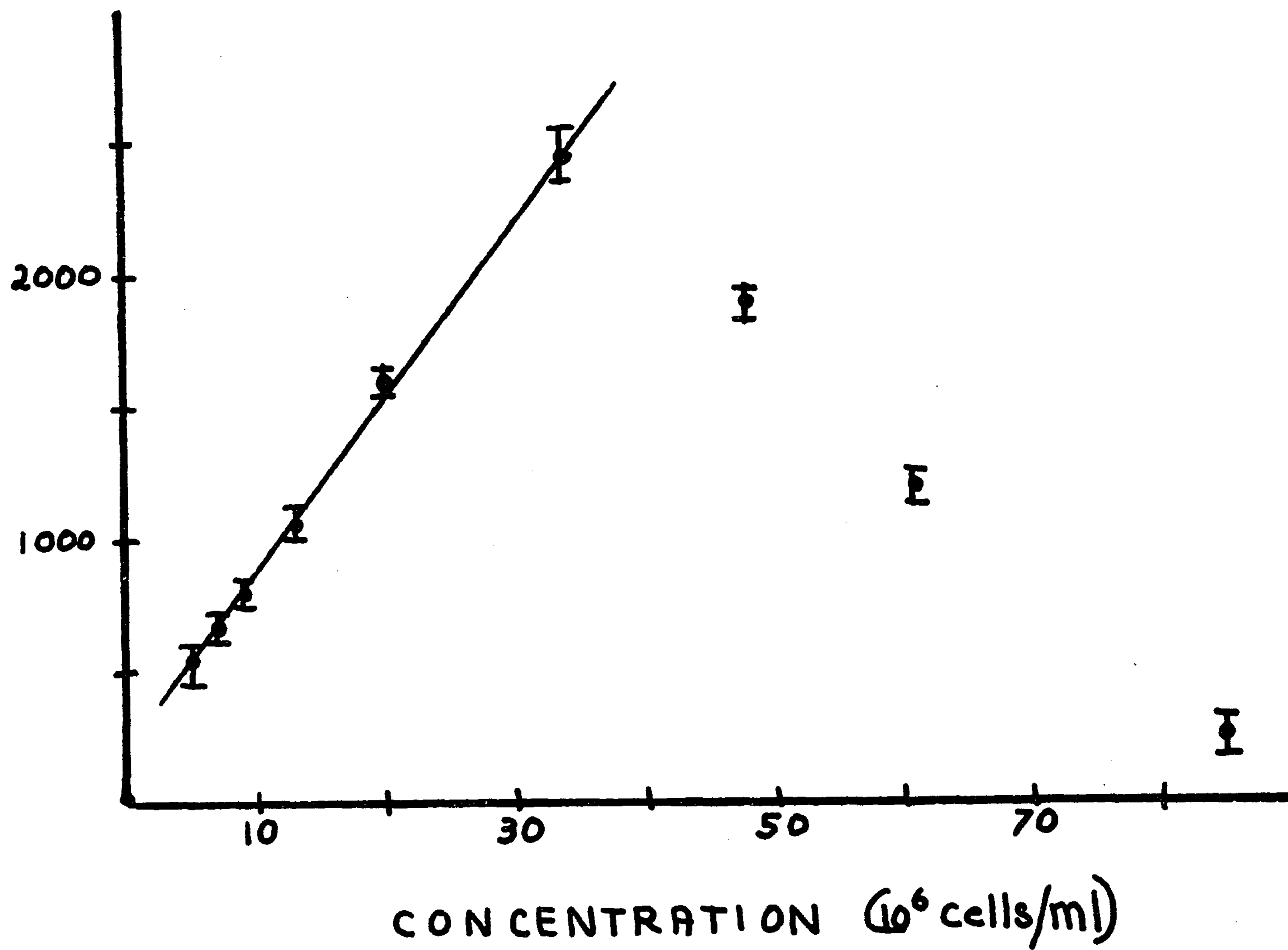
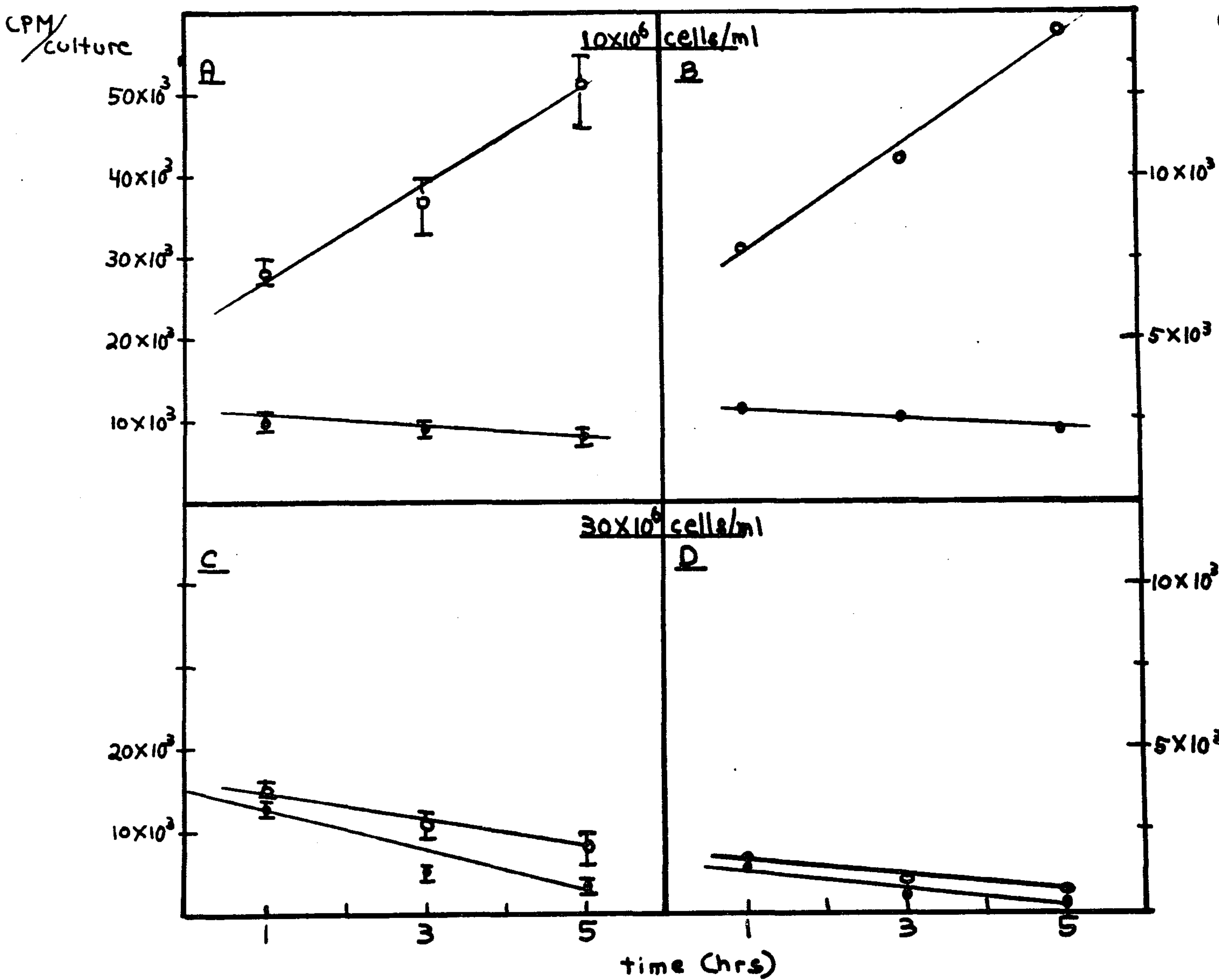

CPM
2000
1000
10
30
50
70
CONCENTRATION (10⁶ cells/ml)

FIGURE 6:

Incorporation of ^{3}H-uridine into RNA by homogenous suspensions of leukemic myeloblasts prepared from one chloroma. Cell suspensions were incubated at two concentrations. In A and C each point represents the mean of triplicate cultures $\pm$S.E.M. In B and D each point is the mean divided by the number of cells per culture.
(●-●-●) 20 min. pulse; (o-o-o) continuous label

20 min. pulse:	continuous label:
A: m=-567, b=10905	A: m=5967, b=20909
B: m=-161, b=3115	B: m=1705, b=5974
C: m=-2963, b=15589	C: m=-1524, b=15617
D: m=-282, b=1494	D: m=-145, b=1487

