# Exhibit 35

to the Declaration of Cullen N. Pendleton in Support of Amgen's Opposition to Roche's Motion for Summary Judgment that Claim 7 of the '349 Patent is Invalid Under 35 USC §112 and is Not Infringed

# Laboratory Methods

## Studies on a radioimmunoassay for human erythropoietin*

JUAN J. L. LERTORA**, PATRICIA A. DARGON, ARVIND B. REGE, and JAMES W. FISHER
*New Orleans, La.*

A highly purified erythropoietin (ESF) preparation (12,000 units per milligram of protein) was labeled with $Na^{125}I$ using the Chloramine-T method. Undamaged immunoreactive labeled ESF was separated from the damaged, nonimmunologically reactive ESF by Sephadex G-150 fractionation. This undamaged immunoreactive ESF was used in a radioimmunoassay for human erythropoietin. Separation of bound from free antigen was achieved using the double-antibody technique. Approximately 55 per cent binding was observed at an antiserum dilution of 1:1500. This assay appears to be sensitive enough to detect as little as 0.025 milliunits of the International Reference Preparation erythropoietin. The estimated levels of this hormone in normal and anemic uremic human subjects suggest that immunoreactive serum erythropoietin levels are elevated above normal in anemia of uremia.

Determinations of erythropoietin (ESF) titers in serum and urine from normal human subjects to assess physiologic levels, as well as the study of the role of ESF in the day-to-day control of erythroid cell differentiation and proliferation have been limited by the lack of sensitivity of current bioassay methods. The ex-hypoxic polycythemic mouse assay[1] is capable of detecting as little as 0.05 unit of the International Reference Preparation (IRP) ESF. However, this assay is not sensitive enough to detect ESF levels in unconcentrated urine or serum from normal subjects. More recently, interest has centered around in vitro techniques for ESF measurement that have been reported to be more sensitive than the polycythemic mouse assay.[2-6]

Soon after the pioneering work of Berson and Yalow,[7] radioimmunoassays were developed for several polypeptide hormones.[8] It is recognized today that this methodology offers a major advantage over the biological assays of all such hormones because of its increased sensitivity, reproducibility, and reduced variability.

From the Department of Pharmacology, Tulane University School of Medicine, New Orleans.
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**The work reported in this paper was completed during the tenure of a Merck, Sharp and Dohme International Fellowship in Clinical Pharmacology. Present address: Department of Internal Medicine, Division of Clinical Pharmacology, University of Iowa Hospitals and Clinics, Iowa City, Iowa 52242.

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With the availability of highly purified ESF preparations[9–12] with specific activities ranging from 7,600 to 12,000 units per milligram of protein, together with the known antigenicity of ESF that allows production of antisera capable of neutralizing its biological activity,[13] it has been possible to prepare $^{125}$I-labeled ESF following the method of Hunter, Greenwood, and Glover[14] and to use this labeled material in the development of a radioimmunoassay for ESF.

The present study describes the methods employed and some basic problems encountered in the development of a radioimmunoassay for human erythropoietin, as well as its application in the estimation of levels of ESF in sera from normal and anemic uremic (end-stage renal insufficiency) human subjects.

## Materials and methods

*Source of purified erythropoietin for radioiodination.* A highly purified ESF preparation obtained from the urine of patients with anemia of hookworm infestation was used in the present studies. The sample of purified erythropoietin* had an estimated specific activity of 11,985 units per milligram of protein and was prepared by Dr. Peter Dukes, Los Angeles Childrens Hospital, Los Angeles, Calif. according to a method previously described.[12]

*Iodination technique.* The purified ESF sample (2.4 $\mu$g of protein) was dissolved in 0.2 ml. of 0.2 M phosphate buffer, pH 7.45. One-half millicurie of $^{125}$I (as sodium iodide, carrier-free) in a volume of 0.025 ml. was added to the protein solution, followed by Chloramine-T (20 to 35 $\mu$g in 0.010 ml.) and the reaction carried on for 10 to 15 seconds. Sodium metabisulfite (60 $\mu$g in 0.025 ml. 0.02 M phosphate buffer, pH 7.45) was then added and allowed to react for 45 to 60 seconds. To provide carrier protein and increase the volume of the mixture, 0.2 ml. of 0.02 M phosphate buffer, pH 7.45, with 0.5 per cent bovine serum albumin (BSA) was added in the final step. All reactions were carried out at room temperature.

*Chromatoelectrophoretic evaluation of $^{125}$I-ESF.* The technique of Berson, and co-workers[7] was used as follows: 0.020 ml. of the iodination mixture was transferred to a vial containing 1.0 ml. of 0.02 M phosphate buffer, pH 7.45, with 0.1 per cent BSA. A 0.025 ml. sample of this dilution was submitted to chromatography using Whatman No. 3 MM paper followed by electrophoresis at 400 volts, 10 milliamps, in 0.05 M Sorensen's phosphate buffer, pH 7.5. The procedure was carried out in a refrigerated room at 4° C. The chromatoelectrophoretogram was scanned for radioactive peaks using a Packard Model 7201 strip scanner.

