centration and diafiltration steps and followed by a gel filtration processing step.

1. Concentration and Diafiltration

First and second (7-day) growth cycle supernatants were separately concentrated thirty-fold using a Pellicon ultrafiltration device (Millipore, Bedford, Mass.) with a 10,000 MW cutoff. Concentrated first and second cycle media were pooled and diafiltered on the Pellicon device against 10 mm Tris at about pH 7.0. Pellicon device against 10 mm Tris at about pH 7.0. (The diafiltered media may optionally be made 20 μm in $CuSo_4$ before ion exchange chromatography.) It may be noted that any ultrafiltration device with a 10,000 or 30,000 MW cutoff may be used and that the diafiltration step may be performed against any suitable low ionic strength buffer at a pH of from about 6.0 to 8.5.

2. Ion Exchange Chromatography

The concentrated, diafiltered media from step 1 was pumped on a relatively low density DEAE agarose column (Bio-Rad, Richmond, Calif.). The column was then washed with three volumes of 5 mm acetic acid/1 mm glycine/6 M urea at about pH 4.5. Optionally, the wash may include 20 μm $CuSO_4$ to assist in oxidation of sulfhydryl groups on the desired protein. Glycine was incorporated to react with any cyanate present. Urea serves to stabilize against acid activation of proteases at low pH and to assist in solubilization of proteins. Following the washings which serve to elute off bound materials with greater pKa's than erythropoietin, the column was washed with 25 mm NaCl/10 mm Tris at about pH 7.0 to return to neutral pH and remove urea. Biologically active erythropoietin was eluted with 75 mm NaCl/10 mm Tris at about pH 7.0. $CuSo_4$ (20 μm) can optionally be included in both the neutralizing wash and/or the elution step.

3. Reverse Phase Chromatography

The procedure applied was essentially as in Example 1 except that an open column, low pressure mode was employed. Following identification of the erythropoietin "peak" in gradient fractions at about 60% ethanol, it is preferred to dilute the collected fraction(s) five-fold with, e.g., 10 mm TRIS at pH 7.0 to reduce ethanol concentration and facilitate ethanol with a small amount of buffer (20 mm sodium citrate/100 mm sodium chloride.

4. Gel Filtration

Products of step 3 from which ethanol has been removed was loaded on a column of Sephacryl S-200 (Pharmacia, Piscataway, N.J.). The column was developed using a projected pharmaceutical formulation buffer of 20 mm sodium citrate/100 mm sodium chloride at pH 6.8 to 7.0.

EXAMPLE 3

Radioimmunoassay and in vivo bioassay procedures as described in the above-mentioned U.S. patent application Ser. No. 675,298 were performed using the recombinant erythropoietin recovered by the procedures of Example 1 and 2. The experimental data indicated yields of 52 and 16 percent, respectively, for the Example 1 and 2 products, with ratios of in vivo to RIA activity of 1.02 and 1.3. Subsequent repeats of the Example 2 procedure on different supernatants have provided yields on the order of 48-50 percent.

While the foregoing illustrative examples have described procedures of the invention as practiced for recovery of erythropoietin from mammalian cell culture sources, the procedures are believed to be suitable for recoveries practiced on other culture fluids such as mammalian lysate/supernatant combination and similar fluids derived from yeast cell cultures. Similarly, the individual and composite procedures (and especially the ion exchange chromatographic procedures) are expected to be useful in recovery of erythropoietin from natural sources such as urine.

It will be apparent to those skilled in the art that the procedures above applied to recovery of erythropoietin can be expected to find applicability in recovery of other complex proteins, especially glycoproteins produced by recombinant methodologies. Glycoproteins whose recovery is within the contemplation of the invention include such distinct products as recombinant tissue plasminogen activator, Factor VIII and Herpes Simplex Virus Glycoprotein D.

What is claimed is:

1. A process for the efficient recovery of erythropoietin from a fluid, said process comprising the following steps in sequence:

subjecting the fluid to ion exchange chromatographic separation at about pH 7.0, thereby to selectively bind erythropoietin in said sample to a cationic resin;

stabilizing materials bound to said resin against degradation by acid activated proteases;

selectively eluting bound contaminant materials having a pKa greater than that of erythropoietin by treatment with aqueous acid at a pH of from about 4.0 to 6.0; and

selectively eluting erythropoietin by treatment with an aqueous salt at a pH of about 7.0; and

isolating erythropoietin-containing eluent fractions.

2. The process of claim 1 applied to recovery of recombinant erythropoietin from a cell culture derived fluid.

3. The process of claim 2 applied to recovery of erythropoietin from a mammalian cell culture derived fluid.

4. The process of claim 3 applied to recovery of erythropoietin from a mammalian cell culture supernatant.

5. The process of claim 1 applied to recovery of erythropoietin from urinary fluids.

6. The process of claim 1 wherein the cationic resin is a DEAE agarose resin.

7. The process of claim 1 wherein said stabilizing step comprises treatment with urea.

8. A process for the efficient recovery of erythropoietin from a fluid, said process comprising the following steps in sequence:

(1) subjecting the fluid to ion exchange chromatographic separation at about pH 7.0, thereby to selectively bind erythropoietin in said sample to a cationic resin;

(2) stabilizing materials bound to said resin against degradation by acid activated proteases;

(3) selectively eluting bound contaminant materials having a pKa greater than that of erythropoietin by treatment with aqueous acid at a pH of from about 4.0 to 6.0;

(4) selectively eluting erythropoietin by treatment with an aqueous salt at a pH of about 7.0;

(5) subjecting eluted, erythropoietin-containing fluids to reverse phase liquid chromatographic separation involving an immobilized C_4 or C_6 resin, thereby to selectively bind erythropoietin in said fluid to said resin;

(6) selectively eluting bound erythropoietin from said resin with an aqueous ethanol solution of from 50 to 80 percent at a pH of from about 4.5 to about 8.0; and,

(7) isolating erythropoietin-containing fractions of the eluent.

9. The process of claim 8 applied to recovery of recombinant erythropoietin from a cell culture derived fluid.

10. A process for the efficient recovery of recombinant erythropoietin from a mammalian cell culture supernatant fluid, said process comprising the following steps in sequence:

(1) subjecting the fluid to ion exchange chromatographic separation at about pH 7.0, thereby to selectively bind erythropoietin in said sample to a DEAE agarose cationic resin;

(2) stabilizing materials bound to said resin against degradation by acid activated proteases through treatment with urea;

(3) selectively eluting bound materials having a pKa greater than that of erythropoietin by treatment with aqueous acid at a pH of about 4.3.

(4) selectively eluting erythropoietin by treatment with an aqueous salt at a pH of about 7.0;

(5) subjecting erythropoietin-containing eluent fractions to reverse phase liquid chromatographic separation involving an immobilized C_4 resin, thereby to selectively bind erythropoietin in said fluid to said resin;

(6) selectively eluting bound erythropoietin from said resin with an aqueous ethanol solution of about 60 percent at a pH of about 7.0; and,

(7) isolating erythropoietin-containing fractions of the eluent.

11. The process of claim 10 further including the step of removal of ethanol from isolated erythropoietin-containing fractions.

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Purification of Human Erythropoietin*

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Human erythropoietin, derived from urine of patients with aplastic anemia, has been purified to apparent homogeneity. The seven-step procedure, which included ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography, yielded a preparation with a potency of 70,400 units/mg of protein in 21% yield. This represents a purification factor of 930. The purified hormone has a single electrophoretic component in polyacrylamide gels at pH 9, in the presence of sodium dodecyl sulfate at pH 7, and in the presence of Triton X-100 at pH 6. Two fractions of the same potency and molecular size, by sodium dodecyl sulfate gel electrophoresis, but differing slightly in mobility at pH 9, were obtained at the last step of fractionation. The nature of the difference between these two components is not yet understood.

Erythropoietin is an acidic glycoprotein that is present at a very low concentration in plasma under normal conditions. Under anemic or anoxic stress, it is found in relatively large amount in the plasma and is also excreted in the urine. Erythropoietin is the substance that is responsible, in large part, for the regulation of normal red blood cell differentiation. Because of this function, and because it may have a role in replacement therapy of some kinds of anemia, it is important to have pure erythropoietin in an amount sufficient for chemical characterization. Reports on the purification of human (1) and sheep (2) erythropoietin have been published. In the former, the evidence for homogeneity was not convincing, and in the latter, the total amount was too low for adequate characterization. We report in this paper on the preparation of milligram quantities of human urinary erythropoietin in a state of apparent homogeneity.