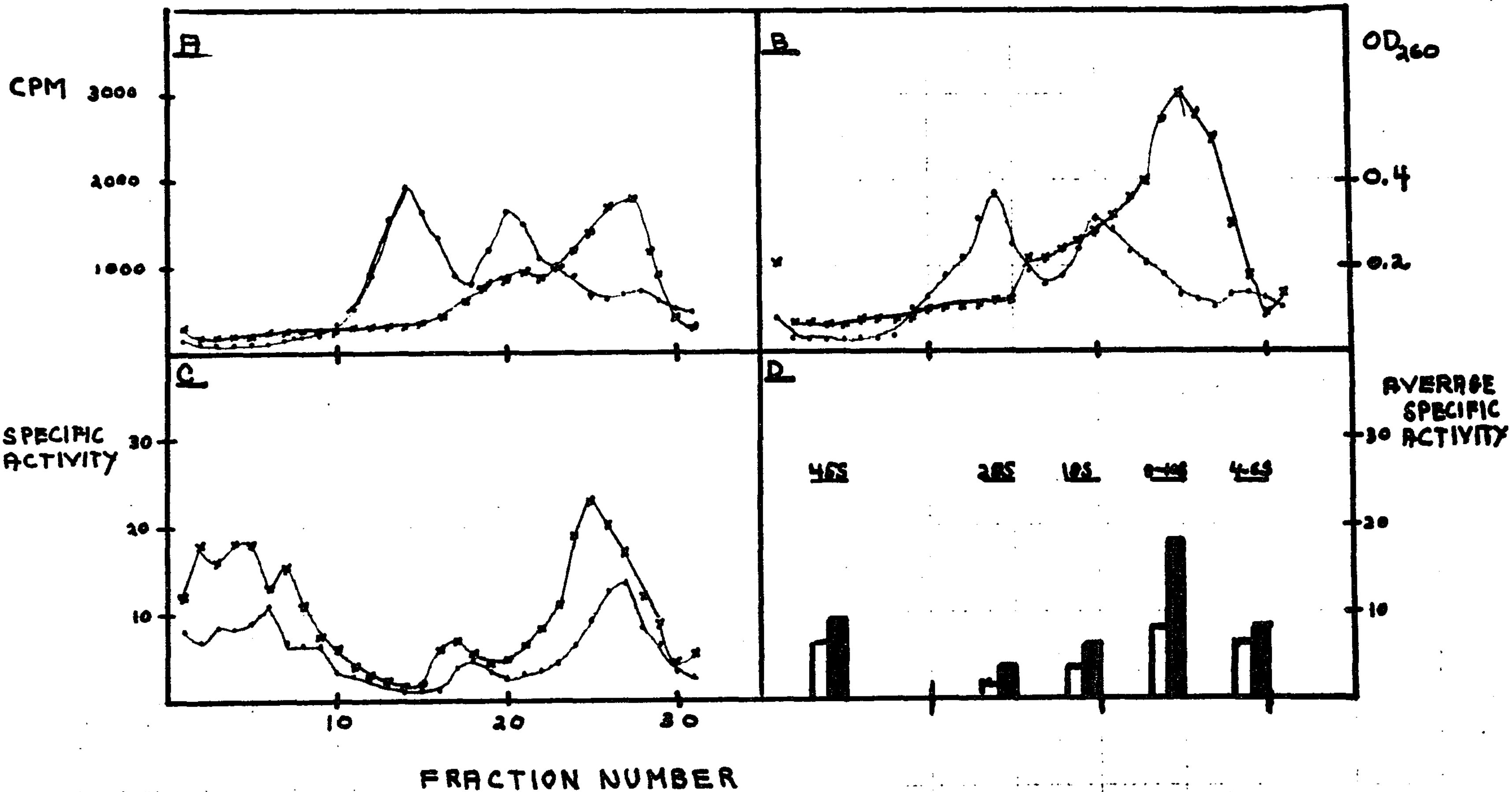


CPM/culture
CPM/10⁶ cells
10x10⁶ cells/ml
30x10⁶ cells/ml
A
B
C
D
50x10³
40x10³
30x10³
20x10³
10x10³
10x10³
5x10³
1
3
5
time (hrs)

FIGURE 7:

RNA extracted from cell suspensions of normal bone marrow. Replicate one ml cultures were incubated for 3 hours and pulse labeled for 20 minutes with 30uCi/ml of ^3H-uridine at the end of the incubation period.

A: Incubation without EPO
B: Incubation with EPO, (●-●-●)OD_{260}, (x-x-x) cpm
C: Specific activity (SA: cpm/OD_{260}X10^3) for each fraction of parallel gradients. (●-●-●) -EPO; (x-x-x) +EPO
D: Average specific activity (AvSA) of each region of the gradient (open bars, -EPO; closed bars, +EPO)



A
B
C
D
CPM
3000
2000
1000
OD260
0.4
0.2
SPECIFIC ACTIVITY
30
20
10
AVERAGE SPECIFIC ACTIVITY
10
20
30
FRACTION NUMBER

FIGURE 8:

Average specific activity of classes of RNA extracted from normal bone marrow. A and B represent separate experiments using cell suspensions prepared from pooled marrow of three rats. Three hour incubation; 20 minute pulse labeled with 30uCi/ml ^{3}H-uridine at the end of incubation period. (open bars, without EPO; closed bars, with EPO).

Average Specific Activity

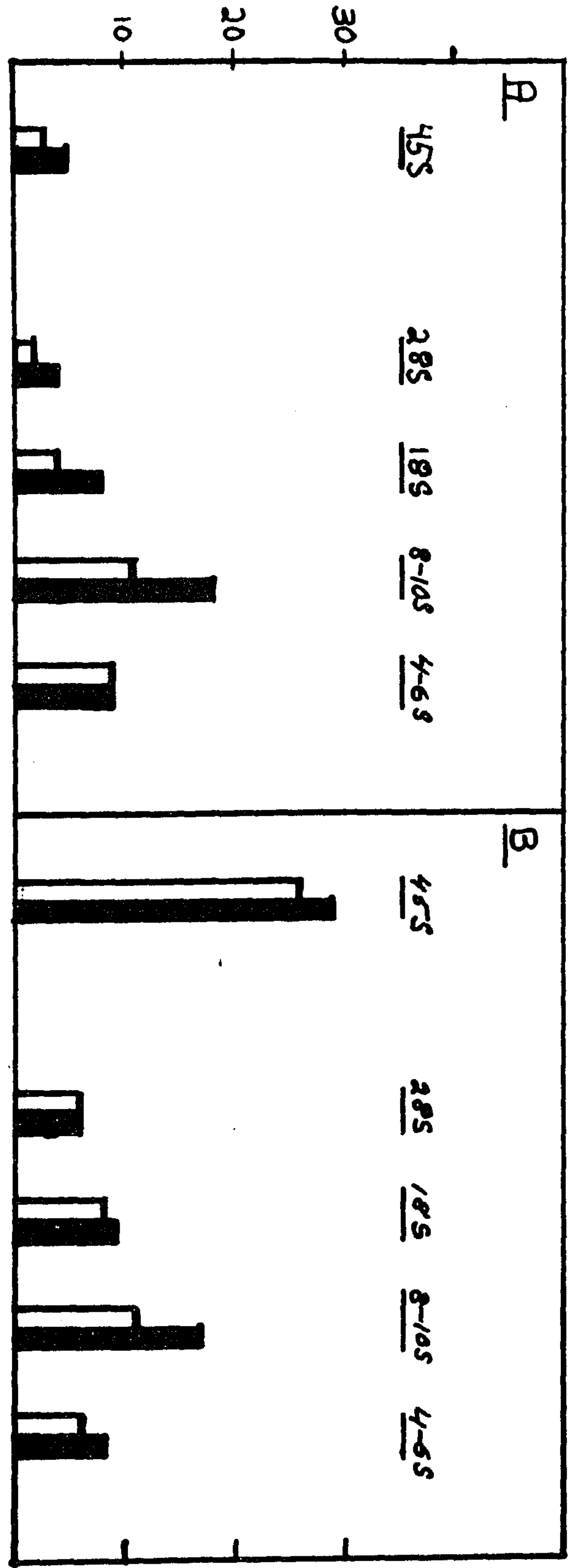
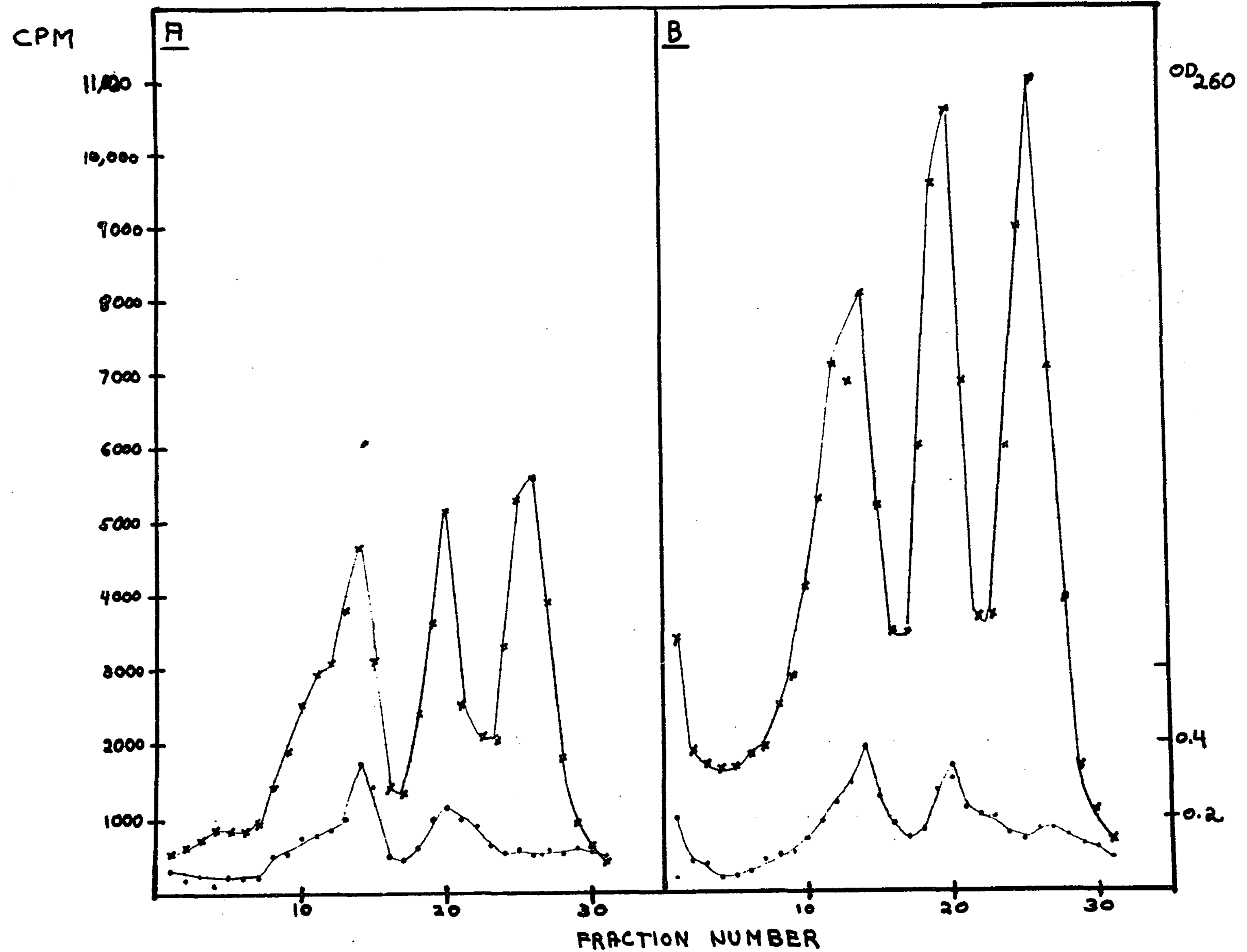


