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RADIOIMMUNOASSAY OF ERYTHROPOIETIN

CIRCULATING LEVELS IN NORMAL

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ABSTRACT

Techniques are described in detail for the radioimmunoassay of human erythropoietin in unextracted plasma or serum. Using 100 microliters of sample, the assay is sensitive at an erythropoietin concentration of approximately 4 milliunits per milliliter and when required the sensitivity can be increased to 0.4 milliunits per milliliter, a range considerably less than the concentration observed in normal human beings. This is approximately 100 times more sensitive than existing in vivo bioassays for this hormone. Studies concerned with the validation of the erythropoietin radioimmunoassay show a high degree of correlation with the polycythemic mouse bioassay. Dilutions of a variety of human serum samples show a parallel relationship with the standard reference preparation for erythropoietin. Validation of the radioimmunoassay is further confirmed by observations of appropriate increases or decreases of circulating erythropoietin levels in physiological and clinical conditions known to be associated with stimulation or suppression of erythropoietin secretion. Significantly different mean serum concentrations of 17.2 milliunits per milliliter for normal male subjects and 18.8 milliunits per milliliter for normal female subjects were observed. Mean plasma erythropoietin concentrations in patients with polycythemis vera are significantly decreased and those of patients with secondary polycythemis are significantly increased as compared to plasma levels in normal subjects. These results demonstrate an initial practical value of the erythropoietin radioimmunoassay in the hematology clinic which will most certainly be expanded with its more extensive use.

INTRODUCTION

Until recently, the quantitation of erythropoietin (Ep)[1] in serum and plasma has been dependent on relatively insensitive bioassay systems. A number of different bioassays have been used. Initially, studies consisted of measurements of hematocrit, hemoglobin concentration, reticulocyte concentration (1-3) or total red cell mass in assay animals (4). Later the incorporation of radioiron into red cells of erythropoietically depressed animals was exploited as an assay for this hormone. For the past 18 years, such an assay employing the polycythemic mouse has been used by most investigators for the bioassay of Ep (5). Polycythemic mice, with a hematocrit of approximately 70% will show essentially no radioiron incorporation into red cells and the injection of as little as 50 mU of Ep will result in a significant increase in incorporation. However, different methods for producing polycythemia and different schedules of hormone administration, radioiron injection and blood sampling have been used, and results from different laboratories are not always intercomparable. Although this bioassay is capable of measuring increased circulating levels of Ep resulting from disease or physiological changes such as extreme hypoxic exposure or severe bleeding, it is not adequate for the measurement of plasma levels in normal humans or animals. Also a variety of materials other than Ep, such as testosterone (6), cyclic-AMP (7), and prostaglandins (8) stimulate erythropoiesis as measured by increase in radioiron uptake. This bioassay is also subject to the possible effect of postulated enhancing (9) or inhibiting (10) factors

which may be present in body fluids. Thus the polycythemic mouse bioassay for Ep is not sensitive enough for the measurement of normal levels in unextracted serum or plasma and can be influenced by a variety of factors other than Ep. However, recently, Erslev et al (11), using a highly concentrated plasma Ep extract, have been able to extend the polycythemic mouse bioassay to cover ranges less than 5 mU/ml. Using this technique, they have arrived at a mean normal plasma Ep concentration of 7.8 mU/ml and a concentration of less than 5 mU/ml for patients with proven polycythemia vera.

A variety of in vitro bioassay systems for Ep measurement have been developed utilizing cultures of bone marrow (12-18), spleen (19, 20) and fetal liver (21, 22). Many of these systems are subject to the effect of non-specific factors which are present in plasma, serum or crude Ep preparations. When they have been applied to normal serum, the values are generally high, which is inconsistent with results obtained with the polycythemic mouse bioassay. However, de Klerk et al (22) in a recent modification of the fetal mouse liver assay obtained a mean of 38 mU/ml for normal human sera, which is close to Ep concentrations observed with recent immunological methods (23, 24). Also, when asialo-Ep has been tested using such in vitro bioassay methods, the Ep activity is retained, whereas all the activity is lost in the polycythemic mouse bioassay (18). This latter discrepancy is also a problem with recent immunological assay methods (23, 24).

Several investigators (25-29) have been concerned with immunological techniques for the measurement of Ep for some time; however, it is only recently, with the isolation of human Ep in

pure form (30), that valid Ep immunoassays, using the classical Berson and Yalow (31) radioimmunoassay (RIA) technique have been demonstrated (23, 24). These radioimmunoassays can be used to measure Ep in unextracted human plasma or serum with a sensitivity approximately 100 times that of the polycythemic mouse bioassay. In addition to human Ep, a variety of animal erythropoietins are measurable (23). Using such radioimmunoassays, appropriate Ep responses in normal humans to such basic physiological manipulations as bleeding and transfusion, and in animals to hypoxia (23) and transfusion (32) are observed. The subject of this communication is to further validate the Ep RIA and to extend its use to a large series of normal humans and some polycythemic patients.

METHODS

Erythropoietin Iodination

The Ep used for iodination was obtained in pure form (30). Some was kindly supplied by Dr. Eugene Goldwasser[2], coded HT 6-30-76 Fr2, and consisted of approximately 1μg lyophilized in the bottom of a polyethylene microfuge tube. The rest was obtained from a distribution program through the National Heart, Lung and Blood Institute[3], coded M-7-72-2, and consisted of approximately 0.7μg lyophilized in glass capillary tubing 1-2mm in diameter and approximately 4cm long. The Ep was obtained by extraction of urine from severely anemic humans and purified to a specific activity (S.A.) of approximately 70,400 U per mg protein (30). The iodinations were carried out using either a modification of the lactoperoxidase method (33) or a method by Fraker and

Speck (34) which utilized 1, 3, 4, 6-Tetrachloro-3a, 6a-diphenylglycoluril (IODO-GEN, Pierce Chemical Co., Rockford, IL, 61105). Erythropoietin iodinated with either of these methods was equally immunologically reactive. Since considerable detail of the lactoperoxidase Ep iodination has been presented earlier (23), more detail of the use of IODO-GEN as an iodinating reagent will be presented here.

Fifty micrograms of IODO-GEN, dissolved in 20 μl chloroform, were dried in the bottom of a conical bottomed reaction vial (Reacti-vial, Pierce Chemical Co.). The lyophilized Ep was dissolved in 10 μl of distilled water and transferred to the reaction vial. Ten microliters of 0.5M PO_4, pH 7.5 was used, as a wash, to transfer any remaining Ep into the reaction vial. This was followed by 1 μl ^{125}iodide (0.5mCi), obtained from New England Nuclear (NEZ-33L), as carrier-free Na ^{125}I, at a concentration of approximately 500 mCi/ml. The reaction was allowed to proceed for 30 sec., at which time the total contents were transferred to a second vial containing 200 μl KI (10mg/ml) in 0.5MPO_4, pH 7.5) plus 5 μl 5% bovine serum albumin (BSA). The total contents of this vial were then transferred immediately to a 1.5 x 5cm Sephadex G-25 column (prepacked by Pharmacia) that had previously been equilibrated with BSA to minimize adsorption of the labeled Ep, and eluted with 0.05M PO_4, pH 7.5. Approximately 1 ml fractions were collected in one dram glass vials containing 5 drops of 5% BSA. It was possible to estimate the radioactivity in these vials, at a much reduced efficiency, by placing them 50cm from the crystal of a well type gamma counter with the top removed. Such an elution is shown in Figure 1 with most of the protein-

bound ^{125}I in fraction 4 and the unreacted ^{125}I in fractions 8-9. This procedure is relatively rapid, with the reaction from iodination to the separation of the labeled Ep requiring less than 5 min. Between 25 and 60% of the ^{125}iodine was transferred to the Ep with each iodination, the iodine transfer being lower for the Ep supplied in the glass capillary tubes as compared to the polyethylene microfuge tube supplied material. The total contents of fraction 4 were further purified on a 1.5 x 30cm Sephadex G-150 column (also previously equilibrated with BSA) by elution in 20 drop fractions with 0.05M PO_4, pH 7.5 at 4°C. This is shown in Figure 2 with two peaks of radioactivity, the first consisting of damaged labeled Ep, and the retarded second peak consisting of the purified undamaged labeled Ep. Material from this second peak was diluted and used in the RIA without further manipulation.

Erythropoietin Antibody

Details of Ep antibody production have been presented elsewhere (27). The anti-Ep used here was obtained from a rabbit which received a booster immunization of a partially purified human Ep preparation[4], with a S.A. of 8000 U/mg (35). This rabbit had been previously immunized with a crude extract of human urinary Ep prepared by pressure filtration through a collodion membrane (36). This antiserum has the ability to neutralize more than 300 U of human Ep per ml (27). Although neutralization studies against other species erythropoietins have not been carried out with this antiserum, it does crossreact with a variety of animal erythropoietins using the RIA system. In addition to

human Ep, crossreactions with mouse, rat, rabbit, sheep, dog, baboon, and monkey erythropoietins have been observed (23). This antiserum was used, purposefully unabsorbed, in the RIA at a final incubation dilution of 1:50,000.