EXPERIMENTAL PROCEDURES

Bioassay—The fasted rat method of bioassay (3), in which the incorporation of labeled iron into circulating red cells is measured, was used routinely to quantitate the amount of erythropoietin activity. Samples for assay were dissolved in 0.1% bovine serum albumin in 0.15 M NaCl, 0.01 M $CaCl_2$. Over the 15-month period covered by this work, the in dose-in response curve obtained when 1, 1.5, 2, and 3 units of erythropoietin[1]/rat were used had the following characteristics: slope, 1.11 ± 0.34; intercept, 0.75 ± 0.39; correlation coefficient, 0.96 ± 0.10. The assay values found for the two final hydroxylapatite fractions were confirmed by the polycythemic mouse method (3) which agreed closely with the other two assay methods. We are indebted to Dr. Walter Fried of the Michael Reese Hospital for doing the mouse assays. For the iodinated preparation and for the assay of activity recovered from polyacrylamide gels, biological activity was measured by the marrow cell culture method (4). This procedure, in which both the total uptake of radio-iron and its incorporation into hemoglobin are used as quantitative indicators of erythropoietin activity, is about 1000 times more sensitive than the fasted rat method, but does not distinguish between native erythropoietin and the asialo form, which is inactive in vivo.

Materials—Sodium dodecyl sulfate and DEAE-agarose were bought from Bio-Rad Laboratories, Richmond, Calif., as was hydroxylapatite (Bio-Gel HT, Control 12746); we found no significant difference between several different lots which we used. Sulfopropyl Sephadex (Lot 79631) and Sephadex G-100 (Lot 50111) were bought from Pharmacia Inc., Piscataway, N. J. Materials for gel electrophoresis (acrylamide, *N,N,N',N'*-tetramethylethylenediamine and *N,N'*-methylenebisacrylamide) and Triton X-100, scintillation grade, were bought from Eastman Kodak Co., Rochester, N. Y. Labeled iodide was obtained from Amersham-Searle Corp., Arlington Heights, Ill. Other reagents used were of the best quality commercially available. Ultrafilters were bought from Amicon Corp., Lexington, Mass. PBS is used to designate a solution consisting of 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0.

Iodination—Labeling with ^{125}I (5, 6) was done as follows. To 20 µl of an erythropoietin solution containing 20 µg of protein, 2 µl of 0.5 M phosphate, pH 7.0, and 20 µl of dimethylsulfoxide were added. One microliter of $Na^{125}I$ (100 µCi, equivalent to 7.14 ng of iodide or 57 pg atoms) was then added, followed by 1 µl of freshly prepared chloramine-T (10 mg/ml in water). The mixture was allowed to stand at 24° for 10 min, after which 10 µl of $Na_2S_2O_5$ (25 mg/ml in water) were added. The solution was mixed and allowed to stand for 1 min; then 200 µl of KI (10 mg/ml in 0.05 M phosphate, pH 7.4) were added and mixed for 1 min at 24°, followed by addition of 50 µl of 7% (w/v) bovine serum albumin. The mixture was put on a Sephadex G-10 column (25 × 0.9 cm diameter), which had been equilibrated with PBS, being washed over to the column with two 200-µl washes of KI solution (10 mg/ml). The erythropoietin was separated from unreacted iodide by elution with PBS and collection of 0.3-ml fractions. The major peak material of large molecular weight label (Tubes 13 to 25) was pooled and dialyzed. The final volume of 4.1 ml contained 5.8 × 10^7 cpm of ^{125}I (2.8 × 10^6 cpm/µg of protein, equivalent to 0.1 g atom of iodine/mol of protein).

Because our previous experience showed that sheep erythropoietin was completely inactivated upon iodination using chloramine-T, we used the method of Stacy *et al.* (5), in which the presence of

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[1] One unit of erythropoietin is defined as the biological activity present in one-tenth of the contents of an ampule of the International Reference Preparation distributed by the World Health Organization. In the routine assay, we used, as a working standard, a preparation of sheep erythropoietin that had been standardized against the International Reference Preparation.

EXHIBIT B

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dimethylsulfoxide acts to protect methionine residues from oxidation. At the level of 0.1 atom of iodine/molecule, we found no loss of biological activity when the assay was done within 1 day of iodination. One week later, however, there was appreciable loss (30%), indicating that the procedure had labilized the hormone. At a higher degree of iodination (4 atoms/molecule), 75% of the biological activity was lost within 1 day.

Electrophoresis – Polyacrylamide gel electrophoresis was done by the micromethod which we had used earlier (2), with gels that were 5 × 0.2 cm in diameter. The conditions are given below for each experiment. Gels were fixed in 25% isopropyl alcohol, 10% acetic acid overnight, stained in 0.25% Coomassie blue in 10% acetic acid for 1 h, and destained in 10% acetic acid.

Source of Erythropoietin – Urine, 2550 liters, was collected from two groups of patients with aplastic anemia of unknown origin, in several hospitals in Kumamoto City, Japan. These groups included some patients with moderately severe, chronic anemia for whom the urine titer was about 1 unit/ml, and others with severe, chronic anemia for whom the urine titer was 2 to 6 units/ml. The urine was collected in 11 pools and filtered under suction; 2.5-liter batches were deionized on a Sephadex G-50 column (57 × 15 cm diameter; bed volume, 10 liters). The effluent (3.5 liters) was made 0.025 M with respect to both NaH_2PO_4 and NaCl, and 2.5 g (dry weight) of DEAE-cellulose, previously equilibrated with 0.025 M NaH_2PO_4, were stirred into the solution. After 30 min of stirring at 4°, the DEAE-cellulose was allowed to settle for 2 h in the cold and then collected on a sintered glass filter with the aid of gentle suction. The adsorbed activity was immediately eluted four times with 25 ml of 0.05 M Na_2HPO_4, 0.15 M NaCl. The eluate was dialyzed against deionized water (two changes of 2 liters each) overnight and lyophilized. The total yield of this fraction was 6,376 million units of activity with a mean potency of about 90 u/A.[1] Previous experience had shown that the desalting step on Sephadex G-50 had a yield of 80%, and that the adsorption and elution from DEAE-cellulose yielded 90% of the input activity (7).

RESULTS

All of the urine concentrates were treated with phenol *p*-aminosalicylate, as described by Chiba *et al.* (8), so that the loss of activity due to enzymic degradation was reduced. This procedure was carried out on 18 batches which consisted of a total of 7,059,670 units[2] and a mean potency of 91 u/A (range, 16 to 160). There were 5,115,110 units recovered, with a mean potency of 109 u/A. In spite of the fact that 28% of the activity was lost and the mean purification factor was only 1.20, it was necessary to use this technique to avoid major losses later in the purification process.

The purification method described below was developed as a result of many trials of various standard techniques. For example, we found that use of gel permeation chromatography early in the procedure did not lead to any significant purification, probably due to the large amount of glycoprotein with similar sizes in the crude urine concentrate; stepwise elution of ion exchange columns was used throughout the procedure since we found that gradient elution decreased the resolution.

Ethanol Precipitation – Sixteen separate batches were precipitated with ethanol by the following procedure. The sample, *e.g.*, 111,600 units at 52 u/A, was dissolved in 50 ml of PBS at 4°; 5 µl were removed for assay, and 12.5 ml of 10 M LiCl[3] were added. Absolute ethanol (62.5 ml) at 4° was added slowly with stirring, which was continued for 30 min after the addition was complete. After the flocculent precipitate had been allowed to settle for 15 min, it was removed by centrifugation at 21,000 × *g* for 10 min at −15°. The pellet was washed three times with 10 ml of 50% ethanol, 1 M LiCl and the supernatants were pooled. The washed precipitate was dissolved in 20 ml of PBS, yielding a turbid solution (50% precipitate).

Sixty-seven milliliters of absolute ethanol were added slowly to the combined supernatants; stirring was continued for 30 min and settling for 15 min. The precipitate was collected as before and washed twice with 10 ml of 65% ethanol, 0.7 M LiCl, and the supernatants were pooled. The washed precipitate was dissolved in 20 ml of PBS (65% precipitate).

To the pooled supernatants, 96 ml of ethanol were added slowly, and stirring was continued for 30 min, after which the precipitate was allowed to settle for 14 h at 4°. We have found that this long period in 75% alcohol is required for optimal further fractionation. The precipitate was washed twice with 10 ml of 75% ethanol, 0.5 M LiCl, the supernatants were pooled, and the precipitate was dissolved in 20 ml of PBS (75% precipitate).

The combined supernatant was brought to 90% ethanol by addition of 540 ml of absolute alcohol, stirred for 30 min, and stored at −20° for 48 h before the precipitate was collected, dissolved in 50 ml of cold water, and immediately frozen (90% precipitate). The results of one representative ethanol fractionation procedure are given in Table I.

For the 16 experiments, the range of yields in the 90% ethanol precipitate was 28 to 100%. The range of potency was 133 to 880 u/A.