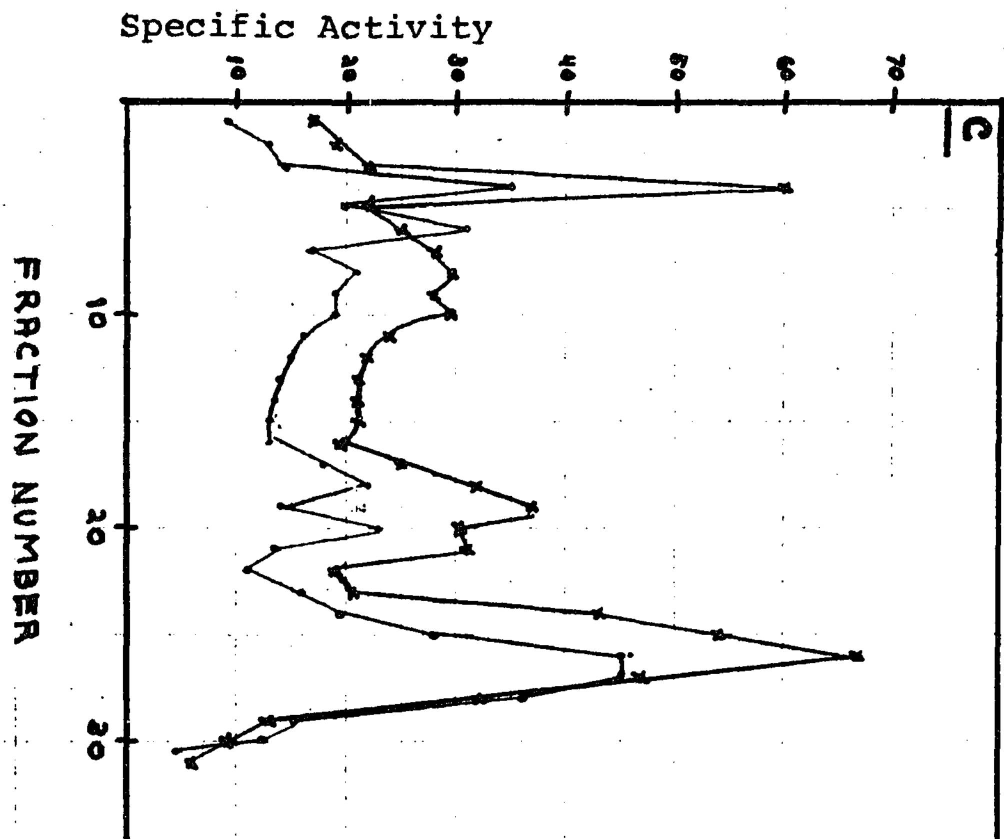
FIGURE 9:

RNA extracted from cell suspensions of normal bone marrow. Replicate one ml cultures were incubated for 3 hours and labeled from the onset of incubation with 30uCi/ml ^{3}H-uridine.

A: Incubation without EPO
B: Incubation with EPO, (●-●-●)OD_{260}, (x-x-x) cpm
C: Specific activity (cpm/OD_{260}X10^3) for each fraction of parallel gradients (●-●-●) -EPO; (x-x-x) +EPO
D: Average specific activity of each region of the gradient (open bars, -EPO; closed bars, +EPO)



CPM
A
B
11,000
10,000
9000
8000
7000
6000
5000
4000
3000
2000
1000
OD260
0.4
0.2
10
20
30
FRACTION NUMBER


Specific Activity
C
FRACTION NUMBER

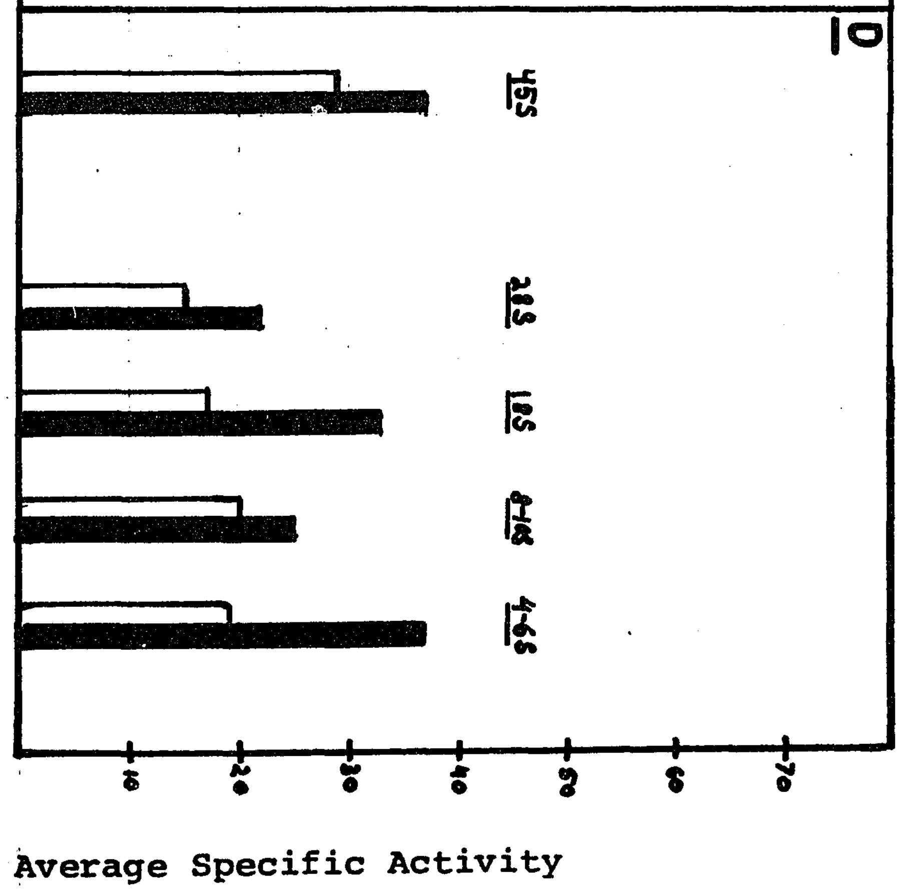

D
45S
28S
18S
8-18S
4-6S
Average Specific Activity

FIGURE 10:

Average specific activity of classes of RNA obtained from normal bone marrow. RNA was prepared as described in Fig. 9. open bars, without EPO; closed bars, with EPO.

