Purification of Human Erythropoietin to Homogeneity by a Rapid Five-Step Procedure

By Gerald Krystal, H. Robert C. Pankratz, Neal M. Farber, and John E. Smart

Human urinary erythropoietin (Ep) has been purified using a simple five-step procedure to yield preparations with potencies of 80,000 U/mg in 25% yield. The five steps involve: (1) affinity chromatography on CM Affi-Gel Blue, (2) chromatofocusing, (3) wheat germ lectin (or hydroxylapatite) chromatography, (4) reverse-phase high-performance liquid chromatography (HPLC) using a phenyl column, and (5) preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ep activity was determined at each stage using a highly sensitive and specific in vitro assay that measures [3H]-thymidine incorporation into erythroid cells from spleens of phenylhydrazine-treated mice. The step 5 material was also tested with the in vivo polyethyleneimine mouse assay procedure and was found to have a similar potency to that obtained in the [3H]-thymidine in vitro assay. SDS-PAGE analysis of the step 5 material revealed a single 38.5-kd band that co-migrated with Ep bioactivity. Homogeneity was confirmed by amino acid sequence analysis. Starting with urine containing ~13 U/mg of protein, the cumulative degrees of purification achieved with each step were: step 1, 25-fold; step 2, 75-fold; step 3, 300-fold; step 4, 1,500-fold; and step 5, 5,000-fold. Corresponding overall recoveries after each step were: >100%, 70%, 45%, 30%, and 25%. These recoveries could be obtained when as little as 5,000 U of starting urinary Ep were processed because of the introduction of Tween 20 and SDS into buffers used at various stages of the purification procedure. In addition, a rapid method for determining Ep purity which involves reverse-phase HPLC of trypsinized 125I-labeled Ep is presented. This allows the establishment of purity with far less material than is required for amino acid sequencing. © 1986 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Materials

Polybuffer exchanger 94 (PBE94), polybuffer 74 (PB74) and wheat germ Lectin-Sepharose 6MB (~5 mg lectin/mL sedimented gel) were purchased from Pharmacia Fine Chemicals, Montreal. CM Affi-Gel Blue, hydroxylapatite (Bio-Gel HTP), and the electrophoresis purity reagents, acrylamide, bis acrylamide, TEMED, and ammonium persulfate were obtained from Bio-Rad, Richmond, Calif. The µBondapak phenyl HPLC column (0.39 x 30 cm) was acquired from Waters Associates, Milford, Mass. The Spectra-Physics 8000 HPL chromatograph was purchased from Technical Marketing Associates, Vancouver, Canada, and the UA-5 absorbance/fluorescence monitor used for HPLC was obtained from Isco, Lincoln, Neb. Insoluble neuraminidase (N-2382), polyethylene glycol (PEG) 4000 and 6000, Triton X-100 (TX100), sodium deoxycholate (DOC) (D-6750), Tween 20 (monolaureate polyoxyethylene-sorbitan), human transferrin (T-2252), bovine serum albumin (BSA) (A-7638), and silver nitrate were obtained from Sigma, St Louis. Nonidet P40 (NP40) was purchased from Particle Data Laboratories Ltd, Elmhurst, Ill; SDS, specially pure, was purchased from BDH Chemicals, Toronto. For HPLC, n-propanol (spectrophotometric grade) was purchased from Aldrich Chemical Co, Milwaukee.

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Urine Collection and CM Affi-Gel Blue Chromatography

Urine was collected from aplastic anemic patients whose urinary Ep titers had been previously established as >0.5 U/mL. The urine was frozen immediately after excretion and was stored at −15 °C. Subsequent preparation for CM Affi-Gel Blue chromatography and the details of this first step in the purification procedure have been described previously.

Assay for Ep

A microassay procedure for Ep, based on the incorporation of [3H]-thymidine into spleen cells from phenylhydrazine-treated mice, was used throughout this study. Ep content of fractions obtained at different stages of purity were determined by comparison with either the World Health Organization (WHO) standard preparation No. 2 or CM Affi-Gel Blue purified human urinary Ep (100 to 350 U/mg) calibrated against the WHO standard.