*Sephadex fractionation of the iodination mixture.* The iodination mixture was applied to a Sephadex G-150 column, with the following characteristics: size: 1.2 by 50 cm; flow-rate: 10.9 to 11.0 ml. per hour; buffer: 0.02 M phosphate, pH 7.45, with 0.1 per cent BSA; temperature: 4° C. Fractions of 0.8 ml. were collected in siliconized glass tubes. The Sephadex G-150 was presaturated with BSA prior to utilization. In order to monitor the radioactivity peaks, 0.010 ml. aliquots of each fraction were counted in a Packard Auto-gamma Spectrometer (Model 5022).

*Immunochemical reactivity of $^{125}$I-ESF.* Fractions of the Sephadex G-150 eluates were evaluated for immunological binding to ESF antiserum using an antiserum dilution curve (a fixed concentration of labeled protein was reacted with decreasing concentrations of anti-ESF).

*Preparation of ESF antiserum.* Male New Zealand albino rabbits were injected intradermally into the foot pads or on the back of the neck with a suspension of ESF in complete Freund's adjuvant every 3 days for 2 weeks. The ESF preparation was obtained from the urine of a patient with hypoplastic anemia, and had an estimated specific activity between 1 and 2 units per milligram of protein. Blood was obtained via the ear artery and serum was collected 5 days after the last injection. The biological activity of 0.2 unit of ESF was completely neutralized by 0.05 ml. of ESF antiserum, as determined in the ex-hypoxic polycythemic mouse assay.[1]

*Standard ESF preparation.* The standard ESF used both in the bioassay and in the radioimmunoassay systems was the IRP-ESF (Standard B) obtained from the National Institute of Medical Research, Bureau of Biological Standards, Mill Hill, London, England. This preparation is crude human urinary ESF with a specific activity of 1.0 unit per 1.48 mg. of protein.

*The erythropoietin was collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina, further processed and assayed by the Hematology Research Laboratories, Childrens Hospital of Los Angeles, under Grant HE10880 (National Heart and Lung Institute).

*Table I.* Experimental format for radioimmunoassay

| *Step* | *Control* | *Tracer* | *Standards* | *Unknown samples (urine or serum)* |
|---|---|---|---|---|
| 1 | 0.1 ml. Diluent | 0.1 ml. Diluent | 0.1 ml. Diluent | 0.1 ml. Diluent |
| 2 | 0.05 ml. Blank | 0.05 ml. Blank | 0.05 ml. Standard | 0 |
| 3 | 0 | 0 | 0 | 0.05 ml. Sample |
| 4 | 0.1 ml. NRS control diluent | 0.1 ml. AS | 0.1 ml. AS | 0.1 ml. AS |
| 5 | 0.05 ml. Label | 0.05 ml. Label | 0.05 ml. Label | 0.05 ml. Label |
| 6 | First incubation: 3 to 4 days at 4° C. | | | |
| 7 | 0.05 ml. GARGG | 0.05 ml. GARGG | 0.05 ml. GARGG | 0.05 ml. GARGG |
| 8 | Second incubation: 18 to 24 hours at 4° C. | | | |

Diluent-0.02 M $PO_4$ buffer, pH 7.45, with 0.1 per cent bovine serum albumin.
Blank = same as diluent (to replace standard volume).
NRS control diluent = diluent with 1 per cent normal rabbit serum used as carrier.
AS = antiserum (Final dilution made with NRS control diluent).
Label = $^{125}$I-hormone diluted to desired counts per minute with diluent to be delivered as 0.05 ml.
Sample = 0.05 ml. of several dilutions of urine or serum. Dilutions made with diluent.
Standard = known dose of erythropoietin (International Reference Preparation, Standard B) in 0.05 ml.
GARGG = goat anti-rabbit gamma-globulin, (final dilution made with diluent).

*Radioimmunoassay experimental format.* The Sephadex G-150 fractions of $^{125}$I-ESF to be used in the radioimmunoassay were diluted to obtain a concentration of 5,000 to 7,000 counts per minute (c.p.m.) in a 0.05 ml. volume. The diluent used was 0.02 M phosphate buffer, pH 7.45, with 0.1 per cent BSA. The same diluent was used for IRP Standard ESF, urine, and serum samples. The protocol used for these studies is presented in Table I. The radioimmunoassay samples were incubated at 4° C. for 3 to 4 days (first incubation period) and the reactions carried out in 6 by 50 mm. disposable glass tubes.