Average Specific Activity

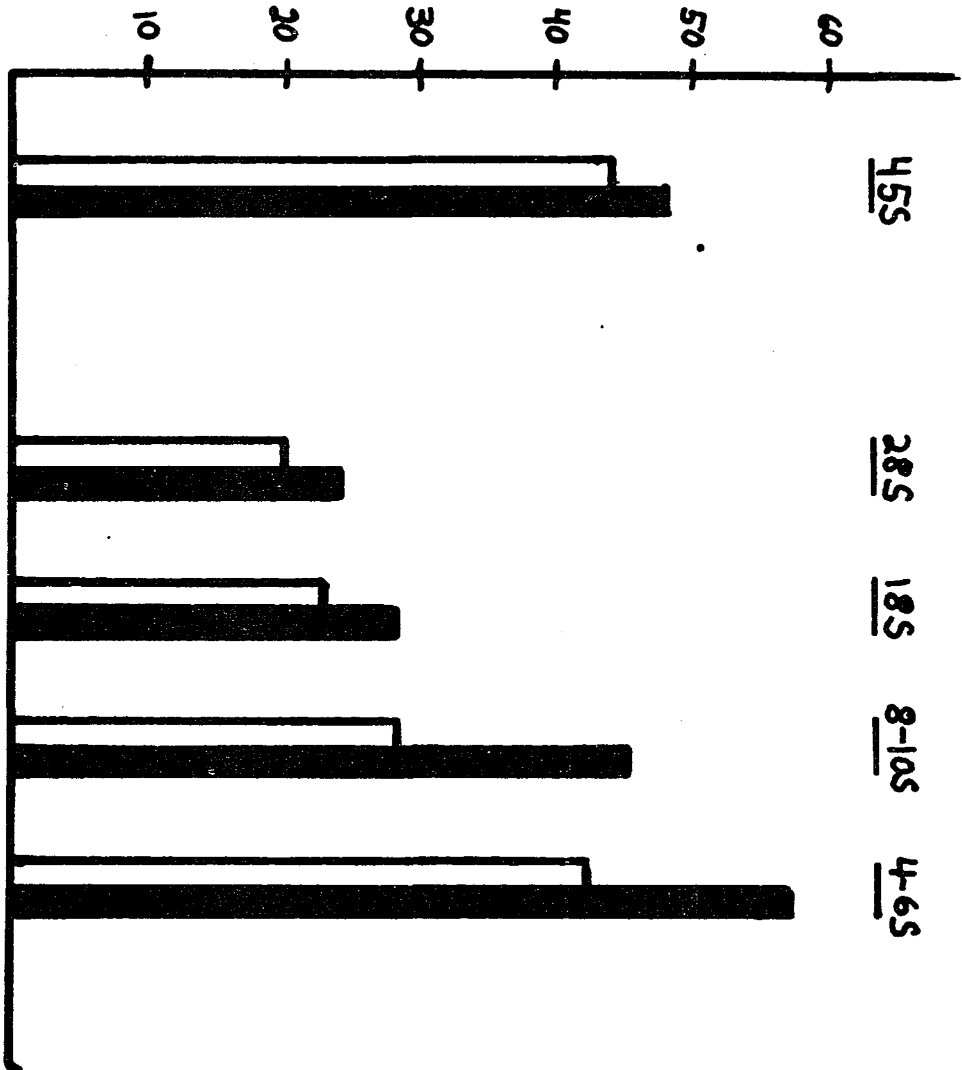


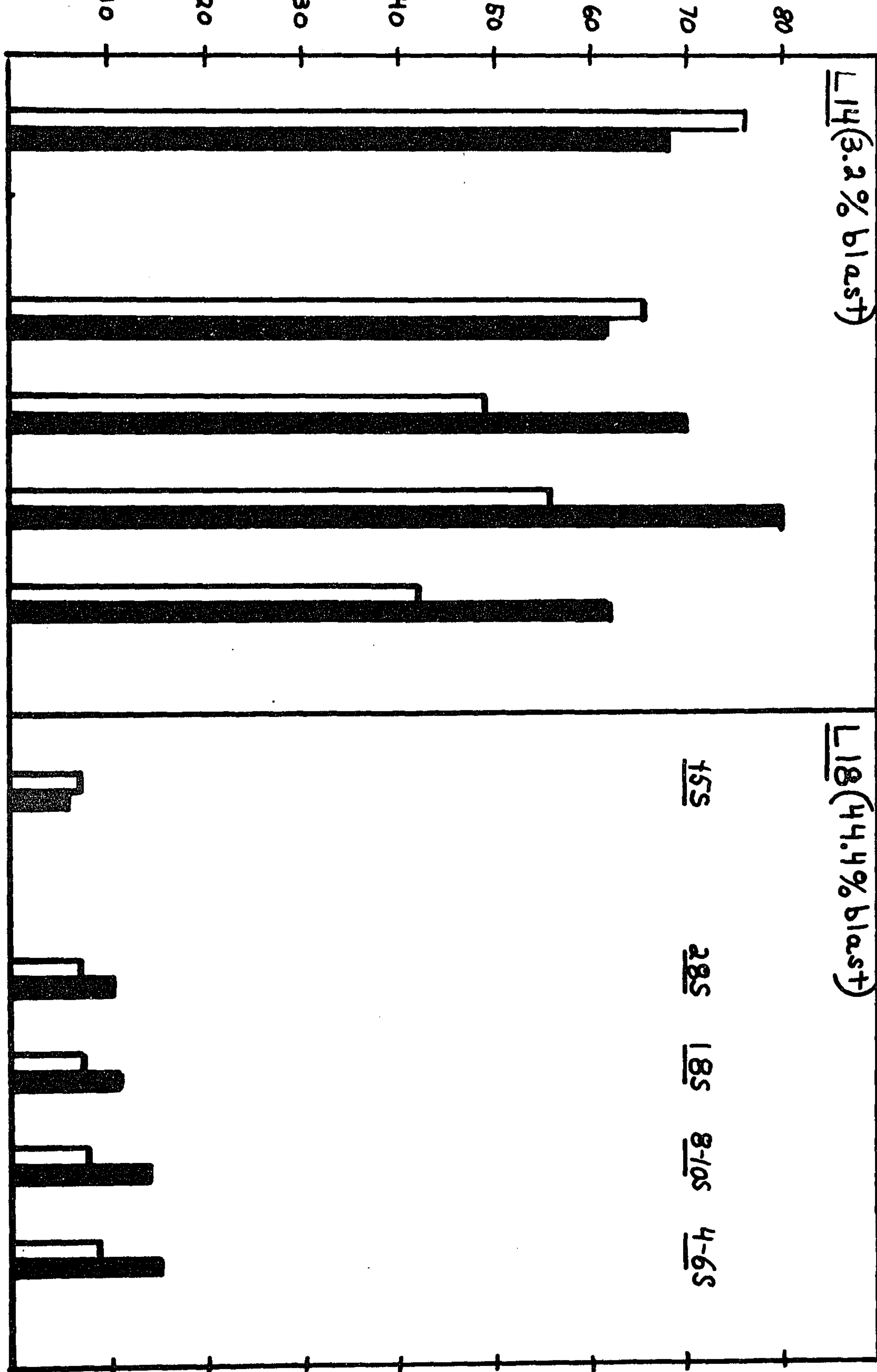
FIGURE 11:

Average specific activity of RNA extracted from bone marrow of 8-10 day leukemic rats. Leukemic myeloblast percentage for each rat is given in parentheses. Three hour incubation with 25-30X10^6 cells/ml; pulse labeled for 20 minutes with 30uCi/ml ^{3}H-uridine at the end of the incubation period. (open bars, -EPO; closed bars, +EPO).

L13 (1.3% blast)
L20 (47.3% blast)
L21 (52.6% blast)
L23 (61.5% blast)
45S
28S
18S
8-10S
4-6S
Average Specific Activity
10
20
30
40
50

FIGURE 12:

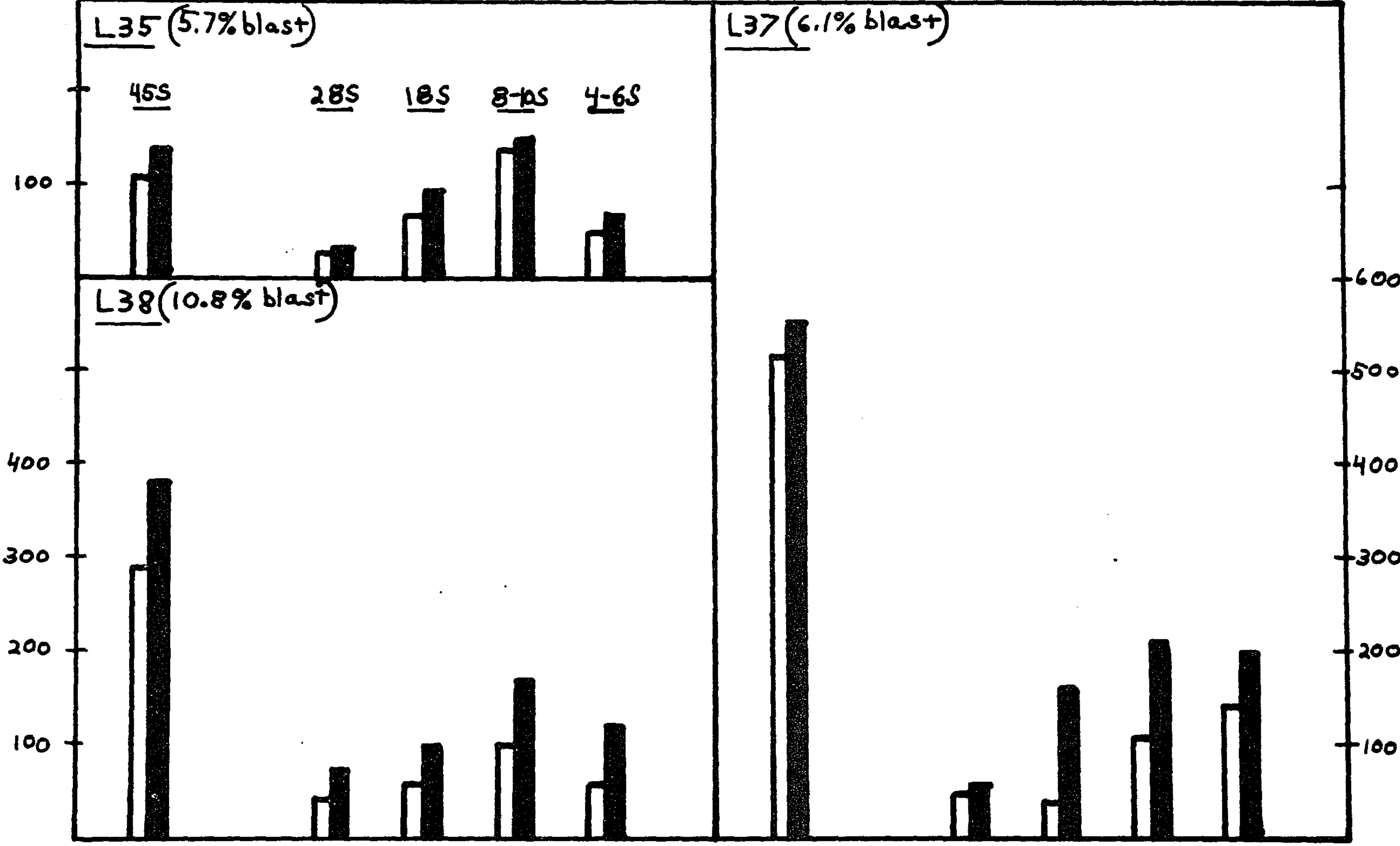
Average specific activity of RNA extracted from bone marrow of 8-10 day leukemic rats. Leukemic myeloblast percentage for each rat is given in parentheses. Three hour incubation with $25\text{-}30\times10^6$ cells/ml; labeled from the onset of the incubation period with 30uCi/ml ^{3}H-uridine. (open bars, -EPO; closed bars, +EPO).