Erythropoietin Radioimmunoassay

Samples of 100 μl of undiluted or diluted plasma or serum were pipetted into disposable polystyrene tubes (12 x 75mm) placed in special racks (Micromedic Systems, Horsham, PA). Similarly, 100 μl of halving dilutions of Ep standard (2nd IRP), from 500 mU/ml down to 1 mU/ml, were pipetted into such tubes. Phosphate buffer, 0.5M, pH 7.5, with 5% human serum albumin (Cutter Lab, Berkeley, CA) added, was used as the diluent for the Ep standard and for any dilution of plasma or serum required. The high human serum albumin (HSA) content was an attempt to maintain similar protein concentrations in all tubes, both standards and unknowns. Two hundred μl of diluted labeled Ep and 200 μl of diluted anti-Ep antiserum were added simultaneously using an automatic pipette (Micromedic Systems, Horsham, PA). The labeled Ep was diluted such that the 200 μl contained approximately 10,000 cpm and the anti-Ep was a dilution of 1:20,000 (Final incubation dilution of 1:50,000). The diluent for both of these reactants consisted of 0.05M PO_4, pH 7.5, with 0.5% BSA added. The tubes were then vortexed and kept at 4°C for an incubation period of 4 days.

Separation of the gamma globulin bound Ep was accomplished with a goat anti-rabbit gamma globulin antiserum. Goats were immunized with rabbit gamma globulin (Miles Lab. Inc., Elkhart, IN) and periodically bled. After the incubation period, 100 μl of

normal rabbit serum, diluted 1:10, and 400 μl goat anti-rabbit gamma globulin, diluted such as to result in maximal precipitation of rabbit gamma globulin (usually 1:8 to 1:12), were added simultaneously with the automatic pipette. The diluent in both cases was 0.05M PO_4, pH 7.5, with 0.5% BSA added. After vortexing, the tubes were returned to 4°C for two hours. Just before centrifugation, 3ml 0.05M PO_4, pH 7.5, were added. The tubes were centrifuged in a refrigerated centrifuge (J-6B, Beckman Inst. Inc., Palo Alto, CA) using a special rotor (JP-3.2, RIA Rack Rotor, Beckman) which allows for centrifugation of the tubes without removal from the racks. Centrifugation was for 30 min. at 2000 rpm (900 xg). The tubes were decanted and then counted using a well-type gamma counter (MS-588, Micromedic Systems, Horsham, PA). The data was collected on paper tape and an electronic recording cassette using an electronic data terminal (Silent 700 ASR, Texas Inst. Inc., Houston, TX). Analysis of the results was done using the sigmoid computer program by Rodbard and Hutt (37) and the Lawrence Berkeley Laboratory CDC 7600 computer which derives the standard curve relating percent bound to log mU Ep/ml and determines values for unknowns in terms of mU/ml.

This protocol is used for most Ep radioimmunoassays and such a curve is presented, labeled A, in Fig. 3. Standard curves are dispersed throughout an assay, so that such a complete curve is set up along with approximately every 200 RIA tubes. A single assay may consist of up to 3000 tubes. Along with each curve, three control tubes are set up. One in which the total amount of Ep-^{125}I which is precipitable by trichloroacetic acid is measured. This is accomplished by the addition of 1 ml of 10% TCA. Another

tube contains all the reactants but does not contain any unlabeled Ep. It is on the basis of this tube that the total amount of antibody precipitable Ep-^{125}I is determined. The anti-Ep binding has ranged from 33 to 39% of the TCA precipitable Ep-^{125}I. Finally, there is a tube with all reactants except anti-Ep. This serves as a background tube, and the amount of Ep-^{125}I trapped in the pellet in this tube is subtracted from the cpm of all tubes. Also along with each standard curve, assays of aliquots of pools of three different serum samples are measured. These serve as internal standards for intraassay and interassay comparison. One is a normal human serum pool, another is a pool of serum from anephric patients, and the third is a pool of serum from patients with iron deficiency anemia. The mean value for the normal human serum pool is 20.03 mU/ml with an intraassay coefficient of variation (CV) of 8.37% and an interassay CV of 9.70% (38). The anephric serum had a mean of 14.39 mU/ml with an intraassay CV of 9.94% and an interassay CV of 13.20%, and the mean for the iron deficiency pool was 97.08 mU/ml with an intraassay CV of 6.38% and an interassay CV of 8.83%. Such comparisons are comforting in going from assay to assay, especially the results with the anephric serum, which has been a problem in the past in giving extremely high values (39).

The curve labeled A in Fig. 3 shows that within the anti-Ep binding limits of 90 to 10% a range of Ep concentrations of approximately 4 to 250 mU/ml can be measured with 50% binding at 32.0 mU/ml. All unknown samples are done in duplicate and values in the range of 100 mU/ml or higher are repeated in a series of halving dilutions, with values assigned from determinations which

fall closer to the middle of the RIA range. Since 100 μl of each standard dilution have been used in the development of this curve it should be pointed out that the range of the Ep-RIA in terms of absolute units is 0.4-25 mU. However, for convenience, since one is usually interested in the concentration of Ep in unknown plasmas or sera, the Ep has been plotted on the abscissa in terms of concentration.

The possibility of extending the range of measurable Ep concentration to a more sensitive range was considered. Since one of the limiting factors in the Ep-RIA is the amount of Ep used in the labeled form, the sensitivity could be extended to a lower range by increasing the relative amount of unlabeled Ep to labeled Ep. This was accomplished by increasing the volume of unknown to one ml and keeping the amount of labeled Ep to that giving a counting rate of 10,000 cpm. Since the original protocol appeared to work well, the same buffers and relative volume relationships were maintained. However, the anti-Ep concentration was reduced and the incubation period increased.

Thus 1 ml of diluted or undiluted plasma or serum was pipetted into disposable polystyrene tubes (16x119 mm). For the Ep standard curve, 1 ml of halving dilutions of Ep (2nd IRP) from a concentration of 100 mU/ml down to 0.2 mU/ml were made and 1 ml was pipetted into each tube. All dilutions of standard Ep and plasma or serum were made with the phosphate buffer with 5% HSA added. Two ml of labeled Ep (approximately 10,000 cpm) and two ml of anti-Ep at a dilution of 1:200,000 made up the remainder of the incubation medium. The incubation period was extended to 10 days at 4°C, after which the anti-Ep was precipitated with goat

anti-rabbit gamma globulin serum as before.

The curve labeled B in Fig. 3 was obtained using this protocol. Now, within the anti-Ep binding limits of 90 to 10%, a range of Ep concentrations of approximately 0.4 to 50 mU/ml can be measured with a 50% binding at 4.5 mU/ml. This curve is more sensitive by a factor of approximately 7; however, the absolute Ep range of this assay is similar to that of the previous assay. The antibody binding with this protocol was approximately 20% as compared to 33-39% for the usual assay. Also it is more cumbersome in incubation time and volume of second antibody and is not as adaptable to our automation system. Thus its use has been reserved for very low Ep concentrations and for dilution studies as shown below.

Erythropoietin Bioassay

Erythropoietin was bioassayed in female LAF_1 mice (Jackson Lab, Bar Harbor, ME) made polycythemic by exposure to increasing levels of carbon monoxide (40). Such animals exposed for 8 hours/day, 5 days per week reach hematocrits of over 70% in about 3 weeks. After exposure, the polycythemic mice are then rested for 7 days prior to being used as assay animals. Erythropoietin preparations were injected subcutaneously and 48 hours later 0.5μCi of ^{59}Fe (NEN, Cambridge, MA) was injected intraperitoneally. Seventy-two hours after the radioiron injection, the mice were anesthetized with ether and bled by cardiac puncture. Results on mice with hematocrits less than 55% at autopsy were discarded. The ^{59}Fe in an aliquot of blood was counted in a well-type gamma counter (Nuclear Chicago) and the percent incorporation into the total red cell volume calculated was based

on a blood volume of 7% body weight. This was compared to a curve generated with standard erythropoietin (2nd IRP). All erythropoietin injections had a volume of one ml and consisted of either whole serum or dilutions of serum or standard erythropoietin made up with 5% HSA. The limit of detection for erythropoietin is about 50 mU with this bioassay.

Normal Subjects and Patients

Normal human plasma and serum samples were collected from healthy volunteer members of the Lawrence Berkeley Laboratory (LBL) and Brookhaven National Laboratory (BNL) staffs. All patient samples were obtained as residual samples from routine outpatient treatment at the Donner Clinic at LBL. The protocols used in these studies were reviewed and approved by the LBL Human Use Committee and the UC Human Use Committee. Informed consent was obtained from all normal subjects and patients.