Since a substantial fraction of the activity was found in the earlier alcohol precipitates, we established conditions for recovery of much of that activity at a potency similar to that of the 90% ethanol precipitate. For example, three groups of pooled fractions (50%, 65%, and 75% alcohol precipitates), with a total volume of 210 ml, were stirred at 4° while 120.37 g of guanidine hydrochloride were added. To the clear solution, 52.5 ml of 10 M LiCl were added with continued stirring. The slightly cloudy solution was stirred for 30 min more, and 790 ml of absolute ethanol was added slowly. After 30 min of stirring, the suspension was kept at 4° for 25 h, and the precipitate was collected by centrifugation at 21,000 × *g* for 15 min at −15°, to yield Supernatant A and a pellet. The pellet was suspended in 50 ml of PBS and stirred for 30 min while 25.66 g of guanidine hydrochloride were added. During the next 20 min, 12.5 ml of 10 M LiCl were added, followed by 187.5 ml of absolute ethanol. Stirring was continued for 30 min, after which the suspension was allowed to settle at 4° for about 14 h. The precipitate was removed at 21,000 × *g* for 15 min, yielding another 75% ethanol supernatant. The re-extraction was repeated; the two supernatant fractions (Supernatant Bi were pooled, but this pool was kept separate from the original Supernatant A. The 75% alcohol precipitate was suspended in 200 ml of PBS and mixed thoroughly.

To Supernatant A, 1052 ml of absolute ethanol were added

TABLE I

Ethanol fractionation

Fraction	A	Activity	u/A	% yield	Purification
Original	2,150	111,600	52	100	
50% precipitate	570	8,900	16	8	
65% precipitate	300	16,100	53	14	3.02
75% precipitate	131	16,500	126	15	2.5
90% precipitate	173	76,500	442	69	8.5

[1] Potency, or specific activity, is expressed as units of biological activity (u) per absorbance unit (A), measured at 278 nm in 1-cm cuvettes.

[2] This figure is slightly different (1.2% higher) from that indicated as the amount obtained from the DEAE-cellulose step. This kind of difference is caused by the uncertainty in the bioassay and will also be seen at subsequent steps.

[3] LiCl was used in the alcohol precipitation procedure in order to increase the solubility of proteins in ethanol (5). Precipitation in the absence of salt resulted in a low potency fraction.

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slowly; the suspension was stirred for an additional 30 min and stored at −20° for 40 h. The precipitate was then collected at 21,000 × *g* for 15 min at −15°, washed twice with 40 ml of 90% ethanol, 0.2 M LiCl, and suspended in 30 ml of PBS. After stirring for 30 min, the suspension was centrifuged at 16,000 × *g* for 10 min at 4°; the small amount of precipitate was washed with 20 ml of PBS, and the supernatant solutions were pooled (90% ethanol precipitate). The pellet fraction was suspended in 20 ml of PBS. Supernatant B was treated in an analogous manner, *i.e.* the 90% alcohol precipitate was collected and both Supernatants A and B were assayed. The fraction precipitated at 90% alcohol from Supernatant A contained 246,840 units (54% of the input activity) with a potency of 560 u/*A*. The fraction precipitate from Supernatant B contained 69,300 units (15% yield) with a potency of 565 u/*A*. When all of the available ethanol precipitates were re-extracted, we recovered 1,515,200 units with a mean potency of 570 u/*A* (range, 220 to 680). This material was pooled with the original 90% alcohol precipitates for further fractionation, making a total of 4,750,740 units at a mean potency of 633 u/*A*.

DEAE-Agarose Fractionation – The solution, in water, of a 90% ethanol precipitate was concentrated to about 5 ml on an Amicon UM-10 ultrafilter, then brought to 25 ml with 0.01 M Tris, pH 7.0, and a 50-µl aliquot was removed. The DEAE-agarose, 100 to 200 mesh, was degassed under reduced pressure, suspended in 0.01 M Tris, pH 7.0, and packed into a column 9.2 × 2.5 cm in diameter (bed volume, 45 ml). The gel was washed with 1.5 liters of 0.01 M Tris, pH 6.9; the ratio of absorbance units added to bed volume (ml) was 6.65. The sample was added to the column over a period of 40 min, and 150-drop fractions were collected. The column was washed with 211 ml of 0.01 M Tris pH 7, and then eluted with the following buffers: 366 ml of 0.01 M Tris, pH 7.0; 5 mM $CaCl_2$; 270 ml of 0.01 M Tris, pH 7.0; 17 mM $CaCl_2$; 194 ml of 0.01 M Tris, pH 7.0; 30 mM $CaCl_2$; and 65 ml of 0.1 M $CaCl_2$. The elution pattern can be seen in Fig. 1, and the results are given in Table II.

Of the 4,566,240 units of total input, we recovered 4,052,710 (89%) in the 17 mM $CaCl_2$ eluate at a mean potency of 1,110 u/*A*, representing a mean purification factor of 1.97. From this point on in the fractionation calcium was added to all buffers except those used with hydroxylapatite columns because there were inconsistent results and appreciable losses of activity when buffers without calcium were used. For the next step in purification, we selected three eluates from DEAE-agarose columns, amounting to 2,480,400 units (61% of the total yield) with a mean potency of 1,750 u/*A*.

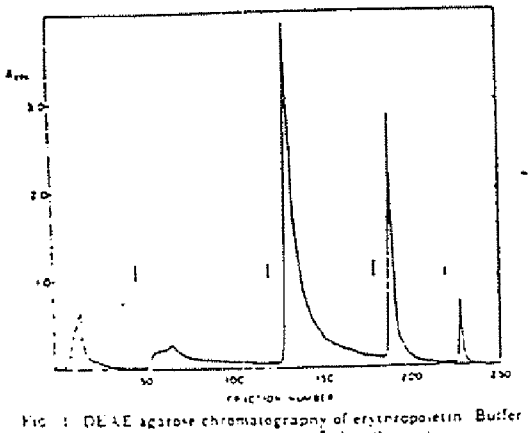

FIG. 1. DEAE agarose chromatography of erythropoietin. Buffer changes are indicated by arrows as specified in the text.

Sulfopropyl-Sephadex Chromatograph – The three eluates (17 mM $CaCl_2$) from DEAE-agarose columns were desalted and concentrated on a UM-10 ultrafilter and then dialyzed against 2 liters of 5 mM $CaCl_2$, pH 7.5, overnight. In the sample run described below, 30 ml of dialyzed solution were brought to pH 4.50 by dropwise addition of 0.1 M HCl; the small amount of precipitate formed was removed by centrifugation and washed with 5 ml of 5 mM $CaCl_2$, pH 4.5. The wash, pooled with the supernatant, was applied to a sulfopropyl-Sephadex column (15.0 × 2.5 cm in diameter; bed volume, 78.3 ml) which had been equilibrated with 5 mM $CaCl_2$, pH 4.50. The absorbance units to bed volume (ml) ratio was 2.47. We found that a low value for this ratio is critical for optimal fractionation on sulfopropyl-Sephadex; for example, if the absorbance unit to bed volume ratio was greater than 10, almost all of the activity was found in the effluent fraction. The following buffers were used in developing the column. Input was: 5 mM calcium acetate, pH 4.50, specific conductivity = 1,075 µmho cm^{-1}. Eluting buffers were: 7.5 mM calcium acetate, pH 4.70, specific conductivity 1,500 µmho cm^{-1}; 12.5 mM calcium acetate, pH 5.25, specific conductivity = 2,100 µmho cm^{-1}; 15 mM calcium acetate, pH 5.5, specific conductivity = 2,400 µmho cm^{-1}; 0.1 M calcium acetate, 0.01 M Tris, pH 7.24, specific conductivity = 11,500 µmho cm^{-1}. The column was run at 0.4 ml/min at 4°, and 200-drop fractions were collected. After a reading was taken at 278 nm and the appropriate pools were made, the solutions were neutralized (within 1 h after elution), and aliquots were removed for assay and stored at −20°. The elution pattern is presented in Fig. 2 and results of the fractionation are shown in Table III.

The overall results of this step in the purification were: 55% recovery (1,352,810 units) in the 12.5 mM calcium acetate, pH 5.55 fraction, at a mean potency of 11,170 u/*A*, and with a mean purification factor of 6.38.

Gel Filtration – The 12.5 and 15 mM calcium acetate eluates from the sulfopropyl-Sephadex column separations were run in two separate batches on the same gel column. The pools were concentrated on Amicon UM-2 ultrafilters to about 5 ml and equilibrated with 10 mM $CaCl_2$, 10 mM Tris, pH 6.87, before application to the column. The Sephadex G-100 gel was degassed under reduced pressure and equilibrated with the same buffer before the column was poured. The column (100 × 2.5 cm diameter) was calibrated with markers of known molecular size before being used for the erythropoietin fractions. The void volume was 135 ml; bovine serum albumin monomer eluted at 224 ml, ovalbumin at 258 ml, and cytochrome *c* at 368 ml. The sample was added to the bottom of the column, as was the buffer which was passed through the column at 21 to 22 ml/

TABLE II

DEAE-agarose fractionation

Fraction	*A*	u	u/*A*	% recovery of activity	Purification factor
Input	209	164,030	549	100	
0.01 M Tris	23	51	2		
0.01 M Tris, 5 mM $CaCl_2$	37	9	0.2		
0.01 M Tris, 17 mM $CaCl_2$	158	143,210	907	87	1.65
0.01 M Tris, 30 mM $CaCl_2$	57	36,060	633	22	1.15
0.1 M $CaCl_2$	8	98	12		

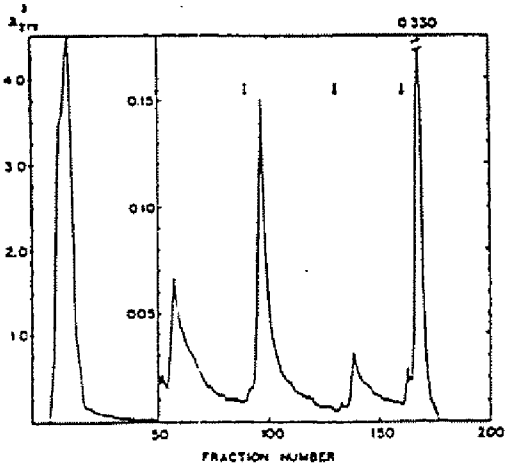

FIG. 2. Sulfopropyl-Sephadex chromatography of erythropoietin. Buffer changes are indicated by arrows as specified in the text.