Average Specific Activity
10
20
30
40
50
60
70
80
L14 (3.2% blast)
L18 (44.4% blast)
45S
28S
18S
8-10S
4-6S

FIGURE 13:

Average specific activity of RNA extracted from bone marrow of 3-5 day leukemic rats. Leukemic myeloblast percentage for each rat is given in parentheses. Three hour incubation with 25-30X10^6cells/ml; pulse labeled for 20 minutes at the end of the incubation period with 30uCi/ml ^{3}H-uridine. (open bars, -EPO; closed bars, +EPO).



Average Specific Activity
L35 (5.7% blast)
45S
28S
18S
8-10S
4-6S
100
L38 (10.8% blast)
100
200
300
400
L37 (6.1% blast)
100
200
300
400
500
600

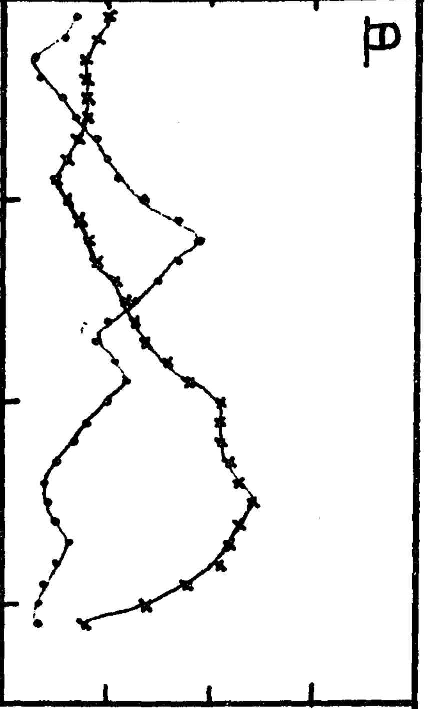
FIGURE 14:

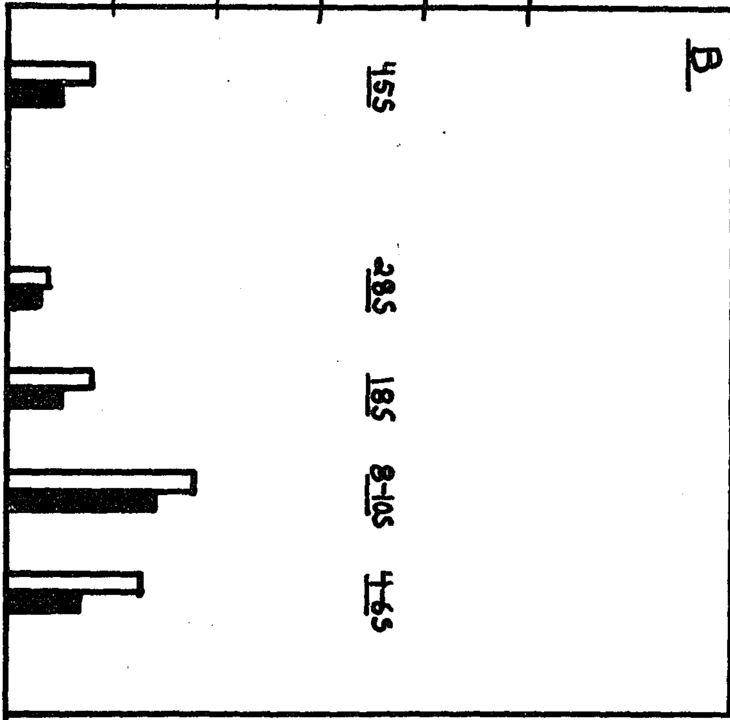
Sucrose density gradient analysis of RNA extracted from a pure population of leukemic myeloblasts harvested from one subcutaneous chloroleukemic tumor. Cell suspensions were incubated for three hours at $15X10^6$ cells/ml and at $30X10^6$ cells/ml with and without EPO and pulse labeled with 30uCi/ml ^{3}H-uridine at the end of the incubation period.

A: Optical density trace and cpm illustrated is of a typical profile of RNA from tumor cell suspensions incubated at $30X10^6$ cells/ml without EPO. (●-●-●)OD_{260}, (x-x-x) cpm.

B: Average specific activity of different classes of RNA isolated from incubations at $30X10^6$ cells/ml with and without EPO.

C: Average specific activity of different classes of RNA isolated from incubations at $15X10^6$ cells/ml with and without EPO. (open bars, -EPO; closed bars, +EPO).


A
OD260
0.2
0.4
CPM
1000
2000
3000


B
Average Specific Activity
10
20
30
40
50
45S
28S
18S
8-10S
4-6S

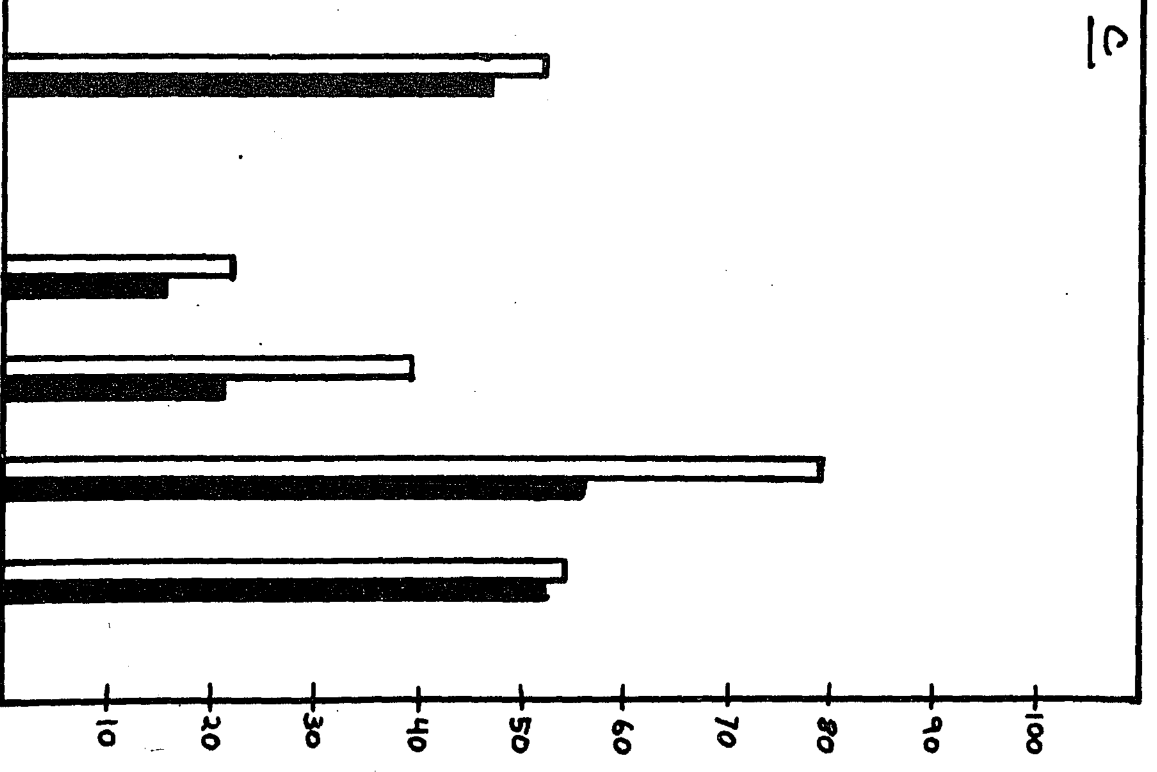

C
10
20
30
40
50
60
70
80
90
100

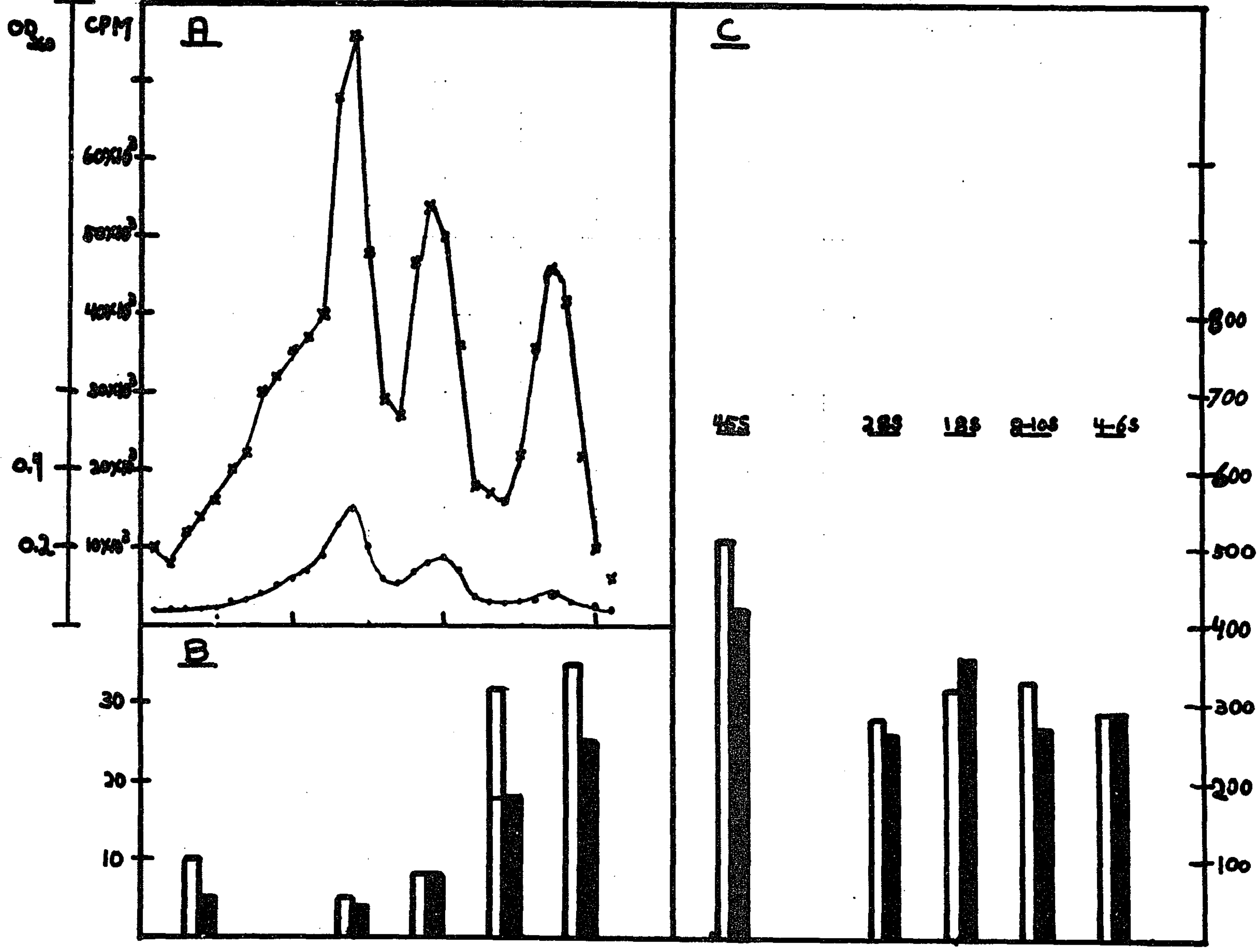
FIGURE 15:

Sucrose density gradient analysis of RNA extracted from a pure population of leukemic myeloblasts harvested from one subcutaneous chloroleukemic tumor. Cell suspensions were incubated from 3 hours at 15×10^6 cells/ml and at 30×10^6 cells/ml with and without EPO and labeled from the onset of incubation with 30uCi/ml ^{3}H-uridine.

A: Optical density trace and cpm illustrated is of a typical profile of RNA from tumor cell suspensions incubated at 15×10^6 cells/ml without EPO. (●-●-●) OD_{260}, (x-x-x) cpm.

B: Average specific activity of different classes of RNA isolated from incubations at 30×10^6 cells/ml with and without EPO.

C: Average specific activity of different classes of RNA isolated from incubations at 15×10^6 cells/ml with and without EPO. (open bars, -EPO; closed bars, +EPO).



Average Specific Activity

FIGURE 16:

Average specific activity of RNA extracted from 50:50 mixtures of normal bone marrow and tumor cell suspensions. Tumor cell suspensions were prepared from one subcutaneous chloroma. Normal bone marrow cell suspensions were prepared from pooled marrow of three rats. Cell suspensions were incubated for 3 hours at $15X10^6$ cells/ml and at $30X10^6$ cells/ml with and without EPO and either pulse labeled or labeled from the onset of incubation with 30uCi/ml ^{3}H-uridine. $15X10^6$ cells/ml; A) pulse label, B) continuous label $30X10^6$ cells/ml; C) pulse label, D) continuous label open bars, -EPO; closed bars, +EPO.

Average Specific Activity

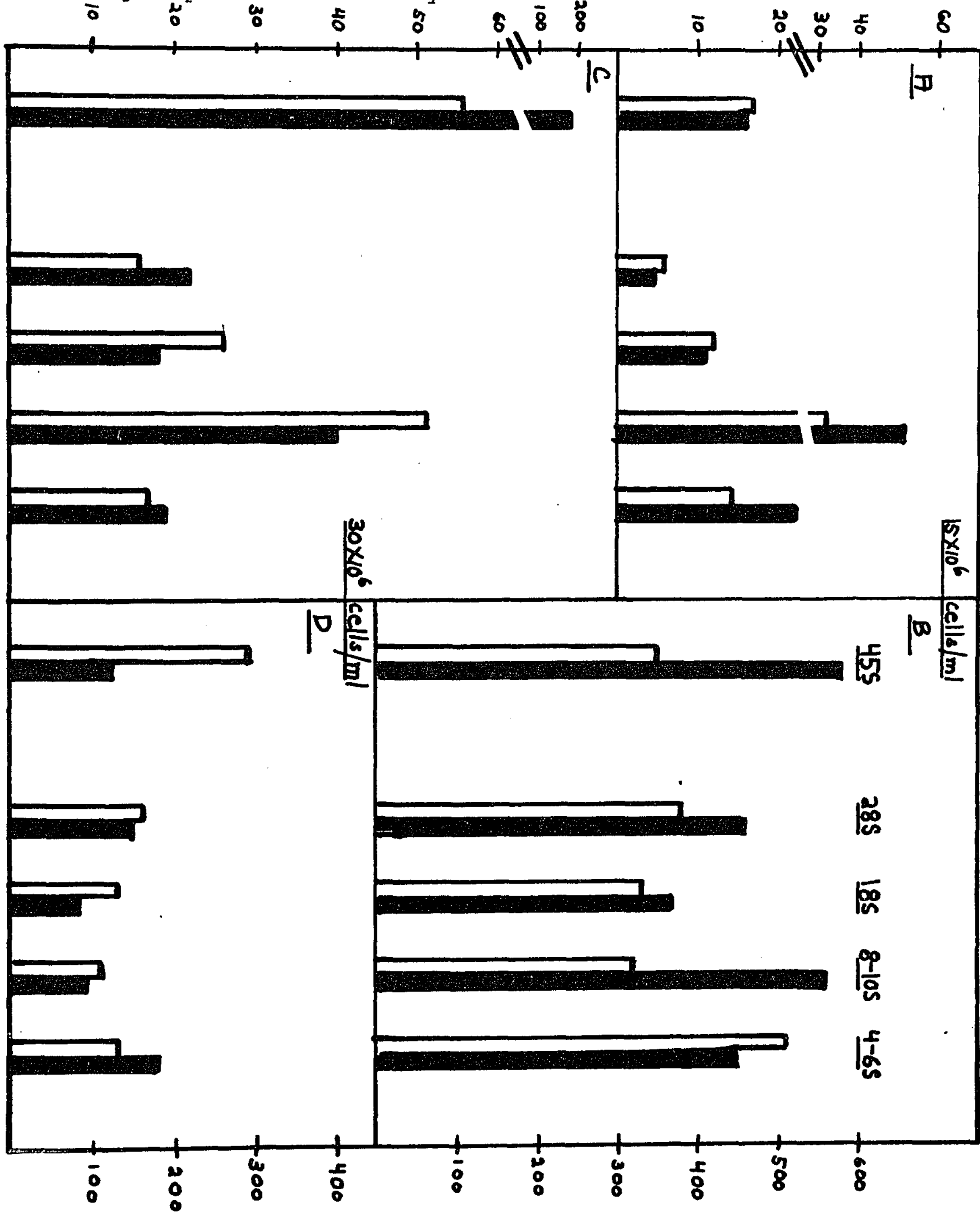


TABLE I

Effect of EPO on ^{3}H-uridine incorporation into RNA of bone marrow cell suspensions prepared from normal rats. Mean percent benzidine positive cells; 24.8±3.0

Cell Concentration (cells/ml)	Number of Experiments	CPM -EPO	CPM +EPO	Percent Change
3-hr. Incubation:				
30X10^{6}	10	1542±190*	2002±230	+29.8
5X10^{6}	6	494±59	625±74	+27.8
5-hr. Incubation:				
30X10^{6}	12	1420±130	2030±240	+42.5
5X10^{6}	5	523±112	666±116	+32.5

*Mean ±S.E.M.

TABLE II

Effect of ^{3}H-uridine specific activity on incorporation into RNA by normal bone marrow cells in culture. - Cells (30X10^{6}cells/ml) were incubated for 3 hrs. and pulse labeled for 20 min. with ^{3}H-uridine at the end of the incubation period. Each number represents the mean of triplicate cultures ±S.E.M.

Specific Activity of ^{3}H-Uridine(uCi/30X10^{6}cells)	3-hr. Incubation CPM -EPO		3-hr. Incubation CPM +EPO		Percent Change
15	4290	±210	5350	±410	+26.3
8	2700	±290	3390	±230	+25.6
4	1480	±200	1870	± 30	+24.5

TABLE III

Effect of EPO on ^{3}H-uridine incorporation into RNA of bone marrow cell suspensions harvested from rats 8-10 days after the onset of the myelogenous leukemia. Cells ($30X10^{6}$ cells/ml) were incubated for 5-hr. and pulse labeled for 20 min. with 4uCi/ml of ^{3}H-uridine at the end of the incubation period.

Animal	Percent Benzidine Positive Cells	Percent Blast	CPM -EPO	CPM +EPO	Percent Change
L1	24.3	<1.0	1360	2150	+58.1
L2	22.3	3.0	2540	1930	-24.1
L3	20.4	5.4	2570	2180	-15.3
L4	22.3	7.0	1890	1890	0
L5	19.8	29.0	4580	4810	+ 5.0
L6	6.8	44.0	2460	2700	+ 9.7
L7	9.2	54.0	2700	2620	- 2.9
L8	3.4	58.5	7760	7450	- 3.9
L9	5.1	63.0	8960	7780	-13.2
L10	<1.0	97.0	5660	5660	0
L11	<1.0	97.0	5960	6710	+12.6

TABLE IV

Effect of EPO on ^{3}H-uridine incorporation into RNA of bone marrow cell suspensions harvested from rats 8-10 days after the onset of the myelogenous leukemia. Cells ($5X10^{6}$ cells/ml) were incubated for 3-hrs. and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-5-uridine at the end of the incubation period. Parallel samples were exposed to isotope from the onset of incubation and terminated at the time indicated in parentheses.