All polycythemic patients were in various stages of treatment; phlebotomy, with or without cytotoxic agents, for polycythemia vera, and phlebotomy alone for secondary polycythemia. Only polycythemia vera patients meeting the NIH polycythemia vera study group criteria (41) were accepted for this study; there were 17 such patients (11 males, 6 females) on whom 192 determinations were done. There were five patients with secondary polycythemia; three males with hypoxia (chronic congestive heart failure, congenital heart disease, pulmonary disease), one male with hemoglobinopathy with high oxygen affinity, and one female of undetermined etiology. In all polycythemic patients, the presence of polycythemia was documented by an increased red cell mass. Most of the samples for Ep were obtained either from patients who

had never been phlebotomized, or in whom it had been more than a month since the most recent therapeutic phlebotomy; only 8 of the Ep measurements were made earlier than this, at times ranging from 4-21 days after phlebotomy. Plasma samples from three severely anemic patients; a Fanconi's anemia, a Diamond-Blackfan's anemia, and a paroxysmal nocturnal hemoglobinuria, who have been longstanding patients treated at the Donner Clinic, were included as part of the Ep RIA verification studies. All samples were separated within 30 minutes of being drawn; and the plasma or serum was frozen and kept at -20°C in polypropylene cryotubes (Vangard International, Inc., Neptune, N.J.) until set up in a RIA.

RESULTS

Comparisons of Ep RIA values with Ep results obtained using the polycythemic mouse bioassay were made with plasma from the three anemic patients studied. These results are presented in Fig. 4 and indicate a high degree of correlation between these two methods with a correlation coefficient of 0.898. The plasma Ep concentration ranged from 500 to 16,700 mU/ml with the RIA and from 950 to 20,000 mU/ml with the *in vivo* bioassay. Dilutions of these plasmas, to bring them within the Ep range of the respective assay systems, were made with 5% HSA. Also, the same Ep (2nd IRP) was used as the reference material for both assays.

Using the more sensitive Ep RIA protocol (Curve B, Fig. 3), selected human sera were serially diluted and the RIA results examined for parallelism with the curve obtained with the 2nd IRP of human Ep. These results are presented in Fig. 5. The three

internal standards of normal, anephric and iron deficiency sera were treated in this way, as was a sample of polycythemia vera serum. The results obtained by serial dilution of these sera are reasonably parallel to those obtained with the 2nd IRP Ep.

Plasma and serum Ep measurements were done on a total of 445 healthy normal subjects with an age range of 18 to 64 years; 293 males and 152 females. Some were unfasted volunteers from the LBL staff (136), and some were volunteers from the BNL staff (309) after an overnight fast. The LBL subjects were sampled twice, between 7:30 a.m. to 9:30 a.m. and 1:30 p.m. to 3:30 p.m. The BNL subjects were all sampled between 9:00 a.m. and 11:00 a.m. All samples were collected as serum except for an additional sampling of EDTA plasma at the morning bleeding of the LBL subjects; at this time, hemoglobin concentrations and hematocrit determinations were made. The serum Ep concentrations are presented in Table I. There was no significant difference in the male and female values of both the LBL and BNL groups. In fact, there was remarkable agreement between the LBL and BNL groups, in spite of the fasting and non-fasting difference. There was no difference between morning and afternoon sampling. However, when the results of the entire serum sampling were pooled, the mean male Ep concentration of 17.2 mU/ml was significantly different from the mean female concentration of 18.8 mU/ml with a p value of <0.005. Results on the plasma samples taken with EDTA, at the same time as the morning LBL serum samples, showed a significant reduction when compared to their respective serum values. The mean for the EDTA male normal plasma was 15.2 mU/ml with a SD of 5.5, and the female normal mean was 15.8 mU/ml with a SD of 4.6. These normal plasma values are presented in

Table II and significantly differ from their respective serum values, as presented in Table I, with a p value of <0.005. Preliminary results on plasma collected using heparin as an anticoagulant do not differ from Ep RIA results obtained on serum collected at the same time.

Also in Table II are presented results for Ep concentrations on plasma samples obtained from patients with polycythemia in various stages of treatment from the Donner Clinic. All plasma results reported here were on plasma samples collected using EDTA as the anticoagulant. The mean hemoglobin concentration for the treated primary and secondary polycythemic males was 15.4 g/dl and 15.9 g/dl respectively, as compared to 15.5 g/dl for the normal males. The mean Ep concentration for 113 polycythemia vera plasma samples was 9.3 mU/ml and for 29 secondary polycythemia plasma samples, 153.7 mU/ml. The mean Ep concentrations for the primary and secondary polycythemic plasma samples were significantly different from each other, as well as from the mean normal plasma value with p values of <0.001. Although the individual secondary polycythemia values are definitely increased over the normal and polycythemia vera values, individual polycythemia vera values are not distinguishable from normal values. This is further depicted in Fig. 6 where no overlap is seen between the Ep values on plasma samples from male patients with secondary polycythemia and polycythemia vera. Results on plasma from the Diamond-Blackfan anemia and a polycythemia with myelofibrosis are also presented in Fig. 6 to emphasize the inverse hemoglobin - Ep concentration relationship. The mean hemoglobin concentration for the treated polycythemia vera and secondary polycythemia females was 15.6 g/dl

and 15.3 g/dl respectively as compared to 13.3 g/dl for the normal females. The mean Ep concentrations for 79 female polycythemia vera plasma samples was 8.7 mU/ml and for 10 secondary polycythemia plasma samples 75.7 mU/ml. These values were significantly different from each other and from the normal female plasma mean with p values of <0.001. Also, as seen in Fig. 7, there is no overlap between the secondary polycythemia Ep values and the values obtained on plasma from patients with polycythemia vera.

DISCUSSION

Details of a RIA for Ep have been presented utilizing pure human Ep (30) radiolabelled with ^{125}I and an antiserum prepared in a rabbit immunized with partially purified human Ep. As a standard reference material, the 2nd IRP of human Ep was used. This RIA is sensitive to an absolute amount of Ep equivalent to 0.4 mU, which is approximately 100 times more sensitive than the polycythemic mouse bioassay. The RIA can be done on as little as 100 μl of material and large numbers of samples can be accommodated. Two protocols have been presented, the most sensitive of which is capable of measuring Ep concentrations in unextracted plasma or serum down to approximately 0.4 mU/ml. This allows for the measurement of not only normal circulating Ep levels, but also Ep levels that are considerably depressed below normal. The RIA shows a high degree of correlation with the polycythemic mouse bioassay and halving dilutions of a variety of human sera show a reasonably parallel relationship to the Ep reference standard. That this parallel relationship exists for human anephric and polycythemia vera serum, as well as for normal serum, supports the hypothesis that the small amount of immunoreactive material present in these

sera is, in fact, Ep. Such studies, although not proof of identity, are necessary requirements for a valid RIA.

In addition to these studies directed to the validation of the Ep RIA, perhaps the most significant information in this respect, comes from detection of appropriate increases or decreases in circulating Ep concentrations in response to physiological and clinical conditions known to be associated with stimulation or suppression of Ep secretion. In this context, bleeding of a normal human subject has been shown to result in increased serum Ep levels, and blood transfusion in decreased levels, as measured by the RIA (23). As seen in Fig. 4, clinical anemias known to be associated with increased Ep levels as measured by in vivo bioassay techniques show correlative increased Ep levels by the RIA. Also increased Ep levels are observed in plasma from patients with secondary polycythemia (Figs. 6 and 7). With the anti-Ep antiserum used here, a variety of animal erythropoietins are also reactive in competing with the labeled human Ep for the anti-Ep. Appropriate increases in serum Ep measured with the RIA have been shown to occur in the hypoxic rat and baboon (23). Acute bleeding of sheep resulted in a prompt rise in radioimmunoassayable Ep 24 hours later (42). Also hypertransfusion of normal mice results in a measurable depression of plasma Ep by the RIA (32). Thus results obtained with the Ep RIA are consistent with the known physiological and clinical information concerning Ep. Perhaps the only discrepancy is that alluded to above regarding the inability to distinguish between intact and asialoerythropoietin (23, 24). This however has also been a problem with certain in vitro bioassay methods for Ep (18).

Using pure human Ep as the labeled reactant in the present Ep RIA, means for normal male serum of 17.2 mU/ml and normal female serum of 18.8 were obtained. These values are approximately 4 times the values seen in a previous Ep RIA attempt (39) which utilized a partially purified Ep preparation as the tracer. Also, it now appears that when a large group of values is analyzed, the female mean is significantly increased over that seen for the male, which is the reverse of that seen with the previous RIA. Using a similar Ep RIA, Goldwasser (43) has obtained normal human serum values similar to those presented here. Extracts of normal plasma assayed in the polycythemic mouse bioassay show a mean of 7.8 mU/ml (11). In a recent review of normal human serum Ep concentration, a value of approximately 40 mU/ml was considered normal (44). Perhaps the absolute value obtained with a particular assay system is not as significant as the ability to demonstrate appropriate relative changes in Ep concentration correlated with physiological and clinical conditions known to be associated with stimulation or depression of Ep secretion. More extensive use of a variety of Ep assay systems will ultimately determine the most appropriate absolute value for normal individuals.