TABLE III

Sulfopropyl-Sephadex fractionation

Fraction	A	u	u/A	% recovery of activity	Purification factor
Input	193	198,160	730	100	
5 mM calcium acetate	138	5,610	27	3	
7.5 mM calcium acetate	8	16,920	3,170	9	4.3
12.5 mM calcium acetate	12	71,240	9,420	36	12.9
15 mM calcium acetate	9	18,600	6,360	9	8.7
0.1 M calcium acetate	19	10,810	3,040	5	4.1

h by means of a Mariotte bottle with a 42-cm hydrostatic head. Each fraction collected was 4.1 ml (120 drops), and the following pools were made: I, 0 to 131.2 ml; II, 131.2 to 184.5 ml; III, 184.5 to 205 ml; IV, 205 to 258.3 ml; and V, 258.3 to 328 ml (Fig. 3). The first four pools were concentrated by ultrafiltration and aliquots were assayed. In one of the runs, pools I and II contained 17% and 5% of the absorbance units, respectively, but no detectable activity; pool III contained 32% of the absorbance units and 104% of the input activity, yielding a fraction with a potency of 38,850 u/A; and pool IV contained 10% of the absorbance units and 2% of the biological activity. Pool V was not assayed.

For the combined two gel filtration runs, the yield in pool III (184.5 to 205 ml) was 100%, the mean potency was 39,060 u/A, and the purification factor was 3.04.

Hydroxylapatite Chromatography—Hydroxylapatite was packed under unit gravity into a column (6.1 × 1.5 cm diameter) and washed with 500 ml of water and then with 400 ml of 0.5 mM phosphate buffer, pH 7.1, conductivity = 69 μmho cm^{-1} (Buffer I), by use of a peristaltic pump which maintained the flow at 0.3 ml/min. After the buffer wash, the length of the column was 3.4 cm and the bed volume was 6.0 ml. The input sample was concentrated and desalted on an Amicon DM-5 ultrafilter by adding water to the concentrate and reconcentrating three times. The final concentrate and the wash of the filter were centrifuged at 6,000 × *g* for 20 min at 4°. The small insoluble pellet was washed once with 0.5 mM phosphate, pH 7.1, and the wash was added to the supernatant. An aliquot for assay was removed and the remainder (22 ml) was added to

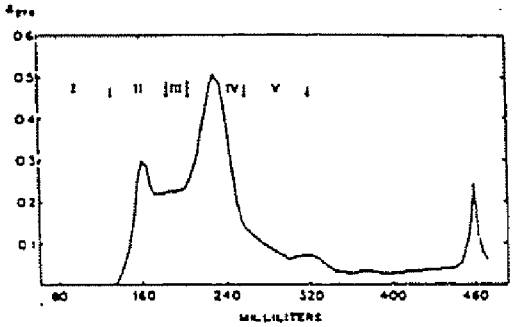

FIG. 3. Gel filtration chromatography of erythropoietin. The *arrows* indicate pools made for assay.

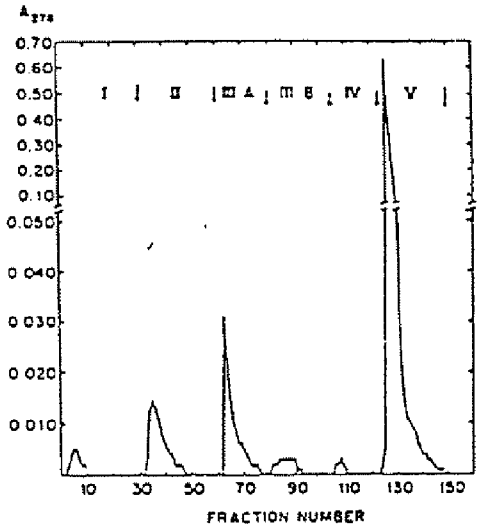

FIG. 4. Hydroxylapatite chromatography of erythropoietin. The *arrows* and *Roman numerals* indicate buffer changes and pools.

TABLE IV

Hydroxylapatite chromatography of erythropoietin

	A	u	u/A	% recovery of activity	Purification factor
Input	11.0	381,480	34,680	100	
Effluent	0.35	490	1,400		
Fraction II	0.96	123,480	128,620	32	3.71
Fraction IIIA	0.85	79,850	93,940	21	2.71
Fraction IIIB	0.32	22,800	71,250	6	2.03
Fraction IV	0.29	6,260	21,590	2	
Fraction V	7.22	17,680	2,450	5	

the column. The ratio of absorbance units added to bed volume (ml) was 1.82. The input buffer was pumped through the column until the effluent A_{278} was less than 0.005 (149 ml), and the following elution schedule was carried out: Buffer II, 1 mM phosphate (pH 7.1, specific conductivity 131 = μmho cm^{-1}), 150 ml (Fraction II); Buffer III, 2 mM phosphate (pH 6.9, specific conductivity = 270 μmho cm^{-1}), 220 ml (Fractions IIIA and

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TABLE V

Purification of erythropoietin: summary

After the DEAE-agarose step, only 61% of the product was used in further fractionation.

Step	Input		Product		Yield (%)		Mean purification factor
	u	Potency u/A	u	Potency u/A	Each step	Overall	
DEAE-cellulose			6,976,170	89	100	100	
Phenol	7,059,670	91	5,115,110	110	72	72	1.21
Ethanol	5,186,590	88	4,750,740	660	92	68	7.50
DEAE-agarose	4,566,240	563	4,052,710	1,107	85	58	1.97
Sulfopropyl-Sephadex	2,480,400	1,750	1,352,810	11,170	55	32	6.38
Sephadex G-100	1,259,040	12,830	1,274,430	39,060	100	32	3.04
Hydroxylapatite	1,083,650	38,770	721,160	82,720	67	21	2.13

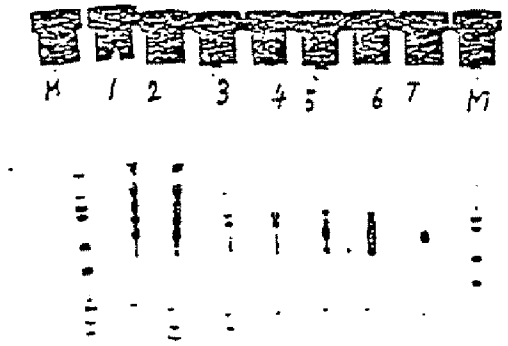

FIG. 5. SDS-polyacrylamide electrophoretic analysis of the most active fractions from each step in the purification of human erythropoietin. The gels marked *M* had serum as marker proteins. *1*, DEAE-cellulose eluate; *2*, phenol-treated; *3*, 90% ethanol precipitate; *4*, DEAE-agarose (17 mM Ca^{++}) eluate; *5*, sulfopropyl-Sephadex (12.5 mM Ca^{++}) eluate; *6*, Sephadex G-100 (pool III); *7*, hydroxylapatite Fraction II. The gel concentration was 7.5%.

IIB); Buffer IV, 2 mM phosphate (pH 6.9, specific conductivity = 402 μmho cm^{-1}, 84 ml (Fraction IV)); Buffer V, 0.1 M phosphate (pH 6.8, specific conductivity = 9.6 mmho cm^{-1}, 134 ml (Fraction VI). The elution pattern is shown in Fig. 4; the results for one such column are listed in Table IV.

The total input for the two runs was 1,083,650 units, with a mean potency of 38,770 u/A. The total recovered in Fractions II and IIIA was 721,163 units (67%) with a mean potency of 82,720 u/A and a mean purification factor of 2.13. Each of the fractions, II and IIIA, from the two experiments was concentrated by means of Amicon DM-5 ultrafilter and stored frozen.

When we examined Fractions II and IIIA from the two hydroxylapatite columns by gel electrophoresis in SDS[1] (7.5% gels), we found single bands, each with a relative mobility (with reference to the Pyronin Y band) of 0.50. No detectable difference in mobility, in the presence of SDS, between Fractions II and IIIA could be found. Fig. 5 shows the SDS-gel electrophoretic analysis of each of the most active fractions throughout the purification procedure, and Table V summarizes the seven-step method. The overall purification factor was 928 (calculated from initial and final potencies).