The Ep activity present in the purest preparation (step 5 Ep) was also determined in vivo using the polycythemic mouse assay (five mice per sample).

Protein Determination

Protein concentrations for all but the last step in the purification procedure were measured using the Coomassie Brilliant Blue G-250 dye-binding technique of Bradford with BSA as the protein standard. For the final electrophoretically pure Ep preparation, protein content was determined by silver-staining analytical SDS polyacrylamide gels using a modified Ohsawa and Ebata procedure, scanning the gels using a Hoefer Scientific GS300 Transmittance/Reflectance scanning densitometer (San Francisco), and comparing the Ep area to known protein standards.

Stabilization Studies

Under sterile conditions, step 2 Ep (refer to Table 1 for definition of steps) at 2,750 U/mg; 1,100 U/mL was diluted to 500 mU/mL with 0.15 mol/L of NaCl, and 100 µL of 0.1% DOC, or PBS. Concentrated step 2 Ep (1,100 U/mL) was also diluted to 500 mU/mL with 0.1% BSA in 10% bovine serum albumin (BSA); 1% SDS, 0.2% Tween 20, 1% BSA, 10% polyethylene glycol (PEG) 6000, 0.1% NP40, 0.1% TX100, 0.1% DOC, or PBS. Concentrated step 2 Ep (1,100 U/mL) was also diluted to 500 mU/mL with 0.15 mol/L of NaCl, and 100 µL was added to a Falcon tube containing 10 µL of 100 µmol/L of CaCl₂. Control tubes were also prepared as above but without Ep. Immediately after the dispensing, the two tubes (with and without Ep) containing 10 µL of 10% BSA were frozen, and the remaining tubes were incubated for 12 hours at 23 °C. For the in vitro assay, aliquots were either tested at a final concentration of 20% (ie, 20 µL/100 µL total vol), or were diluted tenfold, with 0.1% BSA in α minimal essential medium (MEM) without nucleosides and were tested at a final concentration of 2%.

Analytical SDS-PAGE

To assess the electrophoretic purity at each stage of the purification procedure, samples were made at 2% with SDS, 5% with 2-mercaptoethanol, and 10% with glycerol, and were heated to 100 °C for two minutes. They were then subjected to standard SDS-PAGE as described by Laemmli using either 5%, 10%, or 12% acrylamide running gels, 0.85-mm thick by 14-cm long. Following electrophoresis, some gel lanes were sliced into 0.5-cm sections, placed in 1.5-mL polypropylene microcentrifuge tubes (VWR Scientific Inc, San Francisco), and rocked overnight at 4 °C with 0.5 mL of 0.02% Tween 20 in PBS. Microfuged supernatants were then assayed for activity using the [3H]-thymidine assay procedure. The rest of the gel lanes were stained using a modified Ohsawa and Ebata silver-staining procedure.

Tryptic Fragment Preparation and Analysis

Approximately 100 ng of step 5 Ep in 50 mmol/L of sodium phosphate (Ph 7.4), 1% SDS was iodinated with 1 mCi [125I]-NaI (NEN) using 0.5 mg/mL of chloramine T for three minutes at room temperature. The 25-µL reaction mixture was then treated with 5 µL of 5 mg/mL sodium metabisulfite and 50 µL of 2 mg/mL KI and was passed over a 15 × 0.7 cm Sephadex G-25 (medium) column equilibrated with 0.1% SDS. The peak fractions were pooled, lyophilized, and resuspended in 80 µL of Laemmli loading buffer, boiled for five minutes, and electrophoresed in a 15% SDS-polyacrylamide gel. The region of the gel containing the labeled protein was visualized by one-hour autoradiography of the wet gel. The protein band was excised, and the gel piece was shaken in 50 mL of 40% methanol, 10% acetic acid for two hours with one change, to remove SDS. The gel was then minced, lyophilized, and resuspended in 5 mL of 0.1 mol/L of ammonium bicarbonate. Five microliters of 10 mg/mL TPCK trypsin (Worthington Biochemicals, Millipore Corp, Bedford, Mass) was added, and digestion proceeded for 18 hours at 37 °C. Another 5 µL of trypsin was added for another two hours at 37 °C. The digested fragments were washed through a 0.45-µm millipore HA filter with 0.1 mol/L of ammonium bicarbon-