*Separation of bound and free* $^{125}$I-ESF. The double-antibody technique[15] was used as follows: goat anti-rabbit gamma-globulin (GARGG)* was reconstituted in 10 ml. of the radioimmunoassay diluent. After the completion of the first incubation, 0.05 ml. of the GARGG solution was added to each tube. This concentration of GARGG was selected to provide maximal precipitation of antibody-bound $^{125}$I-ESF. The incubation was continued for 18 to 24 hours at 4° C. (Table I). At the end of this period, 0.3 ml. of the radioimmunoassay diluent was added to each tube and the samples counted on the scintillation counter to determine total counts per minute per tube. The samples were then centrifuged in a refrigerated centrifuge for 20 minutes at 2,400 × g. The supernatant was aspirated and discarded and the precipitate counted to determine the per cent $^{125}$I-ESF bound to antibody.

*Bioassay of ESF and sera.* A modification of the ex-hypoxic polycythemic mouse assay described by Cotes and Bangham[1] was used to determine the erythropoietic activity of standard and unknown samples. HAM/ICR strain female mice (18 to 25 grams) were made polycythemic by exposure to 0.42 atmospheres for 22 hours each day for 2 weeks. The mice were injected subcutaneously with one-half the total dose of either saline, human urinary ESF, or test human serum or urine on the fourth and fifth days following their removal from the hypobaric chamber. Each mouse received 0.5 $\mu$Ci of radioactive iron ($Fe^{59}$) citrate intravenously on the sixth posthypoxic day. Two days later (eighth posthypoxic day) each animal was exsanguinated via cardiac puncture and the per cent $Fe^{59}$ incorporation into red blood cells was determined.

*Human sera for bioassay and radioimmunoassay.* Blood was obtained by venipuncture from 5 healthy, hematologically normal human subjects, and from 4 patients with end-stage renal insuffi-

*Schwarz-Mann, 12 mg. of lyophilized antibody protein (Lot Y1247).

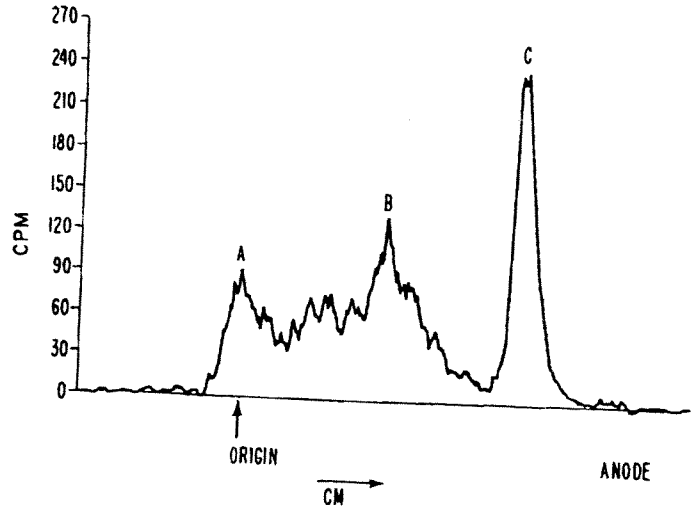


*Fig. 1.* Radiochromatographic scan of iodination mixture, following the technique of Berson and co-workers.[7] Reaction mixture (0.02 ml) was diluted with 1 ml. of 0.02 M phosphate buffer, pH 7.45, with 0.1 per cent BSA; 0.025 ml. of this solution was submitted to chromatography using Whatman No. 3 MM paper followed by electrophoresis at 400 volts and 10 milliamperes in 0.05 M Sorensen's phosphate buffer, pH 7.5, at 0° C.

ciency and anemia undergoing renal dialysis (hematocrit 20 to 24 per cent, creatinine clearance 0 to 2.9 ml. per minute). Serum was collected and frozen (−80° C.). Serum and urine were also obtained from a patient with hypoplastic anemia and stored as above until assayed.

## Results

*Chromatoelectrophoresis of the iodination mixture.* Three peaks of radioactivity were seen in the radiochromatographic scan of the iodination mixture (Fig. 1). Peak A is $^{125}$I-labeled protein bound to the site of application on the Whatman No. 3 MM paper strip and was considered to be the undamaged ESF. Peak B is $^{125}$I-labeled protein migrating anodally and is the protein damaged during the labeling procedure. Peak C is the free $^{125}$I exhibiting the fastest mobility. Control experiments in which the iodination reaction was carried through without adding ESF showed a single peak of radioactivity corresponding to the free $^{125}$I peak when analyzed by chromatoelectrophoresis.

*Sephadex fractionation of the iodination mixture.* Fig. 2 illustrates the elution pattern observed after Sephadex G-150 fractionation of the iodination mixture with detection of 3 peaks of radioactive material. The *first* peak (peak A) represents labeled protein eluted in the void volume (elution volume of blue dextran 2000) and contained a very small proportion of the total counts per minute in the iodination mixture. The *second* peak (peak B) representing the majority of the $^{125}$I incorporated into protein, was eluted with a relative elution volume (volume of elution/void volume) of 1.6 to 1.8. A similar fractionation pattern was obtained using 7,600 to 8,300 units per milligram of ESF preparations with Sephadex G-100 and the biological activity, using ex-hypoxic polycythemic mice, was found to be associated with the first peak, whereas the second peak was devoid of any biological activity.[16–18] The third radioactivity peak (peak C) represents free unreacted $^{125}$I.