The Ep concentrations seen in the plasmas from secondary polycythemia patients were considerably increased over the levels seen in the plasmas of normal subjects and polycythemia vera patients as measured by RIA. Such relationships have also been observed using a plasma Ep extraction technique (11) and a urinary Ep extraction technique (45), with Ep measurements made using the polycythemic mouse bioassay. The mean Ep concentrations from both male and female polycythemia vera patients were significantly

decreased as compared to the plasma levels in the normal subjects. However, whereas in the treatment of male polycythemia vera the hemoglobin concentration was comparable to that present in normal males; the treated female polycythemia vera patients had a mean hemoglobin concentration of 15.6 g/dl as compared to a normal female hemoglobin concentration of 13.3 g/dl. The presence of more lower values in plasma Ep concentration among the female patients with polycythemia vera, as see in Fig. 7, suggests that the inverse hemoglobin - Ep concentration relationship also exists for these patients. Unfortunately, plasma samples from untreated polycythemia vera patients were not available in this study. Such samples, with higher hemoglobin concentrations, could be expected to have even lower Ep concentrations. Ep excretion studies in patients with polycythemia vera indicate that while Ep output is absent or markedly decreased at high hemoglobin concentrations, that when the hemoglobin concentration was reduced to normal levels the Ep excretion increased (45). The high Ep concentrations in the polycythemia vera patient with myelofibrosis shown in Fig. 6 are also supportive of an inverse hemoglobin - Ep relationship in such patients.

Use of pure Ep labeled with radioiodine has removed all discrepancies seen in a previous Ep RIA attempt (39). The problem has not been with the anti-Ep antiserum, as has been suggested (46), since the antiserum used here is the same antiserum used in the previous RIA. The fact that this antiserum is a crude mixture of antibodies against other proteins as well as antibody specific for Ep (47) does not invalidate the RIA. The only reaction of consequence in a RIA is that between the labeled hormone and its

antibody, and the competition of unlabeled hormone with the labeled hormone for the limited amount of antibody. There are several publications in which antibodies to two different hormones have been purposefully mixed in radioimmunoassays simultaneously measuring both hormones in the same sample of serum (48, 49). One hormone was labeled with ^{131}I and one with ^{125}I and care was taken in counting each isotope separately. Results obtained by the simultaneous RIA were equivalent to those obtained by RIA of each hormone separately.

As suggested by Erslev (50), a fast, accurate Ep assay should be of considerable practical importance in the hematology clinic. It is felt that the Ep RIA in its present form fits this need. Large numbers of samples can be handled rapidly. Only a small volume of plasma or serum with no extraction is required, and it is extremely sensitive and reproducible.

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REFERENCES

1. Erslev, A. J. 1960. Humoral regulation of red cell production. Blood. 8: 349-357.

2. Borsook. H. A., A. Graybiel, B. Keighley, and E. Windsor. 1954. Polycythemic response in normal adult rats to a nonprotein plasma extract from anemic rabbits. Blood. 9: 734-742.

3. Hodgson, G. and J. Tohá. 1954. The erythropoietic effect of urine and plasma of repeatedly bled rabbits. Blood. 9: 299-309.

4. Garcia, J. F. and D. C. Van Dyke. 1959. Dose-response relationships of human urinary erythropoietin. J. Appl. Physiol. 14: 233-236.

5 DeGowin, R. L., D. Hofsha, and C. W. Gurney. 1962. A comparison of erythropoietin bioassays. Proc. Soc. Exp. Biol. Med. 110: 48-51.

6. Fried, W., R. DeGowin, and C. W. Burney. 1964. Erythropoietic effect of testosterone in polycythemic mouse. Proc. Soc. Exp. Biol. Med. 117: 839-842.

7. Schooley, J. C. and L. J. Mahlman. 1971. Stimulation of erythropoiesis in the plethoric mouse by cyclic-AMP and its inhibition by anti-erythropoietin. Proc. Soc. Exp. Biol. Med. 137: 1289-1292.

8. Schooley, J. C. and L. J. Mahlman. 1971. Stimulation of erythropoiesis in plethoric mice by prostaglandins and its inhibition by anti-erythropoietin. Proc. Sco. Exp. Biol. Med. 138: 523-524.

9. Gardner, E., C-S. Wright, J. P. Lewis, and R. R. Moores. 1967. Potentiation of purified erythropoietin with serum proteins. Br. J. Haematol. 13: 317-322.

10. Whitcomb, W. H. and M. Z. Moore. 1965. The inhibitory effect of plasma from hypertransfused animals on erythrocyte iron incorporation in mice. J. Lab. Clin. Med. 66: 641-651.

11. Erslev, A. J., J. Caro, E. Kansu, O. Miller, and E. Cobbs. 1979. Plasma erythropoietin in polycythemia. Am. J. Med. 66: 243-247.

12. Friederici, L. 1958. Über den nachweis des erythropoietins in der gewebekultur. Klin. Wochenschr. 36: 132-134.

13. Korst. D. R., E. P. Frenkel, and J. E. Wilhelm. 1962. Studies utilizing short-term marrow culture. I. Ferrokinetics and erythropoietin. In Erythropoiesis. Jacobson, L. O. and M. Doyle, editors. Grune and Stratton, New York. 310-320.

14. Erslev, A. J. 1962. Erythropoietin in vitro. In Erythropoiesis. Jacobson, L. O. and M. Doyle, editors. Grune and Stratton, New York. 275-285.

15. Krantz, S. B., O. Gallien-Lartigue, and E. Goldwasser. 1963. The effect of erythropoietin upon heme synthesis by marrow cells in vitro. J. Biol. Chem. 238: 4085-4090.

16. Krantz, S. B. 1965. The effect of erythropoietin on human bone marrow cells in vitro. Life Sci. 4: 2393-2397.

17. Ward, H. P. 1967. An in vitro assay of erythropoietin. Proc. Soc. Exp. Biol. Med. 125: 370-374.

18. Goldwasser, E., J. F. Eliason, and D. Sikkema. 1975. An assay for erythropoietin in vitro at the milliunit level. Endo. 97: 315-323.

19. Nakao, K., J. Miura, and F. Takaku. 1966. In vitro effect of erythropoietin on the spleen of the polycythemic mouse. I. Blood. 27: 646-653.

20. Miura, Y., F. Takaku, and K. Nakao. 1967. In vitro effect of erythropoietin of the spleen of the polycythemic mouse. II. Radiosensitivity of stem cells. Blood. 29: 852-858.

21. Dunn, C. D. R., J. H. Jarvis, and J. M. Grecman. 1975. A quantitative bioassay for erythropoietin using mouse fetal liver cells. Exp. Hemat. 3: 65-78.

22. de Klerk, G., R. J. W. M. Vet, P. C. J. Rosengarten, R. Goudsmit. 1980. Comparison of the hemagglutination inhibition assay kit for erythropoietin (ESF) with the fetal mouse liver cell bioassay in vitro. Blood. 55: 955-959.

23. Garcia, J. F., J. Sherwood, and E. Goldwasser. 1979. Radioimmunoassay of erythropoietin. Blood Cells. 5: 405-419.

24. Sherwood, J. B. and E. Goldwasser. 1979. A radioimmunoassay for erythropoietin. Blood 54: 855-893.

25. Krugers Dagneaux, P. G. L. C., R. Goudsmit, and H. W. Krijen. 1968. Investigation on an immunoassay of erythropoietin. Ann. N. Y. Acad. Sci. 149: 294-297.

26. Lange, R. D., T. P. McDonald, and T. Jordan. 1969. Antisera to

erythropoietin: partial characterization of two different antibodies. J. Lab. Clin. Med. 73: 78

27. Garcia, J. F. 1972. The radioimmunoassay of human erythropoietin. In Regulation of Erythropoiesis. Gordon, A. S., M. Condorelli, and C. Peschle, editors. Il Ponte, Milano. 132-153.

28. Cotes, P. M. 1973. Radioimmunoassay of erythropoietin. In Methods in Investigative and Diagnostic Endocrinology. Part III. Non-Pituitary Hormones. Berson, S. A. and R. S. Yalow, editors. North-Holland, Amsterdam. 1117-1123.

29. Lertora, J. J., P. A. Dargon, A. B. Rege, and J. W. Fisher. 1975. Studies on a radioimmunoassay for human erythropoietin. J. Lab. Clin. Med. 86: 140-151.