Animal	Percent Benzidine Positive Cells	Percent Blast	CPM -EPO	CPM +EPO	Percent Change	CPM -EPO	CPM +EPO	Percent Change
			Pulse Label			Continuous Label		
L12	28.6	1.0	348	426	+22.3	-	-	-
L13	22.4	1.3	550	672	+22.0	-	-	-
L14	7.4	3.2	1087	1380	+27.0	16530	15724	- 4.9 (3-hr.)
L15	6.7	7.7	1085	1618	+49.1	14545	15975	+ 9.8 (3-hr.)
L16	8.6	24.4	1377	1445	+ 4.9	24426	26655	+ 9.1 (3-hr.)
L17	2.8	34.5	2384	2566	+ 7.6	-	-	-
L18	9.7	44.4	4571	4864	+ 6.4	50829	51473	+ 1.2 (3-hr.)
L19	4.5	46.4	2581	2475	- 4.1	8601	9987	+16.1 (1-hr.)
L20	5.5	47.3	3546	3570	0.0	-	-	-
L21	3.4	52.6	5785	5904	+ 2.1	-	-	-
L22	6.2	59.0	3835	3953	+ 3.1	54330	59830	+10.0 (3-hr.)
L23	3.4	61.5	4148	4548	+ 9.6	-	-	-
L24	3.2	62.0	2948	2702	- 8.3	12553	13235	+ 4.6 (1-hr.)
L25	3.6	72.9	5755	6632	+15.2	78992	78333	0.0 (3-hr.)

TABLE V

Effect of EPO on ^{3}H-uridine incorporation into RNA of bone marrow cell suspensions harvested from rats 3-5 days after the onset of the myelogenous leukemia. Cells ($30X10^{6}$ cells/ml) were incubated for 5-hr. and pulse labeled for 20 minutes with 4 uCi/ml of ^{3}H-5-uridine at the end of the incubation period.

Animal	Percent Benzidine Positive Cells	Percent Blast	CPM -EPO	CPM +EPO	Percent Change
L26	22.1	<1.0	1400	2040	+42.9
L27	24.6	2.1	1500	1800	+20.5
L28	23.3	2.8	1990	2670	+33.8
L29	14.1	3.6	4350	4710	+17.2
L30	19.2	4.4	3000	3650	+17.8
L31	14.6	15.4	2890	3710	+16.1
L32	19.6	26.0	3300	3420	+ 3.8

TABLE VI

Effect of EPO on ^{3}H-uridine incorporation into RNA of bone marrow cell suspensions harvested from rats 3-5 days after the onset of the myelogenous leukemia. Cells ($5X10^{6}$ cells/ml) were incubated for 3-hrs. and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-5-uridine at the end of the incubation period.

Animal	Percent Benzidine Positive Cells	Percent Blast	CPM -EPO	CPM +EPO	Percent Change
L33	24.8	<1.0	931	869	- 6.7
L34	21.3	<1.0	885	1078	+21.8
L35	19.5	2.5	443	536	+21.0
L36	24.1	5.7	693	805	+16.2
L37	11.0	6.1	748	922	+22.3
L38	14.6	10.8	651	876	+43.6

TABLE VII

Effect of EPO on ^{3}H-uridine incorporation into RNA of pure populations of leukemic cells prepared from 10 day subcutaneous tumors. Cells ($30X10^6$cell/ml) were incubated for the indicated incubation period and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-uridine at the end of the incubation period.

Tumor	3-hr. Incubation			5-hr. Incubation		
	CPM -EPO	CPM +EPO	Percent Change	CPM -EPO	CPM +EPO	Percent Change
1	3670	3390	- 8.9	1210	1090	- 9.4
2	3560	3180	-10.8	2810	2760	- 1.8
3	1530	1500	- 1.7	2660	2840	+ 6.8
4	3000	2000	-33.3	3120	1950	-37.3
5	5130	4760	- 7.3	4770	4880	+ 2.3
6	18612	18278	- 1.8			
7	1256	1292	+ 2.8			

TABLE VIII

Effect of ^{3}H-uridine specific activity on incorporation into RNA by tumor cells in culture. Cells ($15X10^6$ cells/ml) were incubated for 3 hrs. either pulse labeled for 20 minutes at the end of the incubation period or labeled from the onset of incubation. Each number represents the mean of triplicate cultures ±S.E.M.

Specific Activity of ^{3}H-Uridine(uCi/$15X10^6$ cells)	3-hr. Incubation CPM	
	20 min.	Continuous
4	6878±398	14000±260
8	13299±1506	24622±3685
15	26331±3196	52400±1030
30	47126±5378	86447±6912

TABLE IX

^{3}H-uridine incorporation into RNA in 50:50 mixtures of normal bone marrow and tumor cells. All cell suspensions were incubated for 3-hrs. and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-5-uridine at the end of the incubation period. One subcutaneous tumor and an aliquot bone marrow cells obtained from 3 rats was used for each assay. Each number represents the mean of triplicate cultures ±S.E.M.

Normal Bone Marrow			Tumor			50:50		
CPM -EPO	CPM +EPO	Percent Change	CPM -EPO	CPM +EPO	Percent Change	CPM -EPO	CPM +EPO	Percent Change
Total Number of Cells ($30X10^{6}$ cells/ml)								
A.								
928±132	1401±96	+51.0	8682±977	-	-	4092±143	5185±223	+26.7
Total Number of Cells ($15X10^{6}$ cells/ml)								
B.								
729±87	1032±130	+40.5	8571±327	-	-	4000±456	4528±205	+13.2
C.								
1435±81	1882±31	+31.1	3468±203	2772±105	-20.1	2765±33	3414±291	+23.5
D.								
1287±83	1832±64	+42.3	7451±1609	7897±1016	+ 6.0	4875±157	6199±49	+27.2

TABLE X

Effect of EPO on ^{3}H-uridine incorporation into RNA of spleen cell suspensions prepared from normal rats. The mean percent benzidine positive cells 3.4 ± 0.8.

Cell Concentration (cells/ml)	Number of Experiments	CPM -EPO	CPM +EPO	Percent Change
3-hr. Incubation:				
30×10^6	4	1410 ± 70*	1480 ± 180	+4.9
5-hr. Incubation:				
30×10^6	4	1470 ± 270	1460 ± 280	-0.7

*Mean $\pm$S.E.M.

TABLE XI:

Effect of EPO on ^{3}H-uridine incorporation into RNA of spleen cell suspensions harvested from rats 8-10 days after the onset of the myelogenous leukemia. Cells (30×10^{6} cells/ml) were incubated for the time indicated and pulse labeled for 20 minutes with 4uCi/ml of ^{3}H-5-uridine at the end of the incubation period.

Animal	Percent Benzidine Positive Cells	Percent Blast	CPM -EPO	CPM +EPO	Percent Change
5-hr. Incubation:					
L1	1.3	0.0	640	760	+19.4
L2	2.0	1.5	1090	920	-15.8
L5	8.0	3.2	570	410	-28.5
L6	5.2	73.5	4740	3780	-20.2
L7	8.3	1.9	320	350	+ 8.7
L9	19.1	2.5	770	380	-50.2
L10	14.7	63.0	5730	5270	- 8.0
L11	6.1	65.3	5520	4640	-16.1
3-hr. Incubation:					
L12	2.4	1.0	673	839	+24.7
L13	16.1	1.0	891	832	- 6.6
L14	1.0	0.0	1231	1135	- 7.8
L15	3.8	<1.0	1311	1205	- 8.1
L16	11.5	<1.0	1050	976	- 7.0
L17	10.6	14.6	1183	1088	- 8.0
L18	3.4	1.0	2372	1751	-26.2
L19	3.6	1.3	2772	2759	0.0
L20	1.0	1.0	4636	5436	+17.3
L21	13.5	6.6	5720	5094	-10.0
L22	14.8	2.4	9986	10263	+ 2.8
L23	10.4	32.0	5334	5389	+ 1.0
L24	14.7	8.1	1764	1762	0.0
L25	16.9	3.8	2244	1683	-25.0

TABLE XII:

Effect of EPO on ^{3}H-uridine incorporation into RNA of spleen cell suspensions harvested from rats 3-5 days after the onset of the myelogenous leukemia. Cells (30X10^{6}cells/ml) were incubated for the time indicated and pulse labeled for 20 minutes with 4 uCi/ml of ^{3}H-5-uridine at the end of the incubation period.

Animal	Percent Benzidine Positive Cells	Percent Blast	CPM -EPO	CPM +EPO	Percent Change
5-hr. Incubation:					
L26	1.2	<1.0	1000	710	-10.9
L27	6.4	<1.0	940	610	-35.0
L28	7.7	1.5	810	890	+ 9.2
L29	7.2	<1.0	1040	990	- 5.3
L30	6.4	<1.0	1480	1470	0.0
L31	11.4	33.0	2260	2800	+ 5.4
L32	4.1	69.0	3410	3850	+12.9
3-hr. Incubation:					
L33	7.5	<1.0	2038	2362	+15.9
L34	8.0	<1.0	2134	2306	+ 8.1
L35	4.2	<1.0	1319	1009	-13.5
L36	6.5	<1.0	1687	1177	-30.2
L37	1.2	<1.0	1683	1700	+ 1.0
L38	5.0	<1.0	1257	1220	- 2.0

TABLE XIII

Addenda to Figs. 7-11. The following table presents the data of the actual amounts of material applied and recovered from sucrose density gradients reported. Within each pair the first values presented are from incubations without EPO; the second values, with EPO. The calculation of 28S/18S values was from maximum peak values.