*Chromatoelectrophoresis of the pooled peaks A and B.* Chromatoelectrophoresis of the pooled peaks A and B was carried out as described in the Methods section. It was observed that most of the $^{125}$I-labeled protein in peak A remained at the origin, whereas peak B

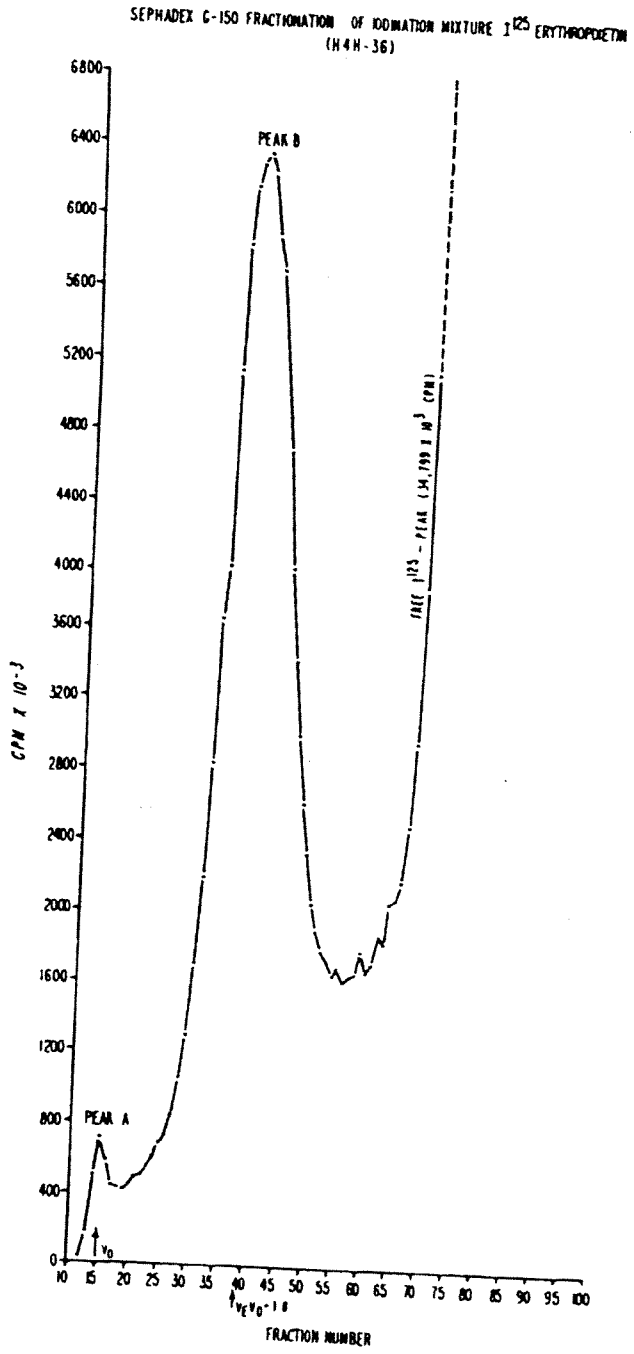


*Fig.* 2. Sephadex G-150 fractionation of the iodination reaction mixture using 0.02 M phosphate buffer, pH 7.45, with 0.1 per cent BSA as an eluent. Fractions of 0.8 ml. were collected and 0.01 ml. aliquots of each fraction were counted.

migrated. This suggested that peak A contains the undamaged labeled protein, whereas peak B represents the damaged labeled protein.

*Antiserum dilution curve with anti-ESF.* Fig. 3 represents the immunochemical evaluation of pooled fractions 13 through 16 (line A) corresponding to peak A and fractions 37 through 49 (line B) from peak B. The pools were diluted such that the total radiolabeled protein reacting with decreasing antiserum concentrations was the same for both protein

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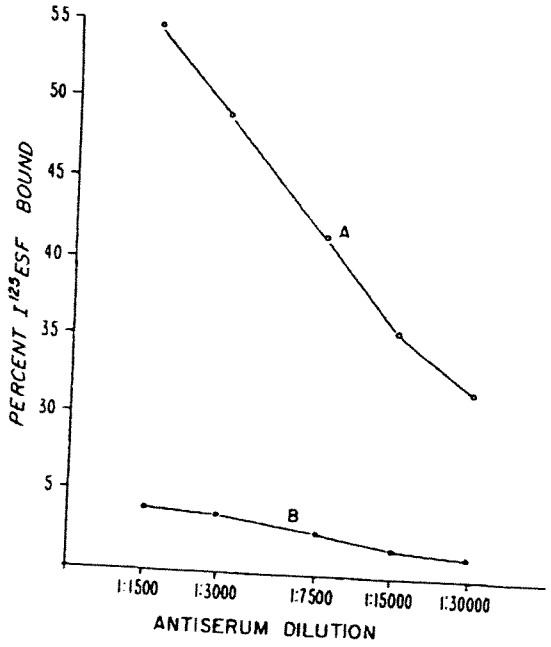