30. Miyake, T., C. K.-H. Kung, and E. Goldwasser. 1977. The purification of human erythropoietin. J. Biol. Chem. 252: 5558-5564.

31. Berson, S. A. and R. S. Yalow. 1968. General principles of radioimmunoassay. Clin. Chem. Acta. 22: 51-69.

32. Moccia, G., M. E. Miller, J. Garcia, and E. P. Cronkite. 1980. The effect of plethora on erythropoietin levels. Proc. Soc. Exp. Biol. Med. 163: 36-38.

33. Thorell, J. I. and B. G. Johansson. 1971. Enzymatic iodination of polypeptide with ^{125}I to high specific activity. Biochem. Biophys. Acta. 251: 363-369.

34. Fraker, P. J. and J. C. Speck, Jr. 1978. Protein and cell membrane iodination with a sparingly soluble chloroamide. 1, 3, 4, 6-tetrachloro-3X, 6X-diphenyl-glycouril. Biochem. Biophys. Res. Commun. 80: 849-857.

35. Espada, J. and A. Gutnisky. 1970. Purificacion de eritropoyetina urinaria humana. Acta Physiol. Lat. Am. 20: 122-129.

36. Van Dyke, D. C., J. F. Garcia, and J. H. Lawrence. 1957. Concentration of highly potent erythropoietic activity from urine of anemic patients. Proc. Soc. Exp. Biol. Med. 96: 541-544.

37. Rodbard, D. and D. M. Hutt. 1974. Statistical analysis of radioimmunoassays and immunoradiometric (labeled antibody) assays. A generalized weighted, iterative, least-squares method for logistic curve fitting. In Radioimmunoassay and Related Procedures in Medicine. I. A. E. A. Vienna. 165-192.

38. Snedecor, G. W. 1956. Statistical Methods. The Iowa State College Press, Ames, Iowa. 5th edition. 44.

39. Garcia, J. F. 1974. Radioimmunoassay of human erythropoietin. In Radioimmunoassay and Related Procedures in Medicine. I. A. E. A. Vienna. 275-287.

40. Fogh, J. 1966. A sensitive erythropoietin assay on mice exposed to CO-hypoxia. Scand. J. Clin. Lab. Invest. 18: 33-44.

41. Berlin, N. I. 1975. Diagnosis and classification of the polycythemias. Semin. Hematol. 12: 5-17.

42. Mohandas, N., M. R. Clark, J. L. Wyatt, J. F. Garcia, P. D. Eisenberg, and S. B. Shohet. 1980. Erythropoietic stress, macrocytosis, and hemoglobin switching in Hb AA sheep. Blood. 55: 757-761.

43. Goldwasser, E. Personal communication.

44. Dunn, C. D. R. and R. D. Lange. 1980. Erythropoietin titers in normal human serum: An appraisal of assay techniques. Exp. Hemat. 8: 231-235.

45. Adamson, J. W. 1968. The erythropoietin hematocrit relationship in normal and polycythemic man: Implications of marrow regulation. Blood. 32: 597-609.

46. Nathan, D. G. 1977. Regulation of erythropoiesis. N. Engl. J. Med. 296: 685-687.

47. Schooley, J. C. and J. F. Garcia. 1965. Some properties of serum obtained from rabbits immunized with human urinary erythropoietin. Blood. 25: 204-217.

48. Glick, S. M. and A. Kagen. 1967. Combined immunoassay of insulin and human growth hormone. J. Clin. Endocr. 27: 133-136.

49. Leclercq-Meyer, V., J. Marchand, O. Rebolledo, W. J. Malaisse, and R. Leclercq. 1975. A combined radioimmunoassay for glucagon on insulin. Diabetologia. 11: 419-425.

50. Erslev, A. J. 1977. The clinical usefulness of erythropoietin measurements. In Kidney Hormones. Vol. II. Erythropoietin. Fisher, J. W., editor. Academic Press Inc., New York. 571-584.

FOOTNOTES

1. Abbreviations used in this paper: Ep, erythropoietin; RIA, radioimmunoassay; HSA, human serum albumin; BSA, bovine serum albumin; PO_4, phosphate buffer; S.A., specific activity; CV, coefficient of variation.

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FIGURE LEGENDS

Figure 1. Separation of erythropoietin, labeled by the IODO-GEN technique, from unreacted iodide on a Sephadex G-25 column.

Figure 2. Separation of labeled erythropoietin from erythropoietin damaged as a result of iodination. Only material in the second peak was used in the radioimmunoassay.

Figure 3. Typical curves for the erythropoietin radioimmunoassay. The protocol for curve A was used for most assays and the protocol for curve B was reserved for very low erythropoietin concentrations.

Figure 4. Correlation of erythropoietin radioimmunoassay results with polycythemic mouse bioassay results using plasma from anemic patients. The correlation coefficient was 0.898.

Figure 5. Serial dilution of various human sera demonstrating a parallel relationship with the standard reference preparation of human erythropoietin.

Figure 6. Plasma erythropoietin concentration as a function of hemoglobin concentration. The solid circle represents the mean for normal male plasma with the dashed line enclosing 2 SD of the mean.

Figure 7. Plasma erythropoietin concentration as a function of hemoglobin concentration. The solid circle represents the mean for normal female plasma with the dashed line enclosing 2 SD of the mean.

Table I

Serum Erythropoietin Concentrations in Normal Human Beings

		Hemoglobin Concentration*	Hematocrit*	Erythropoietin Concentration*
		g/dl	%	mU/ml
Male ‡				
LBL (81)§	7:30 - 9:30 A.M.	15.5 ± 1.0	44.5 ± 2.8	17.6 ± 5.5
LBL (81)	1:30 - 3:30 P.M.			17.5 ± 5.0
BNL (212)	9:00 - 11:00 A.M.	15.4 ± 1.0	44.4 ± 2.8	17.0 ± 5.7
Entire Male Sampling (364)				17.2 ± 5.5
Female				
LBL (55)	7:30 - 9:30 A.M.	13.3 ± 1.0	38.7 ± 2.5	19.0 ± 5.8
LBL (55)	1:30 - 3:30 P.M.			18.7 ± 4.7
BNL (97)	9:00 - 11:00 A.M.	13.5 ± 0.8	39.5 ± 2.5	18.7 ± 7.0
Entire Female Sampling (199)				18.8 ± 6.2

* Each value represents mean ± SD

‡ The Lawrence Berkeley Laboratory (LBL) samples were from non-fasted individuals, whereas the Brookhaven National Laboratory (BNL) samples were obtained after an overnight fast