Table 1: Purification of Human Urinary Ep

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (mL)</th>
<th>Protein Concentration (mg/mL)*</th>
<th>Ep Concentration (U/mL)</th>
<th>Specific Activity (U/mg)</th>
<th>Total Units (U)</th>
<th>Overall Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CM Affi-Gel Blue concentrate</td>
<td>91</td>
<td>0.82</td>
<td>280</td>
<td>340</td>
<td>25,500</td>
<td>114</td>
</tr>
<tr>
<td>2. Chromatofocusing concentrate</td>
<td>9.0</td>
<td>0.89</td>
<td>1,750</td>
<td>1,970</td>
<td>15,700</td>
<td>70</td>
</tr>
<tr>
<td>3. Wheat germ concentrate</td>
<td>2.2</td>
<td>1.10</td>
<td>4,600</td>
<td>4,180</td>
<td>10,120</td>
<td>45</td>
</tr>
<tr>
<td>4. Phenyl-HPLC concentrate</td>
<td>4.0</td>
<td>0.08</td>
<td>1,650</td>
<td>21,000</td>
<td>6,600</td>
<td>30</td>
</tr>
<tr>
<td>5. Preparative SDS-PAGE</td>
<td>14.0</td>
<td>—</td>
<td>400</td>
<td>80,000†</td>
<td>5,630</td>
<td>25</td>
</tr>
</tbody>
</table>

Ep, Erythropoietin; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

*Protein concentrations were determined using the Coomassie Brilliant Blue G-250 dye-binding technique.
†Deduced from gel-scanning analyses of silver-stained gels.
ate and lyophilized. For the reverse-phase chromatography of tryptic peptides, the peptides were dissolved for ~ten minutes in 100 μL of 90% formic acid at 23 °C and were then diluted with 400 μL of H2O. The sample was then applied to a 4.6-mm x 25-cm Spherisorb 10 ODS (C18) reverse phase column (Spectra-Physics). The column was run at 40 °C and at a flow rate of 1.4 mL/min. After injection of the sample, the column was washed for five minutes with 4.5% formic acid (PSC Reagents, J.T. Baker, Phillipsburg, NJ) and was then developed with a linear gradient from 0% to 62.5% ethanol (Photrex Reagents, J.T. Baker) in 4.5% formic acid over a period of 95 minutes on a Beckman 345T HPLC system. Fractions of 0.7 mL were collected in 0.5 minute each, yielding a total of 200 fractions; each fraction was counted in a Beckman 5500 gamma counter.

**Amino Acid Sequencing**

Approximately 10 μg of step 5 Ep was dialed against 0.01% SDS, lyophilized, and resuspended in 100 μL of H2O. The sample was boiled, and 10 μL was removed for amino acid analysis. The remaining aliquot was sequenced on an Applied Biosystems Gas Phase Sequencer model 470A using MeOH/HCl conversion. The PTH amino acids were analyzed on a Hewlett Packard 1084B HPLC with model 1040A diode array detector.

**RESULTS**

**'Stabilization' Studies**

During our initial studies into the purification of Ep, we discovered that substantial losses in activity were incurred as homogeneity was approached. This was particularly evident when purifications were carried out with <10,000 U of starting material (human urine). Subsequent experiments suggested that losses were owing, in large part, to sticking of Ep to glass and plastic surfaces, and that this adsorption could be prevented by the addition of 0.1% BSA or 0.1% SDS to our Ep preparations.