Since the hydroxylapatite Fractions II and IIIA appeared to have a single identical component on SDS gels, we examined

[1] The abbreviation used is SDS, sodium dodecyl sulfate.

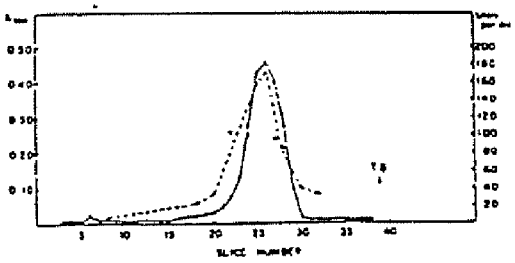

FIG. 6. Gel electrophoresis (pH 9) of erythropoietin (hydroxylapatite Fraction II). ● represents absorbance of the Coomassie blue-stained material, and ○ represents biological activity. The gel concentration was 8%. *T. D.*, tracking dye.

these fractions further for evidence of heterogeneity. When these fractions were compared by gel electrophoresis at pH 9, it was clear that there was a small, but significant difference in mobility. Fraction II had a mobility relative to the bromphenol blue tracking dye of 0.49, and the value for Fraction IIIA was 0.52. In spite of our finding of similar potency and molecular size, these two preparations must be considered different. The chemical basis for this difference is now being studied.

Fraction II was run on two gels at pH 9; one was fixed, stained, and scanned, and the other was sliced into 1.1-mm slices which were put into 0.5 ml of 0.10% bovine serum albumin, 10 mM $CaCl_2$ in 0.15 M NaCl, and the hormone was allowed to diffuse out of the gel at 4° for 18 h. On assay by the *in vitro* method, we found the biological activity coincident with the single band of stained protein (Fig. 6).

In view of our previous finding that native sheep erythropoietin was very poorly fixed to polyacrylamide gels and was largely lost during the staining procedure, we adopted the expedient used earlier for the sheep hormone. Fraction II was iodinated with ^{125}I, run on a gel at pH 9 which was then cut into 1.1-mm slices, and counted before and after fixation. The results in Fig. 7 show a single peak of labeled hormone, only a fraction (44%) of which was fixed. The iodinated hormone was then run on a gel at pH 6 in order to confirm the apparent homogeneity. It became clear that there was a large degree of aggregation at the lower pH, since only a small amount of the radioiodine could be found in the gel, with the major fraction remaining at the origin. We then used the observation of Kawasaki and Ashwell (10), who found that aggregation of a liver glycoprotein could be reduced by the use of Triton X-100. When both the native and asialo forms of erythropoietin were run on gels in the presence of 0.05% Triton X-100 (Fig. 8), we found for the former a single symmetrical peak and for the

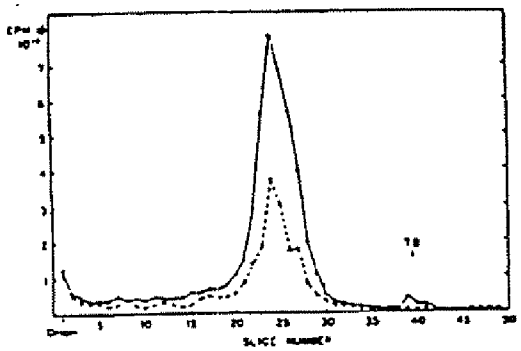

FIG. 7. Gel electrophoresis (pH 9) of ^{125}I-labeled erythropoietin (hydroxylapatite fraction II). ●——● represents total radioactivity; ○- - -○ represents radioactivity remaining in the gel slices after fixation. The gel concentration was 8%. *T. D.*, tracking dye.

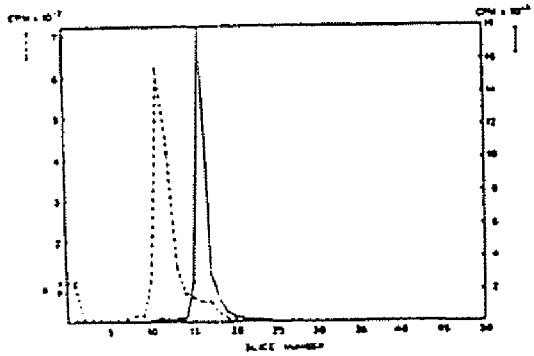

FIG. 8. SDS-gel electrophoresis of ^{125}I-labeled erythropoietin (hydroxylapatite Fraction II). ●——● represents native erythropoietin; ○- - -○ represents asialoerythropoietin. The gel concentration was 10%.

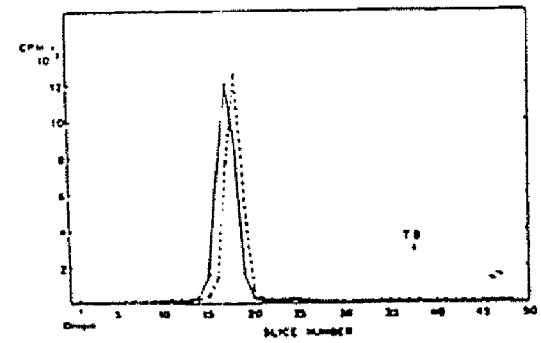

FIG. 9. Gel electrophoresis (pH 6, 0.05% Triton X-100) of ^{125}I-labeled erythropoietin (hydroxylapatite Fraction II). ●——● represents native erythropoietin; ○- - -○ represents asialoerythropoietin. The gel concentration was 10%. *T. D.*, tracking dye.

latter, one major symmetrical peak with only a trace of the native hormone contaminating the asialo form. At a lower concentration of Triton X-100 (0.01%), there was still appreciable aggregation, as detected by label that remained at the origin.

Examination of iodinated Fraction II, both native and asialo, on SDS gels (11) showed single, symmetrical peaks (Fig. 9) with no evidence of heterogeneity with respect to size.

We measured the absorbance at 278 nm and at 191 nm, using crystalline bovine serum albumin as a standard and correcting for stray light at 191 nm, and found that $A^{1\%}_{1cm}$ for erythropoietin is 8.51. Using this value, the mean potency of homogeneous human erythropoietin can be expressed as 70,400 units/mg of protein.

DISCUSSION

Espada and Gutnisky (1) isolated a fraction, from urine of patients with anemia due to hookworm, that had a potency of about 8,000 units/mg of protein. They claimed, on the basis of a gel permeation experiment, that this fraction was homogeneous; the poor resolution characteristic of this method of analysis, however, makes it necessary to use additional kinds of information to establish purity. In a subsequent paper, Espada *et al.* (12) claimed that the same preparation was homogeneous by gel electrophoresis at pH 9, although they pointed out that the stained band was diffuse. In addition, these authors showed an immunodiffusion pattern that was inconclusive with respect to immunological homogeneity. Our finding that human erythropoietin has a minimal potency of 70,400 units/mg of protein suggests that Espada's preparation is either about 11% pure or, if it is homogeneous, is largely in the asialo form that has no activity *in vivo*.

Our previously reported data (2) indicated that the preparation of sheep plasma erythropoietin, with a potency of 9,200 u/*A*, was free of any contaminant except for a small amount of asialoerythropoietin. If this is truly the case, then human urinary erythropoietin is 7 to 8 times more active than the sheep hormone, when assayed by the same method. This may be due to a greater sensitivity of rats to the human than to the sheep hormone, or it may indicate that human urinary erythropoietin is intrinsically more active than sheep plasma erythropoietin.

The appearance of two fractions with the same potency, as a result of hydroxylapatite fractionation, suggests a degree of heterogeneity which is not detected upon electrophoresis in SDS, and which might be accounted for by a small difference in the number of terminal sialic acids or of amide groups, or of both. Our findings of single peaks upon electrophoresis at pH 9, pH 6, and pH 7 in SDS constitute reasonable evidence of homogeneity with respect to charge and molecular size for each of the two fractions. At pH 6 in the presence of Triton X-100, the native and asialo forms are clearly separated (Fig. 9), and we could expect to be able to detect an appreciable amount of the latter mixed with the former. With the exception of the small amount of native erythropoietin found in the asialo preparation, both forms appear to be homogeneous.

Without added surfactant, there is a considerable tendency for native and asialo erythropoietin to aggregate at pH 6 and for the asialo form to aggregate at pH 9, but at pH 7 in the presence of SDS and dithiothreitol, both forms appear to be monomeric. The human asialo hormone has an apparent molecular weight of 34,000 in SDS, whereas the native form has an apparent molecular weight of 39,000.* These values contrast with the molecular weight of 41,000 found for sheep plasma asialoerythropoietin by the SDS-gel electrophoretic method and the calculated value of 46,000 for the native form

*The molecular weight determined by SDS-gel electrophoresis was the same whether we used 7.5% or 10% gels.