§ Numbers in parenthesis indicate number of erythropoietin measurements

Table II

Plasma Erythropoietin Concentrations in Normal and Polycythemia Human Beings*

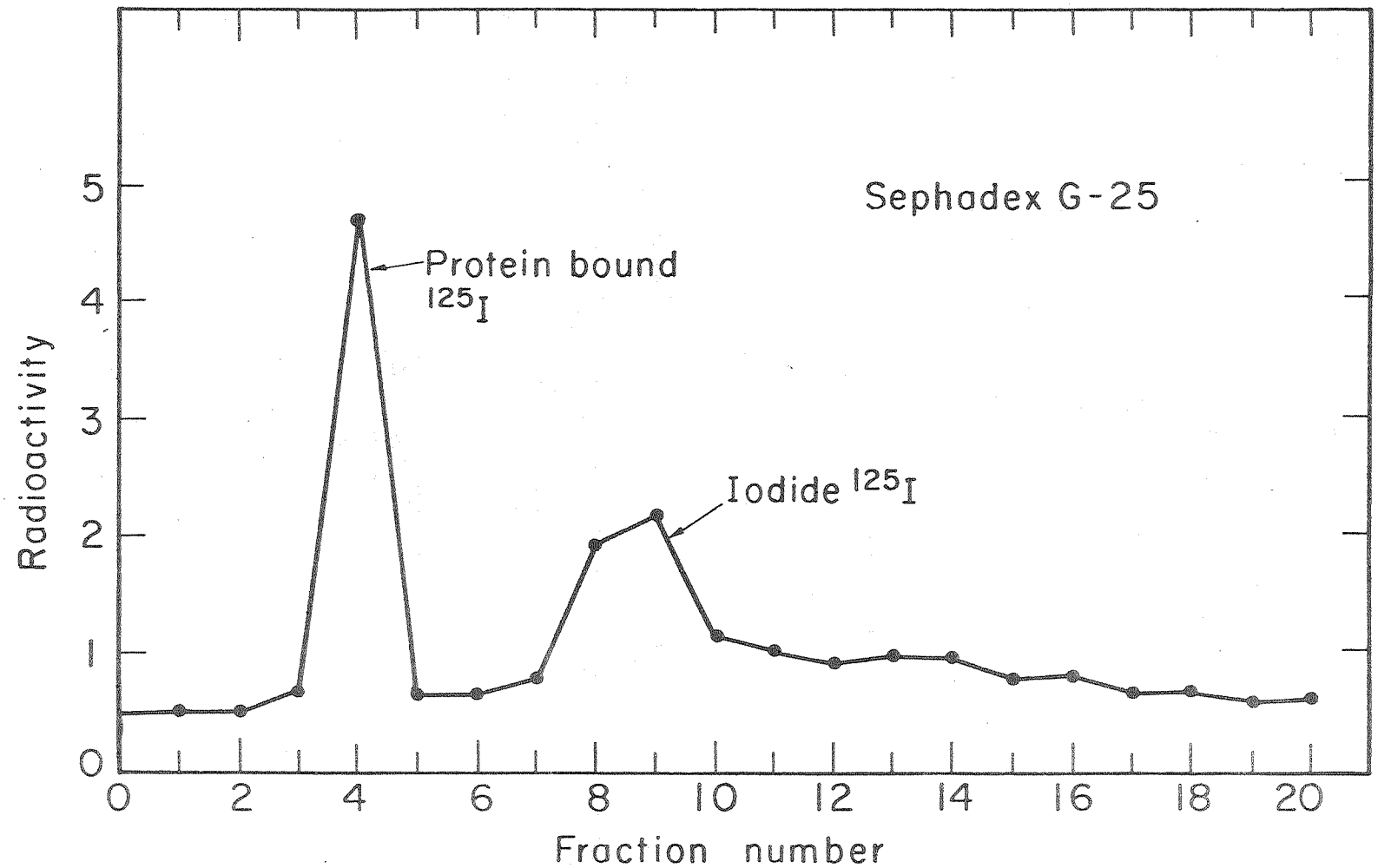
	Hemoglobin Concentration‡	Erythropoietin Concentration ‡
	g/dl	mU/ml
Male §		
Normal (81)"	15.5 ± 1.0	15.2 ± 5.5
Polycythemia vera (113)	15.4 ± 1.1	9.2 ± 5.0
Secondary Polycythemia (29)	15.9 ± 0.9	153.7 ± 106.7
Female §		
Normal (53)	13.3 ± 1.0	15.8 ± 4.6
Polycythemia vera (79)	15.6 ± 1.3	8.7 ± 5.6
Secondary Polycythemia (10)	15.3 ± 0.7	75.7 ± 29.8

* All plasma samples collected using EDTA as the anticoagulant

‡ Each value represents mean ± SD

§ Within each sex, all Ep means are significantly different from each other with p values of <0.001

" Numbers in parenthesis indicate number of erythropoietin measurements



XBL786-3242

Fig. 1

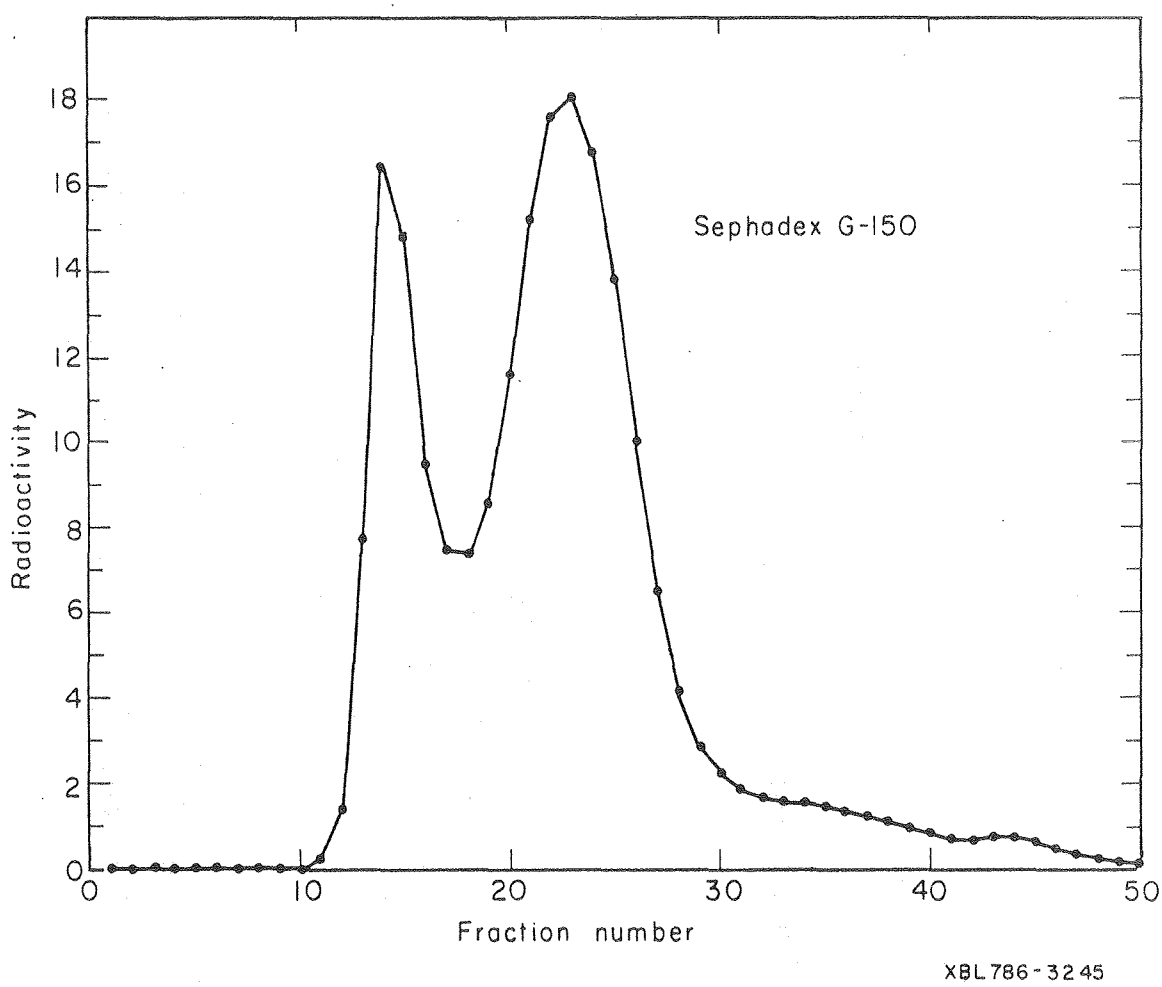
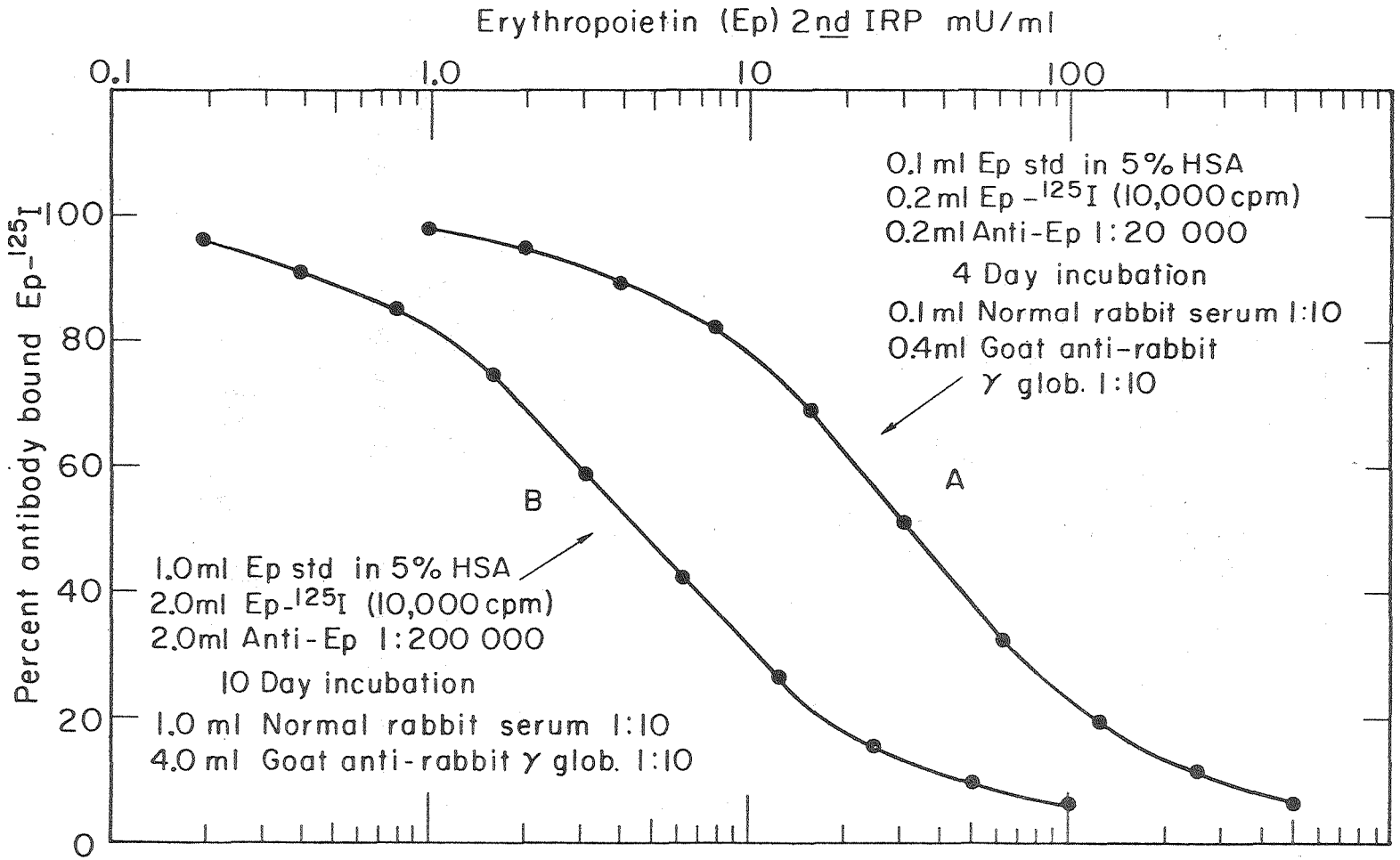
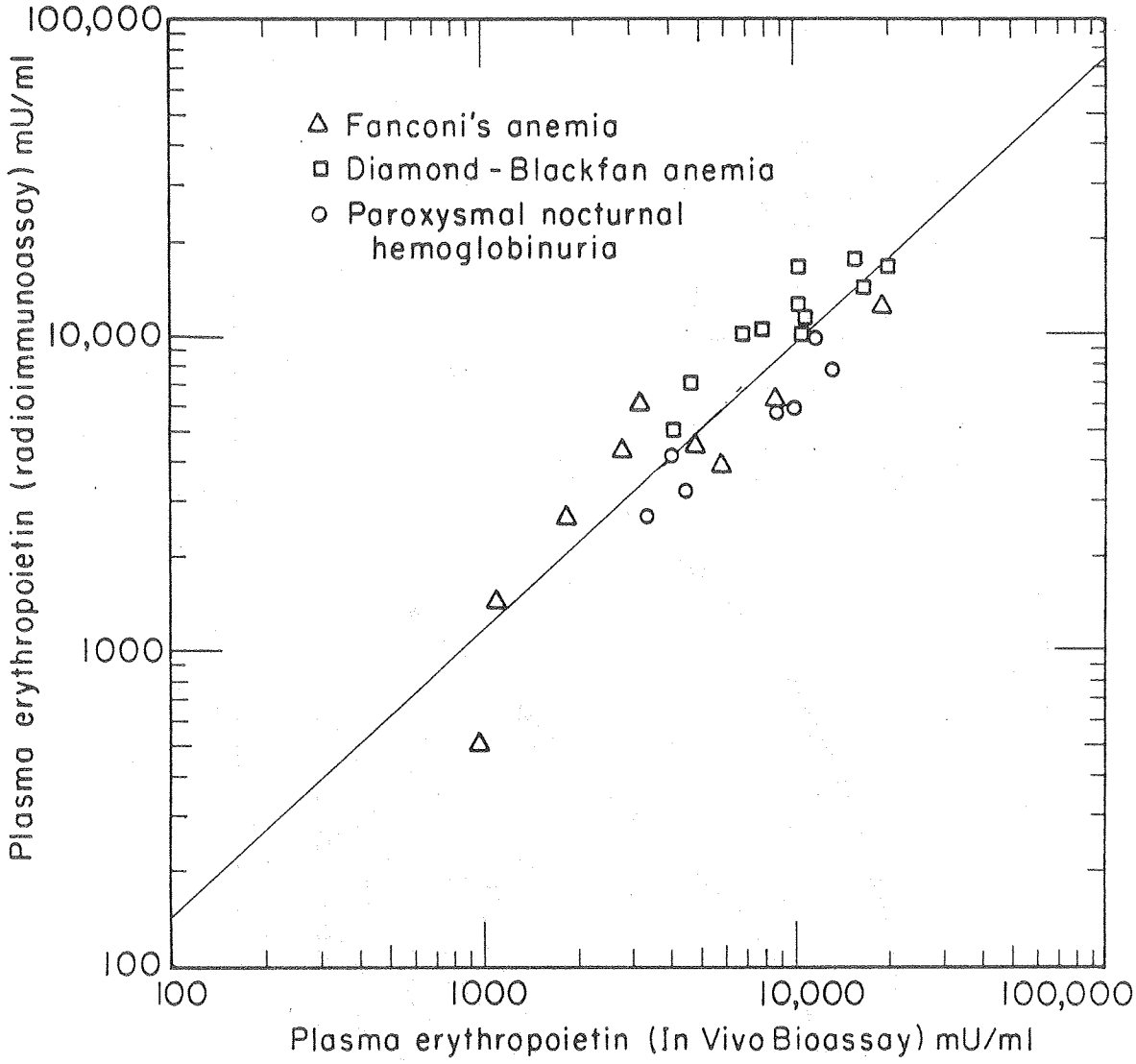