*Fig. 3.* Antiserum dilution curves for pooled fractions 13 through 16 from the first $^{125}$I-labeled protein peak (A) and pooled fractions 37 through 40 from the second $^{125}$I-labeled protein peak (B). (Sephadex G-150 fractionation Fig. 2.)

peaks. Significant binding was observed at an antiserum dilution of 1 : 1500 with fractions 13 through 16, and a linear decrease in the per cent $^{125}$I-labeled bound protein was evident with increasing dilutions of the antiserum. Pooled fractions 37 through 40 were found to have markedly reduced immunoreactivity at all antiserum dilutions.

*International reference preparation dose-response regression line.* Fig. 4 shows the IRP dose-response regression line when pooled fractions 13 through 16 (peak A, Fig. 2) were used as a tracer antigen. The curve was linear between 0.025 and 2 milliunits of IRP-ESF.

*Studies with erythropoietically active urine and serum.* Urine from a patient with a pure red cell aplasia was used in the radioimmunoassay and dilution curves constructed. Using the radioimmunoassay diluent described in the methods section, the urine was studied in the range of 1 : 1 and 1 : 160 dilutions and, as shown in Fig. 5, a graded decrease in binding was observed with increasing urine concentrations. When the slope of the urine dilution regression line (slope = −15.44, S.D. = 0.74) was compared with the slope of the IRP-ESF dose-regression line (slope = −17.12, S.D. = 1.16) no significant difference was observed. The average estimated activity for all of the erythropoietically active urine dilutions in the linear portion of the curve using the radioimmunoassay was 30.7 ± 2.7 (mean± S.E.M.) mU of ESF per milliliter. A serum sample was also studied in the range of 1 : 5 and 1 : 320 dilutions and as seen in Fig. 6 a linear decrease in per cent $^{125}$I-ESF bound to antibody was also seen with decreasing dilutions of the erythropoietically active serum. In this case, the slope of the serum dilution regression line (slope = − 11.14, S.D. = 0.37) was found to be significantly different from the IRP-ESF standard curve ($p < 0.005$) and from the urine dilution curve ($p < 0.005$). Slopes were compared using a one-tailed t-test as described by Dixon and Massey.[19]

*Erythropoietin titers in serum.* Sera from normal human subjects and patients with renal insufficiency had no detectable levels of ESF per 1.0 ml. when assayed in the ex-hypoxic polycythemic mouse, except for one serum from a patient (CK) with renal

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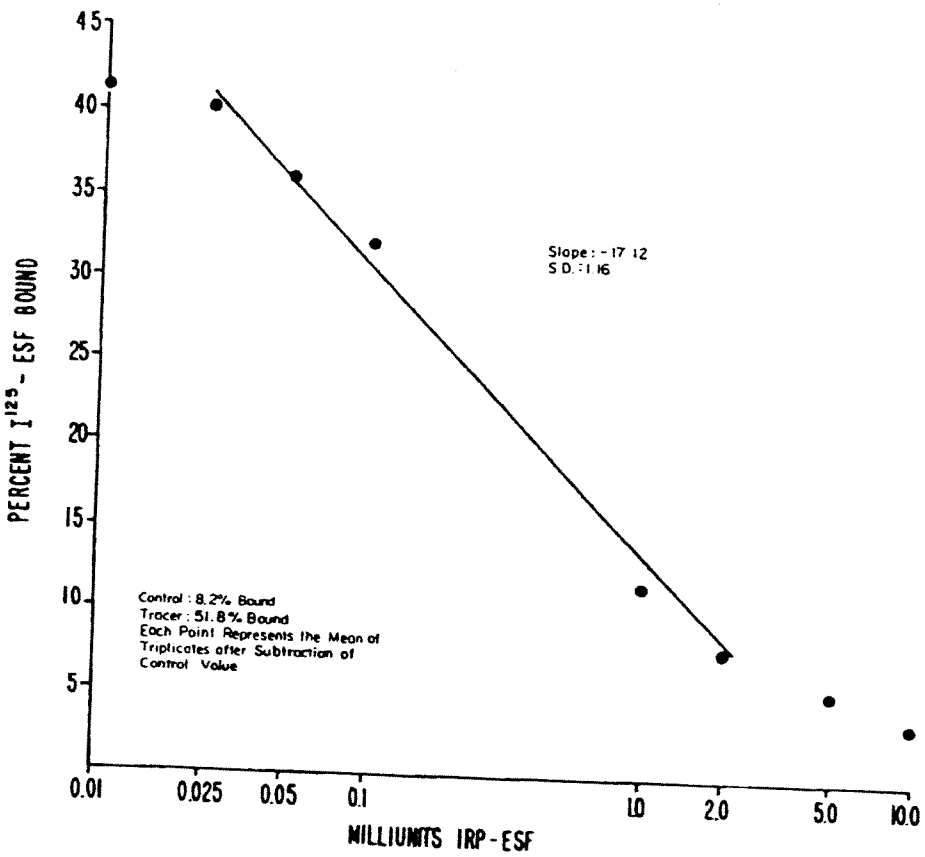