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of the sheep hormone (13). When we studied the sheep hormone, it was clear that the Weber and Osborn method of molecular weight determination (11) by gel electrophoresis in SDS was not accurate for the fully sialylated hormone, possibly because of a substantial contribution by the sialic acids to the net charge. At present, we cannot estimate the molecular size of the native human hormone from that of the asialo form since we do not yet have an accurate estimate of the sialic acid content.

The method of iodination of erythropoietin deserves comment. We found with the sheep hormone that the unmodified iodination method (5), in which chloramine-T is used, caused total loss of biological activity. Following the precedent set by Stanley and Metcalf (14), we used a modification of the method of Stagg *et al.* (6) in which the iodination was carried out in 45% dimethylsulfoxide to protect methionine residues from oxidation. In contrast to the findings of Stagg *et al.*, who found no loss of gastrin activity, and those of Stanley and Metcalf who found no loss of colony-stimulating activity, we found that the iodination method does cause appreciable inactivation of erythropoietin. For the case of the preparation with 4 iodine atoms/molecule, it can be calculated that less than 2% of the hormone would be noniodinated. This would suggest that the 25% of the biological activity that remained was due to the iodinated derivative. Until a method can be found for preparation of a fully active, labeled hormone, this less active, labeled erythropoietin may still be useful for the study of a number of biological characteristics of the hormone which, until now, have not been amenable to experiment.

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Vol. 47, No. 6, 1972 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

STABILIZATION OF URINARY ERYTHROPOIETIN

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SUMMARY

The instability of erythropoietin derived from human urine may be due to protease and/or sialidase action. Extraction of the hormone into phenol and subsequent precipitation with alcohol results in a marked increase in stability without appreciable loss of activity.

INTRODUCTION

Urine from anemic patients has been an important source of large amounts of crude erythropoietin (1). Plasma from anemic animals has yielded a preparation of erythropoietin that is virtually homogenous by gel electrophoresis (2). Although a high potency fraction of the urinary hormone has been described (3), no evidence regarding its homogeneity has been published. One of the impediments in purifying urinary erythropoietin, despite the initial advantage of having starting material with a low protein concentration, is its marked instability. Graham et al. (4) have shown that there are large losses during fractionation, and in our early experience with urinary erythropoietin concentrates, losses were considerably greater than those we found

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EXHIBIT C

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for comparable steps when working with plasma. Loss of activity was even found during storage at -20°C.

We have determined that urine concentrates contain both protease and sialidase activities that probably are the cause of the instability. The method described in this communication makes it possible to prepare from urine crude fractions that are low in proteases and sialidase and are stable. There has been a previous report of protection of erythropoietin activity by use of a small amount of phenol as a bacteriocidal agent (5).

MATERIALS AND METHODS

Starting materials were two different crude precipitates from erythropoietin-rich human urine. They were generously supplied by the National Blood Resources Branch of the National Heart and Lung Institute, Bethesda, Maryland, and were derived from the collection center in Corrientes, Argentina with some processing at the Children's Hospital, Los Angeles, California. One preparation was labeled C-1 and the other TaLSL.

Assay for erythropoietin was done by the fasted rat method (6), using as a standard a plasma fraction that had been standardized against the International Reference preparation. All assays were done with groups of five rats and by interpolation of the log dose-log response standard curve using standard doses of 1, 2, and 3 units per rat. Protein concentrations were determined by measurement of absorbance at 278 nm, and using the value of $A^{1\%}_{278} = 10$ derived from measurements of mixtures of glycoproteins.

Assay for protease activity was done using as substrate an insoluble collagen-dye complex (Azocoll, Calbiochem). A suspension of 1 mg of the substrate in 200 µl of 0.1 M phosphate buffer at pH 7.0, mixed with 1 mg of crude urinary erythropoietin preparations in 200 µl of the same buffer, was incubated for 15 min at 37°, the insoluble material removed and the solution absorbance at 530 nm was read. A blank consisting of the same concentration of substrate but with no source of protease was incubated simultaneously and the activity determined from the difference between the blank and experimental readings.

Sialidase activity was determined using sialyllactose (5 mg/ml in 0.1 M Na acetate, 0.01 M $CaCl_2$, pH 4.5) as substrate. After incubation with the erythropoietin preparations for 3 hr, the free sialic acid was determined by the alkaline Ehrlich's method (7).

Treatment of Erythropoietin Samples

The C-1 material, at 10 mg/ml, was dissolved in 8 M urea and the urea was then removed by pressure ultrafiltration through a UM-10 membrane (Amicon Corporation, Lexington, Massachusetts) in the cold. This preparation is so insoluble that a high concentration of urea is needed to solubulize it; the activity then stays in solution when the urea is removed. After complete removal of urea, the crude erythropoietin in phosphate-buffered saline (PBS) was heated for 5 min at 100°C. The solution was chilled and centrifuged, and the precipitate was washed once with cold PBS. The pooled supernatants were frozen until the next step.

Because it is more easily dissolved, the TaLSL preparation was put directly into cold PBS (10 mg/ml), then heated at 100°C for one min and centrifuged as described the C-1 preparation.

A mixture of one volume of PBS, one volume of 0.2 M phosphate buffer at pH 7.0, three volumes of 0.475 sodium p-aminosalicylate (PAS) and 5 volumes of freshly distilled phenol was shaken vigorously in the cold, and after phase separation the upper (aqueous) phase was discarded. The lower phase, phenol equilibrated with buffer and PAS, was used to treat the erythropoietin preparations.

To each preparation in PBS, enough phosphate buffer at pH 7.0 and PAS were added to make the final concentrations 0.04 M and 0.285 M, respectively. They were then shaken vigorously with an equal volume of phenol and the phases were separated by centrifugation in the cold. The separated fractions (including any interphase material) were dialyzed against PBS in the cold to remove phenol, and concentrated by pressure ultrafiltration. The fraction derived from the phenol phase still contained an appreciable amount of

phenol, which was re
at a final concentra
before assay.

Assays for pro
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Protease and S

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phenol, which was removed by precipitation of the proteins with cold alcohol at a final concentration of 90 %. The precipitate was dissolved in PBS before assay.

Assays for protease and sialidase activities in the treated and untreated erythropoietin preparations are shown in Table I. After the phenol extraction about 75 % of the protease activity was lost; alcohol precipitation did not cause any further loss. The inactivation of sialidase activity in the treated preparations was complete.

Table I

Protease and Sialidase Activities in Erythropoietin Preparations

Sample	Protease activity ΔA_{310}	Sialidase activity Sialic acid freed (ug) in 180 min)
Original C-1	0.057	0.18
Phenol-treated C-1	0.013	0.0
Alcohol precipitated, phenol-treated C-1	0.015	0.0

ΔA_{310} represents the difference between the blank and experimental absorbance.

RESULTS

Results of bioassay of fractions from both erythropoietin preparations are presented in Table II. The data show that more than 90 % of the activity was recovered from the phenol phase with a small increase in potency. When the phenol extraction was done in the absence of PAS, activity could be variably recovered in all three fractions. The stability of the phenol-treated C-1 fraction is compared with that of the heat-treated extract in Fig. 1. For at least the first 16 weeks of storage in the frozen state, the phenol-treated erythropoietin concentrate lost very little activity, while the loss of activity from the starting material was substantial.

Table II

Erythropoietin Activity of Phenol-treated Fractions[a]

Sample	Total units	Protein (units/mg)	Recovery (%)
Heated C-1	3,820	7.5	(100)
Upper and inter phases	0		0
Lower phase	3,660		95.8
Alcohol precipitate	3,540	10.5	92.7
Heated TaLSL	7,220	32	(100)
Upper and inter phases	0		0
Lower phase	7,070		97.9
Alcohol precipitate	6,500	47	90.0

a These are the average results of six experiments using C-1 and three using TaLSL.

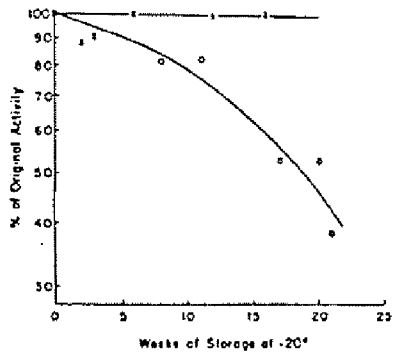

Fig. 1. Stabilization of urinary erythropoietin by phenol treatment.
o Untreated
x Phenol treated

The use of this method of stabilization should permit the development of a more efficient procedure for the isolation of pure human erythropoietin.

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LKB

INSTRUCTION

Ultromould - for casting polyacrylamide gels in thickness between 0.1 and 0.5 mm for electrofocusing with Ampholine carrier ampholytes

EXHIBIT D

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Practical Information

Note: These instructions refer to the items listed below. The use of these conditions with other chemicals, carrier ampholytes and accessories may not give optimal results.