Sephadex G-150
Radioactivity
Fraction number
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
XBL 786-3245

Fig. 2



XBL808-3620

Fig. 3



XBL804-3255

Fig. 4

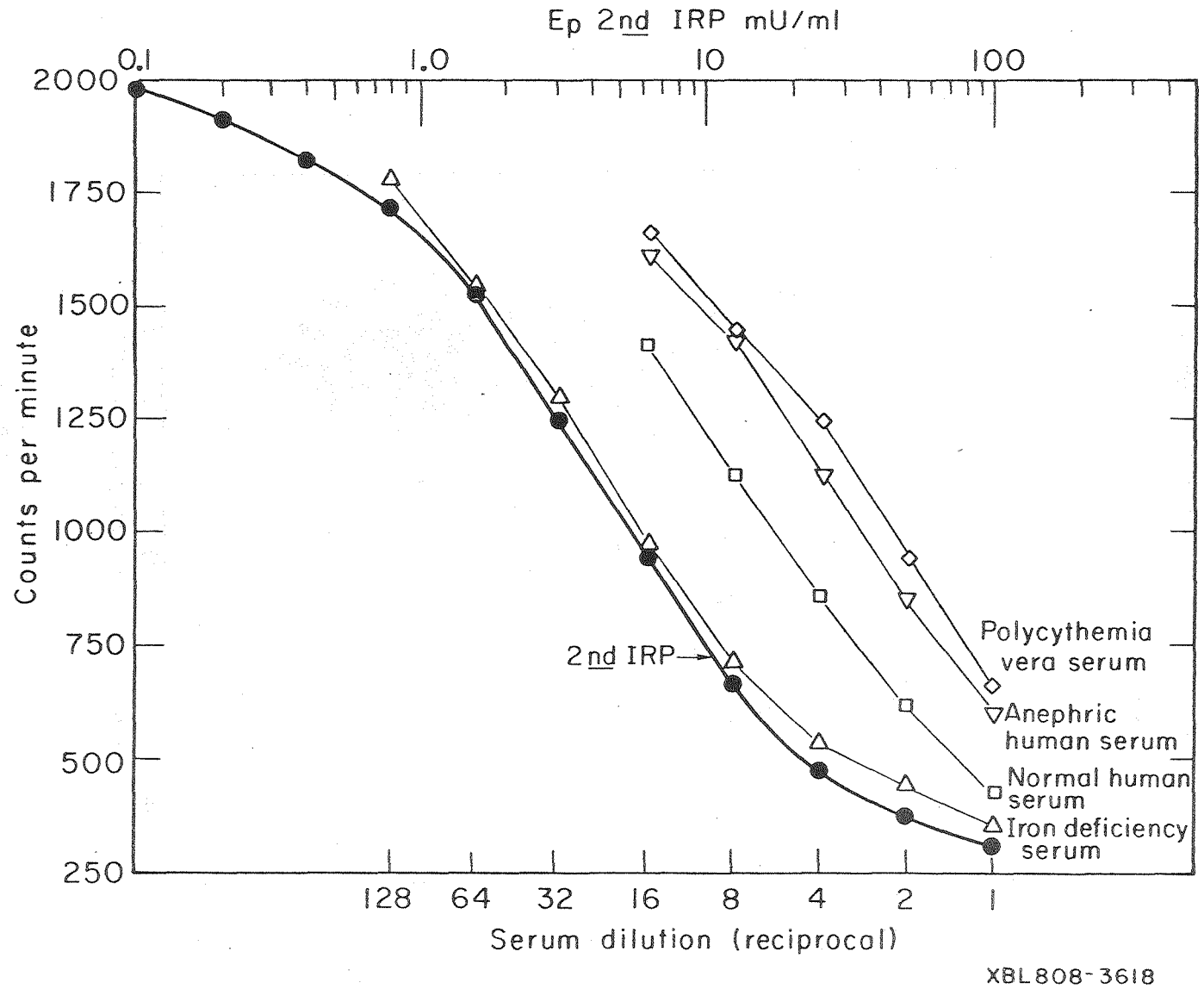

Ep 2nd IRP mU/ml
0.1
1.0
10
100
2000
1750
1500
1250
1000
750
500
250
Counts per minute
2nd IRP
Polycythemia vera serum
Anephric human serum
Normal human serum
Iron deficiency serum
128
64
32
16
8
4
2
1
Serum dilution (reciprocal)
XBL808-3618