*Fig. 4.* Dose-response regression line for International Reference Preparation erythropoietin (Standard B) using pooled fractions 13 through 16 (Fig. 2) as tracer $^{125}$I-labeled erythropoietin.

disease. As indicated in Fig. 7, the sera were assayed in our radioimmunoassay system in the range of 1:10 and 1:40 dilutions, and a linear binding inhibition was observed with increasing serum concentrations. However, since the slopes of the serum dilution curves did not parallel the corresponding standard IRP-ESF curve, only an estimate of ESF levels was carried out (Table II) at a 1:10 dilution, for the purpose of comparing normal with anemic uremic sera. The two groups could be clearly separated with a greater competition with $^{125}$I-ESF seen in the anemic uremic group (Fig. 7).

## Discussion

There are three necessary requirements for the development of a radioimmunoassay. They are: (1) labeled purified antigen (hormone), (2) specific antiserum, and (3) a method for separating the antibody-bound labeled antigen from the free labeled antigen at the completion of the competitive reaction with standard or unknown unlabeled antigen.[20] Recent advances in the purification of human urinary ESF[9–12] provided us with the opportunity to develop a radioimmunoassay for this hormone. The material used in our studies (12,000 units per milligram) is the most pure ESF now available from a human source. Purity of the protein to be labeled, in our case ESF, is necessary in order to give specificity to the assay system.

Production of antisera to ESF has also been achieved during the past several years,[13] and has been used as a tool in studies of erythropoiesis. Anti-ESF can block the biological activity of ESF in the ex-hypoxic polycythemic mouse assay system. A technique

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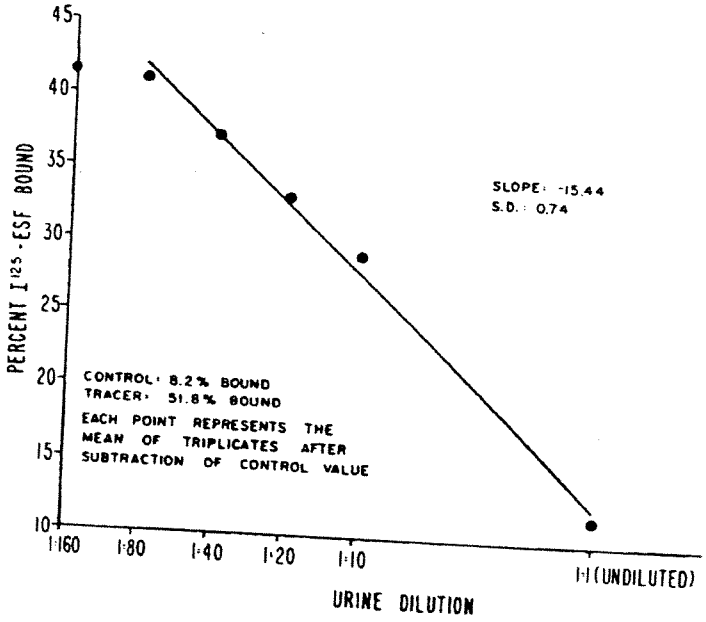


*Fig. 5.* Dilution regression line for erythropoietically active urine from a patient with hypoplastic anemia using pooled fractions 13 through 16 (Fig. 2) as tracer $^{125}$I-labeled erythropoietin.

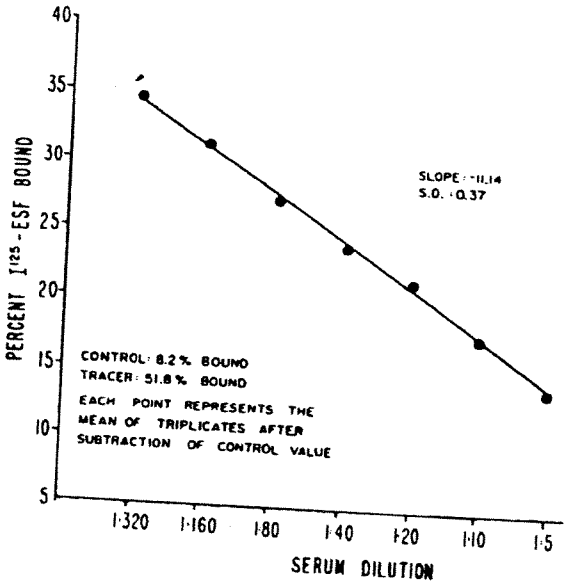


*Fig. 6.* Dilution regression line for erythropoietically active serum from a patient with hypoplastic anemia using pooled fractions 13 through 16 (Fig. 2) as tracer $^{125}$I-labeled erythropoietin.

for the separation of antibody-bound from the free $^{125}$I-ESF is needed since the concentrations of antigen (ESF) and antibody are in a range that would not allow spontaneous precipitation, and the double-antibody technique[15] using goat anti-rabbit gamma-globulin (GARGG) was selected due to its relative simplicity and after considering previous experience which indicated that GARGG will not precipitate free ESF from solution.