Applied			Recovered										
							Percentages Recovered						
							Total	Total	OD_{260} in RNA size classes				
OD_{260}	CPM	$\frac{CPM}{OD_{260}}$	OD_{260}	CPM	$\frac{CPM}{OD_{260}}$	$\frac{28S}{18S}$	OD_{260}	CPM	45S	28S	18S	8-10S	4-6S
Fig.7:													
4.7	20560	4.4	4.6	19220	4.2	1.2	98	93	2	31	27	15	18
4.8	40799	8.5	4.7	31895	6.8	1.3	102	78	3	30	27	13	16
Fig.8(A):													
3.0	17413	5.8	3.1	16290	5.2	1.2	103	93	11	33	22	16	15
2.6	17738	6.8	2.8	17568	6.3	1.5	106	100	18	36	18	9	12
Fig.8(B):													
3.1	35214	11	2.5	23286	9.3	1.3	81	70	5	24	16	15	24
4.0	50346	13	3.5	36742	11	1.4	78	73	5	27	16	11	19
Fig.9:													
4.3	66915	16	4.0	71969	18	1.5	93	107	5	34	24	16	20
5.3	163780	31	4.9	144974	30	1.4	92	89	6	31	23	12	18
Fig.10:													
4.9	144600	30	4.8	127185	27	1.2	98	88	3	30	25	15	17
5.5	186760	34	4.9	151880	31	1.2	89	82	8	33	27	11	8
Fig.11:(L13)													
3.0	24560	8.2	2.9	23485	8.1	1.1	96	96	7	24	16	11	14
2.2	25590	12	1.7	22112	13	1.1	77	86	13	18	13	12	19

TABLE XIII (Contd)

Applied			Recovered						Percentages Recovered				
							Total	Total	OD_{260} in RNA size classes				
OD_{260}	CPM	$\frac{CPM}{OD_{260}}$	OD_{260}	CPM	$\frac{CPM}{OD_{260}}$	$\frac{28S}{18S}$	OD_{260}	CPM	45S	28S	18S	8-10S	4-6S
Fig.11:(L20)													
5.2	77248	15	5.3	72594	14	1.2	102	94	11	19	25	8	6
5.2	74736	14	5.4	68265	13	1.9	104	91	14	38	25	10	7
Fig.11:(L21)													
2.8	39230	14	2.2	39222	18	1.6	79	100	14	31	14	5	19
3.0	27375	9.1	3.0	27320	9.1	1.4	100	100	14	26	20	9	10
Fig.11:(L23)													
1.7	34125	20	1.4	33850	24	1.3	82	99	18	20	18	13	10
1.6	36230	23	1.3	34691	27	1.2	80	95	14	27	17	11	7
Fig.12:(L14)													
4.6	387520	84	4.7	332063	71	1.1	102	86	17	26	23	17	13
4.2	342705	82	4.2	273365	65	1.1	100	80	14	29	25	13	14
Fig.12:(L18)													
3.6	22680	6.5	3.3	20579	6.3	1.4	92	91	7	25	20	16	32
3.5	33355	9.5	3.1	32970	11	1.1	89	99	8	26	26	12	21
Fig.13:(L35)													
1.2	133020	111	0.94	92130	98	1.8	78	69	11	44	12	4	27
1.3	183271	141	0.99	151254	153	1.5	76	83	5	36	15	7	3
Fig.13:(L37)													
3.2	503200	173	3.1	375332	121	0.98	97	75	2	35	41	8	13
2.6	591720	228	2.2	480450	218	1.1	85	81	2	39	31	8	14
Fig.13:(L38)													
1.3	210600	162	1.4	140636	105	1.1	108	70	2	34	35	6	19
1.0	142029	142	0.82	136415	171	1.8	80	96	6	37	19	2	33

TABLE XIII (Contd)

Applied			Recovered										
							Percentages Recovered						
							Total	Total	OD_{260} in RNA size classes				
OD_{260}	CPM	$\frac{CPM}{OD_{260}}$	OD_{260}	CPM	$\frac{CPM}{OD_{260}}$	$\frac{28S}{18S}$	OD_{260}	CPM	45S	28S	18S	8-10S	4-6S
Fig.14:(A)(B)													
4.5	34423	7.7	4.5	34796	7.7	1.7	100	101	13	40	19	13	8
4.7	33149	7.1	4.8	32183	6.7	1.4	102	97	14	37	21	14	3
Fig.14:(C)													
3.3	165620	50	3.1	132496	43	1.7	94	80	13	30	21	10	16
3.3	154836	47	3.5	122321	35	1.5	106	79	9	30	21	7	13
Fig.15:(B)													
3.9	42725	11	4.0	39784	10	1.2	102	93	4	38	33	8	13
3.9	39530	10	3.4	31162	9.3	1.3	87	79	2	41	31	6	12
Fig.15:(A)(C)													
3.3	1491X10^{3}	452	3.2	1209X10^{3}	372	1.8	98	81	8	45	27	9	18
3.5	1588X10^{3}	453	3.6	1242X10^{3}	345	1.5	102	78	7	41	25	9	16
Fig.16:(A)													
2.8	62880	22	2.6	49276	19	1.1	93	78	5	30	24	14	17
3.0	57045	19	2.9	51197	18	1.1	96	90	4	33	24	11	13
Fig.16:(B)													
3.6	1552X10^{3}	431	3.8	1445X10^{3}	380	1.1	105	93	19	31	29	7	8
3.6	1709X10^{3}	474	3.7	1556X10^{3}	420	1.2	103	91	6	29	30	9	14
Fig.16:(C)													
3.1	117102	38	3.1	130759	42	1.4	100	111	16	33	18	11	22
3.5	121914	35	3.8	109063	29	1.4	108	89	3	37	19	8	26
Fig.16:(D)													
5.2	1080X10^{3}	207	5.3	899200	164	1.2	101	81	5	30	25	12	15
5.7	1106X10^{3}	194	5.2	782359	150	1.1	91	71	8	30	26	13	14

TABLE XIV

Addenda to Fig. 7-11. The following table presents the data of the actual amount of radioactivity recovered within each RNA species from sucrose density gradients and the percent change with EPO. Within each pair the first values presented are from incubations without EPO, the second values, with EPO. Numbers in parentheses are the percent change in AvSA with EPO.

	45S	28S	18S	8-10S	4-6S
Fig. 7	917 2301 (+37)	2345 4485 (+93)	4070 7405 (+72)	5425 10585 (+130)	5183 6570 (+38)
Fig. 8(A):	1205 2335 (+36)	1955 3415 (+94)	3015 3850 (+68)	5200 4650 (+73)	4220 2940 (0)
Fig. 8(B):	2345 4950 (+25)	3340 5195 (0)	2950 4800 (+13)	3980 6045 (+51)	3860 5130 (+17)
Fig. 9:	4220 8740 (+21)	20270 36920 (+46)	13875 36610 (+124)	7575 17990 (+20)	18620 30285 (+59)
Fig. 10:	5030 13760 (+16)	29255 39354 (+20)	29790 37316 (+24)	19620 23903 (+71)	35170 23510 (+41)
Fig. 11 (L13):	2175 2310 (-24)	7290 3365 (0)	4122 2095 (+5)	2230 3640 (+21)	5255 2670 (+11)
Fig. 11 (L20):	3374 3600 (+8)	6400 7580 (+10)	14805 17095 (+8)	18990 15440 (+2)	19910 17440 (+6)
Fig. 11 (L21):	3790 1680 (-66)	3645 1325 (-67)	8570 7280 (-55)	7160 7910 (-21)	9610 5760 (-24)
Fig. 11 (L23):	3090 2805 (+200)	5285 2220 (-66)	8420 5995 (-44)	10595 12250 (+28)	6313 4240 (-22)

TABLE XIV (Contd)

	45S	28S	18S	8-10S	4-6S
Fig. 12 (L14):	62485 40735 (-10)	82075 74400 (-9)	59795 75615 (+40)	47710 43680 (+36)	28590 37545 (+50)
Fig. 12 (L18):	1150 1710 (-28)	4830 8380 (+65)	3960 9430 (+83)	3700 5320 (+71)	8075 9635 (+75)
Fig. 13 (L35):	12660 7600 (+27)	13990 13260 (+17)	9200 15020 (+36)	5880 11680 (+7)	14530 2100 (+35)
Fig. 13 (L37):	32240 24200 (+6)	54250 51485 (+20)	50800 109125 (+300)	27280 36960 (+90)	56420 61600 (+43)
Fig. 13 (L38):	8125 22800 (+31)	19040 27745 (+88)	29395 19050 (+67)	8405 3200 (+60)	21280 39605 (+100)
Fig. 14 (A, B):	4390 2154 (-42)	4880 2515 (-33)	6765 5885 (-12)	11270 6530 (-22)	4836 5037 (-38)
Fig. 14(C):	20955 14805 (-10)	20460 15750 (-32)	25390 15435 (-46)	24490 13720 (-29)	26785 23660 (-4)
Fig. 15(B):	1560 340 (-50)	6400 5580 (-20)	9905 8485 (0)	8820 3670 (-44)	13725 10200 (-29)
Fig. 15 (A, C):	130560 105840 (-18)	403200 383760 (-7)	276480 324000 (-13)	95040 87480 (-18)	167040 167195 (0)
Fig. 16(A):	2210 1220 (-35)	4680 4750 (-16)	7455 7595 (-8)	11550 16170 (+62)	6160 8215 (+42)
Fig. 16 (B):	252000 127600 (+65)	444600 492660 (+21)	353100 411440 (+12)	80000 185360 (+75)	148410 230400 (-11)

TABLE XIV (Contd)

	45S	28S	18S	8-10S	4-6S
Fig. 16(C):	27500 19800 (+220)	16390 30820 (+37)	14455 12980 (-30)	17495 12040 (-17)	11560 18660 (+11)
Fig. 16(D):	76270 49320 (-44)	254400 234600 (-6)	171860 108440 (-38)	69520 61480 (-18)	10285 129960 (+38)

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