Fig. 5

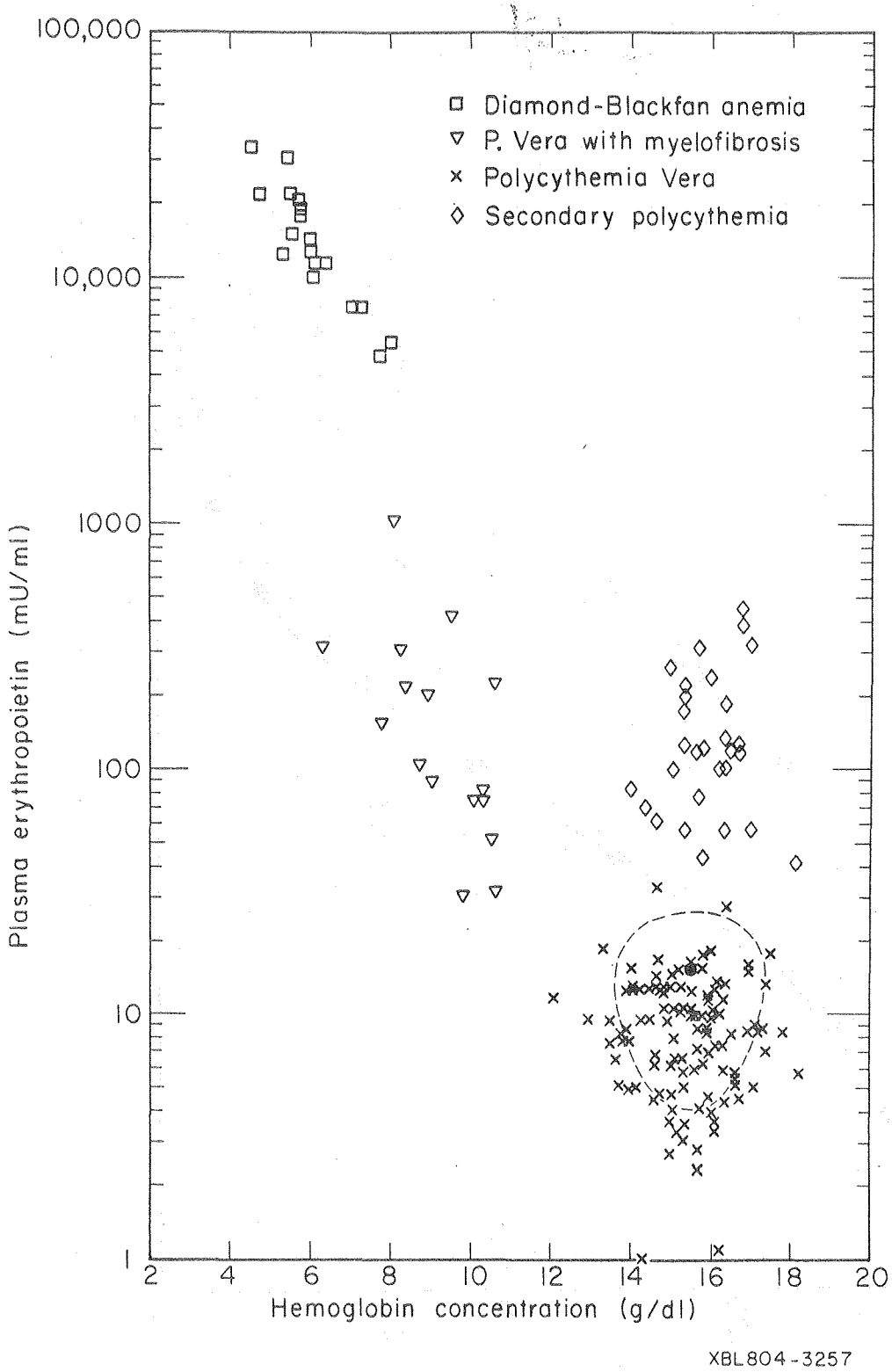
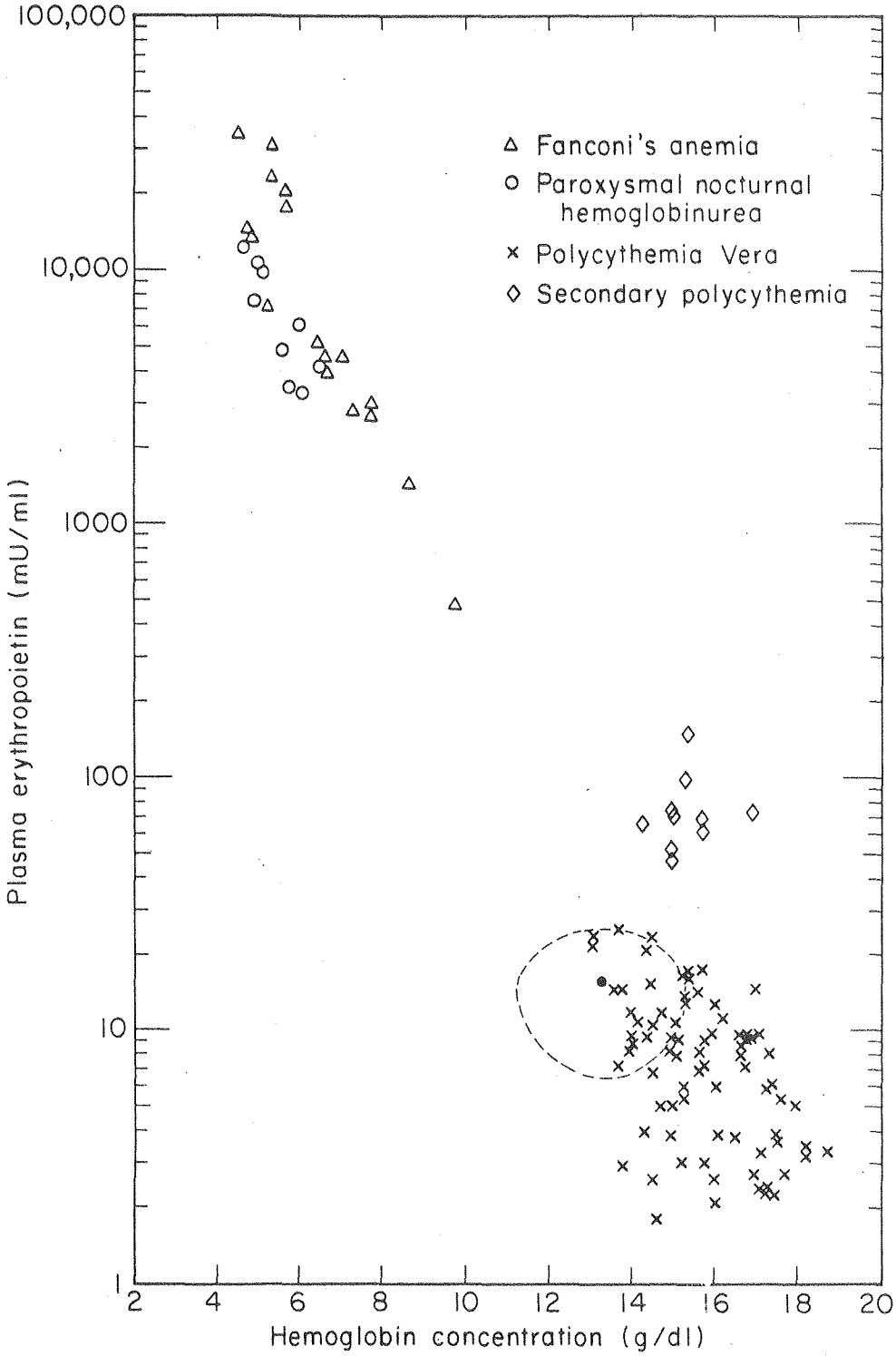

Diamond-Blackfan anemia
P. Vera with myelofibrosis
Polycythemia Vera
Secondary polycythemia
Plasma erythropoietin (mU/ml)
100,000
10,000
1000
100
10
1
2
4
6
8
10
12
14
16
18
20
Hemoglobin concentration (g/dl)
XBL804-3257

Fig. 6



XBL804-3256

Fig. 7