Evidence is now presented, following a more extensive survey of potentially "stabilizing" compounds, that 0.02% Tween 20 is as effective as BSA or SDS for maintaining bioactivity of partially purified Ep preparations (Table 2). One percent PEG 6000, 0.01% NP40, and 0.01% TX100 were also found to be effective, although they were less effective than either Tween 20, BSA, or SDS. Little or no protective effect was observed with 0.01% DOC or 10 mmol/L of calcium chloride. Moreover, TX100 and NP40 were toxic in the phenylhydrazine mouse spleen cell assay at final concentrations of >0.001%, whereas SDS was not, at 0.002%; Tween 20 was nontoxic even at concentrations which effectively prevented adsorption of Ep to plastic ware (0.02%). Because Tween 20 was a nonionic detergent and was therefore unlikely to cause significant changes to the native physical properties of Ep, it was added routinely to buffers used in the purification procedures subsequent to the CM Affi-Gel Blue step.

**Purification Procedure**

Aliquots of frozen urines, collected from patients with potentially high Ep titers, were thawed, dialed against α MEM without nucleosides, and tested in the [3H]-thymidine suspension culture assay at a final concentration of 10%. Those urines containing Ep levels >0.5 U/mL were used for purification. This preliminary screening was essential since, in our experience, only about one-third of the aplastic anemic patients with hemoglobins of <6 g/dL showed urinary Ep titers significantly >0.01 U/mL. Selected urines were then decanted through four layers of Curity Hospital towels, made 0.025 mmol/L with phenylmethyl sulfonyl fluoride and 0.025% with phenol, and were pressure-filtered through a Sartorius Separator and a type SM 13400 membrane filter (Canlab, Vancouver, Canada) as described previously.

**CM Affi-Gel Blue fractionation.** This step has already been described in detail. In brief, filtered urine was applied at 4 °C to a CM Affi-Gel Blue column (40 mL/mL gel) pre-equilibrated with 0.15 mol/L of NaCl and 10 mmol/L of Na phosphate (pH 7.2). Under the conditions used, Ep bound to the column whereas most of the applied protein did not. Ep was subsequently eluted, using a steep salt gradient from 0.15 mol/L to 1.15 mol/L of NaCl in 10 mmol/L of Na phosphate buffer. A typical elution profile is shown in Fig 1. Ep eluted as a broad peak, suggesting heterogeneity, at ~0.9 mol/L of NaCl. The column was then regenerated at 23 °C with 8 mol/L of urea. This fractionation procedure routinely gave a 25-fold to 50-fold purification of Ep, with an apparent Ep recovery in excess of 100%. Typically, specific activities following this step were in the range of 100 to 350 U/mg.

**Chromatofocusing.** The Ep-containing fractions from CM Affi-Gel Blue chromatography were pooled, concentrated ~tenfold by ultrafiltration using an Amicon Model 402 apparatus (Amicon Corp) fitted with a PM10 membrane, and dialyzed overnight at 4 °C against buffer A (20 mmol/L of Tris-Cl, 0.1% PEG 4000 [pH 7.0]). This step 1 Ep was then applied to a PBE94 column (6,000 U/mL of gel) pre-equilibrated with buffer A. Five-milliliter fractions were collected at 4 °C at a flow rate of 10 mL/h. Buffer A was then run through the column until the OD280 returned to baseline, and the column pH was slowly reduced to 3.8 with diluted PB74 (13 mL of PB74 plus 187 mL of distilled water) that had been adjusted to pH 3.8 with 1 mol/L of HCl. Five-milliliter fractions were again collected, this time into tubes containing 0.125 mL of 1 mol/L Tris-Cl (pH 7.5) and 0.10 mL of 1% Tween 20. When the eluate reached pH 3.8,
the column was restored to pH 7.0 with buffer A. To elute most of the Ep activity, 0.3 mol/L of NaCl in buffer A was then applied, and 1.0-mL fractions were collected (into tubes containing 0.02 mL of 1% Tween 20) at 4 °C and at 10 mL/h. Last, 3 mol/L of NaCl in buffer A was put through the column to remove tightly bound proteins and regenerate the gel. A typical profile is shown in Fig 2.