LKB 2217-200	Ultromould gel casting unit with 0.1, 0.2, 0.3, 0.4 and 0.5 mm spacers.
LKB 2217-201	Ultromould spacers 0.1 mm (pkt/5)
LKB 2217-202	Ultromould spacers 0.2 mm (pkt/4)
LKB 2217-203	Ultromould spacers 0.3 mm (pkt/3)
LKB 2217-204	Ultromould spacers 0.4 mm (pkt/2)
LKB 2217-205	Ultromould spacer 0.5 mm (pkt/1)
LKB 1850-211	GelBond PAG-Film
LKB 1850-250	Repel-Silane
LKB 1820-101	Acrylamide
LKB 1820-102	NN'Methylenebis-acrylamide
LKB 1820-103	Ammonium Persulfate
LKB 1820-104	TEMED
LKB 1840-101	Coomassie Blue R250
LKB 2117-710	Roller
LKB 1818	Ampholine® preblended

Table I
Stock Solutions

Acrylamide 29.1% and NN'-Methylenbis-acrylamide 0.9% solution	Dissolve 29.1 g acrylamide and 0.9 g bis in 75 ml of distilled water. Stir until the solution is clear, then make up to 100 ml with distilled water. Filter the solution. It can be stored for up to two weeks at +4°C.
Ammonium Persulfate 10% (w/v)	Dissolve 0.1 g ammonium persulfate in 1 ml of distilled water. This solution should be used fresh.

Note: High quality distilled water should be used.
Note: Acrylamide and bis are very toxic. There is a serious risk of poisoning by inhalation or skin contact. Wear gloves and avoid inhaling dust. Do not pipette solutions by mouth.

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Table II

Composition of gel solution for one polyacrylamide gel (T=7.5%, C =3%, pH range 3.5–9.5, 110×245 mm)

Gel thickness	0.1 mm	0.2 mm	0.3 mm	0.4 mm	0.5 mm
Acrylamide and Bis stock solution	1.25 ml	2.5 ml	3.75 ml	5.0 ml	6.25 ml
LKB 1818-101 pH 3.5-9.5 Ampholine carrier ampholytes	0.38 ml	0.75 ml	1.2 ml	1.5 ml	1.9 ml
Distilled water	3.3 ml	6.7 ml	9.9 ml	13.4 ml	16.7 ml
Deaerate for 5 minutes in a Buchner flask, then add:					
Ammonium persulphate stock solution	35 µl	70 µl	100 µl	140 µl	175 µl
TEMED	5 µl	10 µl	15 µl	20 µl	25 µl
Mix gently by swirling Total gel volume	5 ml	10 ml	15 ml	20 ml	25 ml

Table III

Electrode solutions for pH range 3.5–9.5				
Anode	0.025 M Aspartic acid	MW:132	0.33 g	Make to 100 ml with dist. water
	+			
	0.025 M Glutamic acid	MW:146	0.37 g	
Cathode	2.0 M Ethylenediamine	MW:60	13.2 ml	Make to 100 ml with dist. water
	+			
	0.025 M Arginine	MW:174	0.44 g	
	+			
	0.025 M Lysine	MW:164	0.40 g	

Table IV
General running conditions for pH range 3.5–9.5

Set a cooling temperature of +10°C

General power supply settings. The power settings depend on the gel thickness, the lower settings being used for the thinner gels.

Gel thickness:	0.1–0.5 mm
E (V)	2000
I (mA)	10–50
P (W)	10–25
T (min)	45–60

Note: If only half a gel is used, the power and current settings should be reduced to one half.

Table V
Procedure for fixing, staining and preserving the gel

Solutions	Concentration	Time
Fixing solution	35% Methanol 10% Trichloroacetic acid 3.5% Sulphosalicylic acid	5 min
Destaining solution (must be fresh)	35% Ethanol 10% Acetic acid	5 min
Staining solution (used at room temperature)	0.5% Coomassie Blue R250 35% Ethanol 10% Acetic acid	5 min
Destaining solution	See above	2×10 min
Preserving solution	1% Glycerol 35% Ethanol 10% Acetic acid	5 min
Drying the gel 0.1 mm – 0.3 mm	Dry the gel by using a hair-dryer	5 min
0.4 mm – 0.5 mm	Lay the gel on a glass plate and leave it to dry at room temperature (usually over night).	

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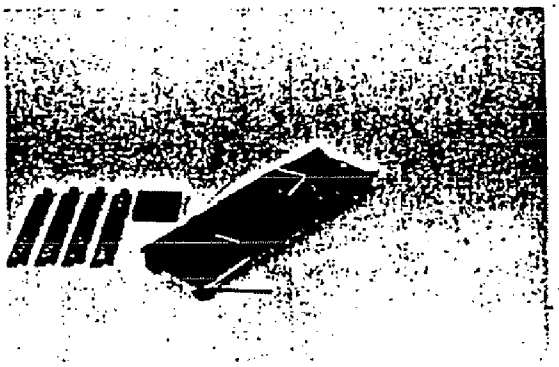
How to cast 0.1 mm - (

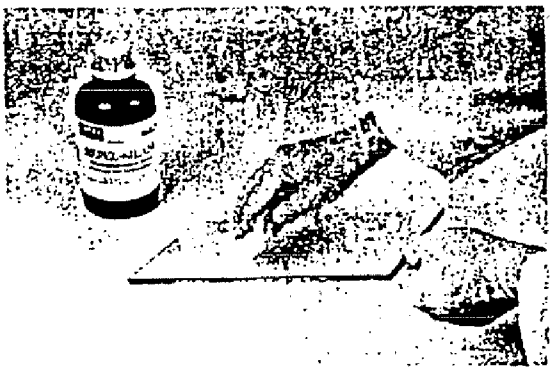
1 **2217-200 Ultromould – gel casting kit**
Contents:
Gel casting table (with levelling feet).
Glass plate (long). Glass plate (short). Holding guide.
Gel spacers for 0.1 mm, 0.2 mm, 0.3 mm, 0.4 mm,
0.5 mm (one for each). Weight. Screw.



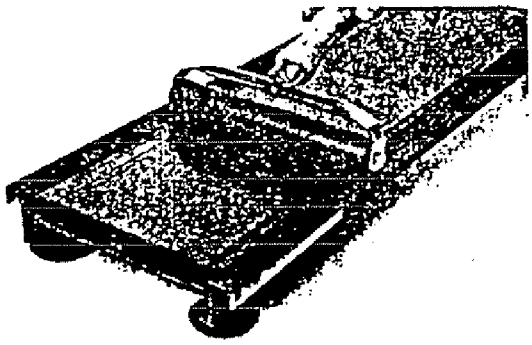
Consumables, Accessories and Chemicals
LKB 1850-211 GelBond™PAG-Film (pkt/50)
LKB 1850-250 Repel Silane
LKB 1820-101 Acrylamide
LKB 1820-102 NN'-Methylenebis-acrylamide
LKB 1820-103 Ammonium Persulfate
LKB 1820-104 TEMED
LKB 1818-101 Ampholine carrier ampholytes, pH 3.5–9.5
LKB 1840-101 Coomassie Blue R250

Recommended Electrofocusing Equipment:
LKB 2217 Ultrophor Electrofocusing Unit
LKB 2197 Power Supply
LKB 2219 MultiTemp II Thermostatic Circulator
LKB 2117-111 Surface pH Electrode

2 One face of the short glass plate is first coated with Repel Silane before polymerization, so that the polyacrylamide gel will not adhere to the surface of the glass plate when the mould is opened. Pour about 2 ml of Repel Silane (LKB 1850-250) onto the clean short glass plate and spread it out with a tissue paper. Let it dry and rinse the glass plate with distilled water.



3 Mount the levelling feet by turning the gel casting table upside down and screwing one foot into each corner. Place the gel casting table in a fume cupboard. (It is very important to work in a fume cupboard as the acrylamide and bis are neurotoxins.) Place the long glass plate on the lower shelf of Ultromould so that it touches the white end screw. Pour a few ml of water on the left-hand half of the plate and roll the GelBond PAG-Film (hydrophilic side upwards) onto it using a clean rubber roller (LKB 2117-710); ensure that you squeeze out all the air bubbles. Remove excess water with tissue paper.