The Chloramine-T method for iodination of proteins is a widely used procedure in the preparation of labeled peptide hormones for radioimmunoassay.[14] In our hands, it allowed the preparation of $^{125}$I-labeled ESF that in turn was used as a tracer antigen in a

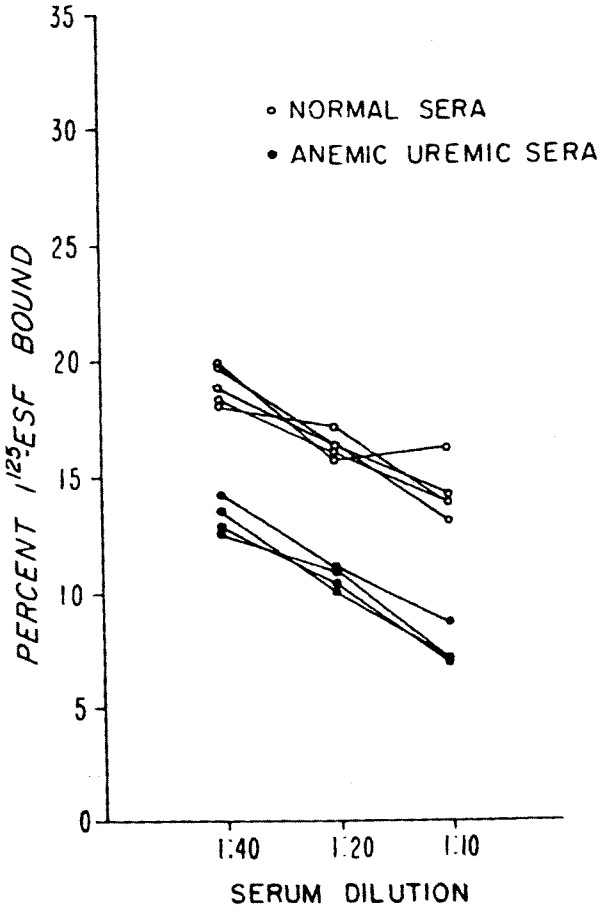


*Fig. 7.* Radioimmunoassay of erythropoietin in sera from normal and anemic uremic human subjects, using pooled fractions 13 through 16 (Fig. 2) as tracer $^{125}$I-labeled erythropoietin.

radioimmunoassay for the hormone. However, as can be appreciated from the chromatoelectrophoretic evaluation of the labeled ESF in our studies, a significant amount of the labeled protein migrates anodally indicating a high degree of protein damage during the labeling reaction.

Sephadex G-150 fractionation of the iodination mixture permitted resolution of two peaks of $^{125}$I-labeled protein. Using the results of chromatoelectrophoresis and immunoreactivity with anti-ESF it was concluded that the material in the first peak in the elution pattern of the iodination mixture represented mainly undamaged $^{125}$I-labeled ESF. Our previous experience with Sephadex G-100 fractionation of the $^{125}$I-labeled ESF indicated that the biological activity was located in the first peak of the $^{125}$I-labeled protein, as determined using the ex-hypoxic polycythemic mouse assay.[16–18] Our assumption of the integrity of the labeled material in peak A from Sephadex G-150 fractionation is based on several criteria: (1) identical elution patterns using Sephadex G-100 and G-150 fractionation were seen; (2) peak A from both fractionations was immunoreactive with ESF antiserum whereas peak B was devoid of any immunoreactivity; (3) if the material in peak A from Sephadex G-100 was fractionated on G-150 and eluted along with peak B, a greater immunoreactivity would have been observed with peak B following G-150 fractionation, and such was not the case; (4) following G-150 fractionation, most of pooled peak A remained at the origin, whereas pooled peak B fractions migrated anodally when

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*Table II.* Estimation of erythropoietin titers in human sera using the radioimmunoassay

| | *Erythropoietin titers* (milliunits/ml).* |
|---|---|
| *Normal subjects* | |
| GF | 84 |
| SR | 74 |
| JL | 74 |
| DB | 72 |
| RN | 52 |
| *Anemic uremic patients:* | |
| CK | 162 |
| DT | 200 |
| TW | 200 |
| SB | 200 |

*Serum (1.0 ml.) was nondetectable when assayed for erythropoietin in ex-hypoxic polycythemic mice, both for normal and anemic uremic subjects, except CK.

chromatoelectrophoresed; and (5) a linear and moderately steep dose-response regression line was seen with the standard IRP-erythropoietin. The antiserum dilution curve constructed with the pooled fractions from peak A indicated approximately 55 per cent binding at an antiserum dilution of 1:1500.

It appears that the majority of the iodinated product loses its ability to bind to the specific antibody as seen from the immunochemical evaluation of the two peaks of $^{125}$I-labeled protein obtained after Sephadex G-150 fractionation and from comparison of their relative size. This is probably a major disadvantage in using the Chloramine-T method for iodinating ESF. Iodination of ESF using microelectrolytic procedures,[21] conjugation-labeling,[22] and enzymatic iodination with lactoperoxidase[23] may reduce the degree of protein damage in the labeling procedure. The reason why the immunoreactive material in our studies is eluted in the void volume is not clear since the erythropoietic activity, as determined in the ex-hypoxic polycythemic mouse assay, after Sephadex G-150 fractionation of unlabeled crude human urinary ESF is not excluded from the gel and is eluted in fractions that would correspond to the second protein peak in our Sephadex G-150 fractionation. It is possible that under our experimental conditions of labeling and fractionation, molecular aggregates of ESF may be formed with greater apparent molecular weight or else some alteration in the shape of the molecule is produced which results in the exclusion of part of the undamaged iodinated protein from the gel. This pattern of elution may, nevertheless, be advantageous, since it permitted separation of the damaged protein in the second peak.

The dose-response regression line with IRP-ESF is an indication of the sensitivity of the radioimmunoassay since as little as 0.01 to 0.025 milliunit of unlabeled ESF was capable of producing a detectable competition with $^{125}$I-ESF. The linear portion of the curve has a slope which is steep and offers a wide workable range for the assay of ESF, since it extended from 0.025 to 2.0 milliunits of IRP-ESF.

The studies of serum and urine from a patient with a pure red cell aplasia, as well as sera from normal and anemic uremic human subjects at several dilutions in our radioimmunoassay, allowed the comparison of the slopes of the sera and urine dilution curves with the standard curve for IRP-ESF. Nonparallelism was observed with the serum dilution curves, and this may indicate a difference in immunological reactivity between

standard IRP-ESF and native ESF in serum. It could also indicate nonspecific protein binding to ESF antibody, with impairment of free competition of native ESF with $^{125}$I-labeled ESF and alteration of the slope. The possibility of hormonal cross-reactivity (particularly with other glycoprotein hormones) should not be ruled out until more extensive studies are completed using this system. Finally, the alteration in the slope may depend upon an interference with the second antibody reaction, since it is conceivable that GARGG may cross-react with human gamma-globulin in serum.[24] Studies are underway to correct the differences in the slopes of the serum dilution regression line and IRP standard dose-response regression line. The first approach will be based on calculations which involve a correction factor applied to each dilution of the IRP-DR regression line.[25] In the second approach, IRP dilutions will be made with serum from patients with polycythemia vera. These IRP dilutions will then be used to obtain the IRP standard dose-response regression line. On the other hand, the lack of significant difference between the slopes of the IRP-ESF dose-response curve and urine dilution regression line points to the essential identity of the immunoreactive ESF in urine and in the IRP-ESF preparation. The IRP-ESF preparation is human urinary erythropoietin.

Comparison of our estimated normal range for ESF levels in serum (52 to 84 mU. per milliliter with that of other workers, indicates somewhat higher levels in our present series. Goudsmit, Krugers Dagneaux, and Krijnen,[2] using an immunochemical double-diffusion method, estimated normal levels of ESF in human plasma to be in the range of 0.5 to 2.0 mU. per milliliter. Jordan, Lange, and McDonald[5] using a hemagglutination-inhibition assay for ESF, have also reported blood levels of ESF in normal human donors to be in the range of 6 to 60 mU. per milliliter. Garcia[6] has reported plasma ESF levels of 3.7 to 11.0 mU per milliliter using a radioimmunoassay. Theoretically, every sample with more than 50 milliunits of ESF per milliliter should have been detected in the ex-hypoxic polycythemic mouse assay and that was not the case except for patient CK (Table I). This could be related to differences in the biologically active and immunologically active binding sites on the ESF. Further work with this system is necessary to explain the differences in these two assay systems.

Nevertheless, a relative comparison between normal and anemic uremic sera was possible, and a greater binding inhibition of $^{125}$I-ESF was observed at all dilutions with the latter group (Fig. 7). This may indicate titers of ESF that are above normal levels (Table II) in the anemia of uremia. It is of interest that Lange and co-workers[4] using the hemagglutination inhibition assay, have reported levels of ESF above the range of normal in sera from anemic uremic patients that were erythropoietically inactive in the polycythemic mouse assay. Garcia has recently reported findings in anemic uremic sera using a radioimmunoassay.[26] However, these patients may still suffer from ESF deficiency relative to their increased demand for red blood cells.[27]

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