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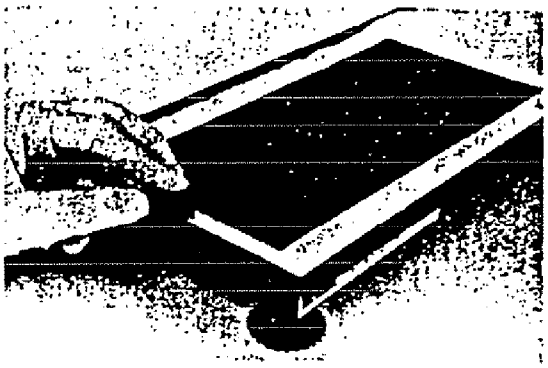
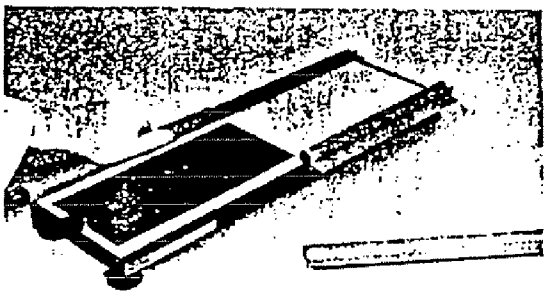
.5 mm ultrathin polyac

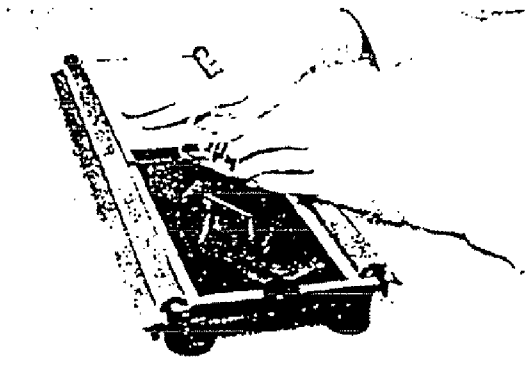
4 Place a spacer of the desired thickness onto the glass plate with the window in the spacer over the GelBond PAG-Film. Hold the gel spacer securely in place by screwing the black end screw firmly into the corresponding hole in the gel casting table. The long glass plate should now be held firmly between the black screw and the white screw at the two ends of the gel casting table.



5 Apply the four holding guides to the rails along the edges of the casting table. They act to guide the movement of the short glass plate when filling the cassette and also to keep the gel spacer fixed in position. To collect any acrylamide solution which may be spilled, the Ultromould gel casting unit can be placed in a plastic try (90 01 5543 Exposure Box).
Level the unit now, when it is ready for use. It is very important to prevent acrylamide solution collecting on only one side, since this may cause leakage.



6 Place the short glass plate, with the side coated with Repel Silane downwards away from the window in the spacer. Prepare the appropriate volume of gel solution, as described in Table 2. Then transfer it to a beaker.
Note: Gloves must be used.
Pour a few ml of gel solution onto the long glass plate just in front of the short plate, and start to push the short glass plate over the window in the spacer. Continue to pour more gel solution in front of the short glass plate at the same time as you push it further over the window. If air bubbles appear between the glass plates draw the short glass plate just far enough back to release them and then continue the forward movement.



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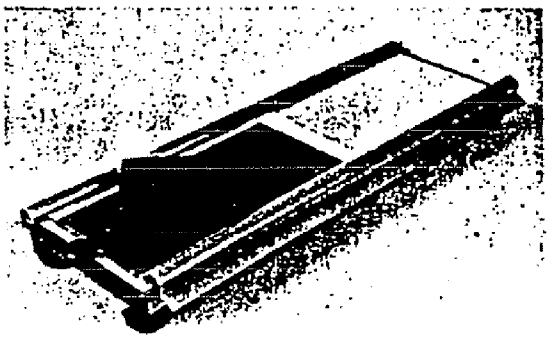
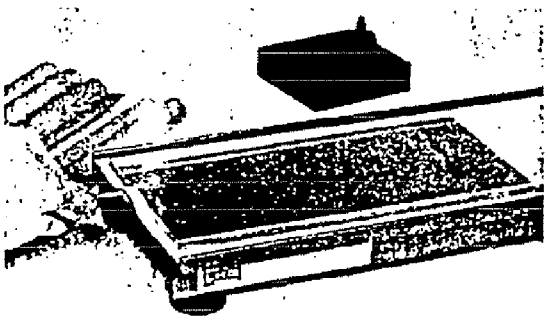
ylamide gels with Ulti

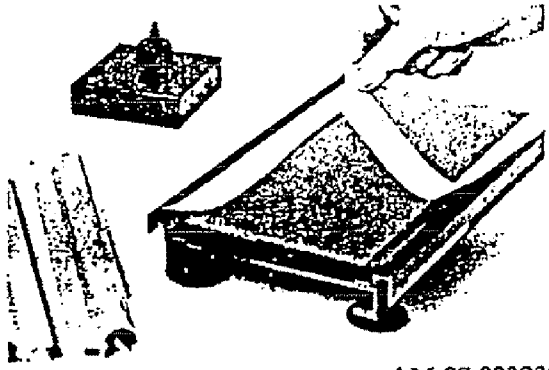
7 When the mould is filled and the short glass plate touches the black end screw, place the weight on the glass plate and make sure that there are no air bubbles between the glass plates or at the edges. Polymerization will be complete in one hour at room temperture. Before separating the glass plates, remove unpolymerized gel solution.



8 Remove the weight, the holding guides and the screw, insert a spatula between the GelBond PAG-Film and the long glass plate and twist slightly to separate them. It is also possible to insert a spatula between the GelBond PAG-Film and the short glass plate, twisting slightly to separate the gel from the short glass plate. Carefully lift the short glass plate up without tearing the gel.



9 When the short glass plate is removed, pick the spacer up at one corner and carefully peel it off. The gel can now be used at once. Otherwise, it can be tored for several days at 4°C in a humid air-tight box. Vash the gel casting table and all the accessories, and he Ultromould is ready for a new gel.



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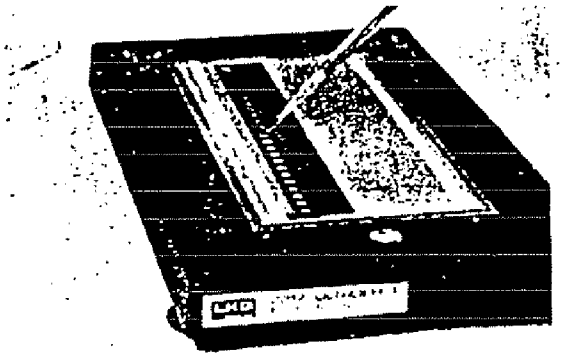
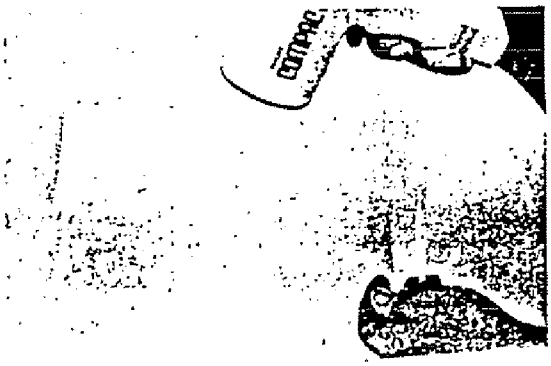
ɔmould. Step by step.

10 Connect the Ultrophor to the MultiTemp Thermostatic Circulator, set a temperature of +10°C and switch on the thermostatic bath. If another circulator is used, make sure it delivers 6–10 l/min. Level the Ultrophor using the three levelling feet.
Spread a small amount of insulating fluid (kerosene or light paraffin oil) over the cooling plate. Place the polyacrylamide gel on the GelBond PAG-Film onto the cooling plate. Avoid trapping air bubbles. Soak (but do not saturate) the electrode strips with the appropriate electrode solutions or use 1818-P Instruction for analytical electrofocusing in 0.5 mm polyacrylamide gels).
Lay the electrode strips on the appropriate sides of the gel, and cut off the parts which protrude beyond the end of the gel. Use the template as a guide when applying the strips. It is possible to run just a part of the gel, but in this case the gel must be placed in the centre of the cooling plate, which gives the best and most even contact with the electrodes.

11 Apply the sample by using LKB 1850-904 Electrofocusing Sample Application Foil, which has cut-outs of different sizes for 2.5, 10 and 15 µl of sample solution. Up to 24 or 48 samples can be run at the same time. If the optional third electrode is employed, the sample capacity is increased to 48 or 96. Sample Application Pieces (LKB 1850-901) can also be used. The application pieces may be dipped in sample solution, or a micropipette may be used to deliver an exact volume of sample to a dry sample application piece previously laid on the gel surface. The foil may be kept in position during the whole run, but the pieces should be removed half way through. Apply the electrodes of the Ultrophor as described in the instruction manual. Place the safety lid in position, connect the cables to LKB 2197 Power Supply and run according to Table IV in Practical Information.



12 After electrofocusing, measure the pH gradient in the gel by means of a surface pH electrode (LKB 2117-111) at 1 cm intervals. Then refocus for 5 minutes. After refocusing, lift off or cut away the electrode strips, then immediately lay the gel plate in fixing solution for 5 minutes. Discard the fixing solution and wash the gel for 5 minutes in destaining solution. Discard the destaining solution and stain the gel in staining solution for another 5 minutes at room temperature. Destain the gel with two or more changes of destaining solution until the background is clear. Finally lay the destained gel in preserving solution for about 5 minutes. Use a minimum of 300 ml of each solution.
Gels which are 0.1 mm – 0.3 mm thick can be placed on a glass plate and dried down completely in a stream of hot air. Gels which are 0.4 mm – 0.5 mm thick should be placed on a glass plate and left at room temperature (usually overnight) in a fume cupboard until the gel is completely dry.



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