Chromatofocusing was found to be superior to diethylaminoethanol (DEAE) Sephacel chromatography in terms of both column capacity and recovery of Ep activity. Moreover, we found that recoveries were far higher when the pH was returned to 7.0 and when the Ep was eluted with high salt rather than when the pH was either lowered below the isoelectric point of Ep (−3.5) (E. Goldwasser, personal communication) or when eluting was performed with high salt at any pH <4.5. Lowering the pH to 2.9 resulted in the release of only ~50% of the Ep activity; removal of the rest required elution with 0.3 mol/L of NaCl. Although most Ep preparations yielded an elution pattern identical to that shown in Fig 2, approximately one preparation in ten released a substantial amount of total Ep activity (ie, 40% to 50% in some cases) during the initial lowering of the pH to 3.8. This latter elution pattern could be duplicated by treating preparations that gave the standard elution profile with neuraminidase-agarose beads, suggesting that some Ep preparations are less sialated than others, possibly due to patient-to-patient variation in Ep glycosylation or increased losses of sialic acid residues during the purification of some preparations.

**Wheat germ Lectin-Sepharose 6MB chromatography.** Chromatofocused Ep preparations that displayed the typical elution pattern shown in Fig 2 could be further purified using wheat germ affinity chromatography. The use of wheat germ lectin to purify Ep was first described by Spivak et al. and the conditions used here were modified only slightly from those described by Dukes et al. Specifically, the 0.3 mol/L NaCl pool following chromatofocusing was loaded directly onto a wheat germ Lectin-Sepharose 6MB gel bed (8,000 U/mL of gel) previously equilibrated with 0.02% Tween in PBS. Chromatography was carried out at room temperature and at a flow rate of 2 mL/h. Most of the Ep activity was eluted with 10 mol/L of N-acetyl-D-glucosamine in PBS containing 0.02% Tween 20, and the column was then regenerated first with 200 mmol/L of N-acetyl-D-glucosamine in the same buffer followed by 20 gel bed vol of 0.02% Tween 20 in PBS. Typically, the bulk of

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**Fig 1.** CM Affi-Gel Blue chromatography of filtered human urine. See ref. 5 for complete details.

**Fig 2.** Chromatofocusing of typical step 1 Ep. To monitor the change in pH, every tenth tube did not contain 0.125 mL of 1 mol/L Tris-Cl (pH 2.5). S.B., starting buffer.
ERYTHROPOIETIN PURIFICATION

the contaminating proteins did not bind to the column, and >90% of the recoverable Ep activity eluted with 10 mmol/L of N-acetyl-D-glucosamine (Fig 3). We found that the best purification was achieved if an amount of Ep that closely approached the capacity of the gel was applied, ie, ~8,000 U of chromatofocused Ep per milliliter of gel, using lot No. IF 32137 from Pharmacia Fine Chemicals. However, gel capacity had to be determined through experimentation because it varied markedly from lot to lot. If substantially less than the column capacity of Ep was applied, a significant proportion of the total activity eluted with the 200 mmol/L of N-acetyl-D-glucosamine buffer. Using this procedure, we obtained, with most chromatofocused preparations, a fourfold purification with an overall yield of 65%.

Hydroxylapatite chromatography. Approximately one in ten Ep preparations gave an anomalous chromatofocusing profile, consistent with the fact that the Ep in these preparations was less sialated than most of the Ep preparations. The 0.3 mol/L NaCl pools remaining after chromatofocusing of these anomalous preparations were not well suited to wheat germ fractionation, ie, most of the Ep activity did not bind to the lectin. For these preparations, an alternative procedure which involved hydroxylapatite was devised. This chromatographic step was first used by Miyake et al13 as part of their purification procedure for Ep. However, using their procedure, we achieved extremely low recoveries. This problem could be alleviated somewhat either by applying very high concentrations of protein to the column as Miyake et al did or by adding 0.02% Tween 20 to the chromatographic buffers. This detergent did not appear to interfere with the elution profile. Specific details of the procedure used are given in the legend to Fig 4. Two distinct fractions containing Ep activity were detected, confirming the heterogeneity previously reported with this gel fractionation procedure.3 When anomalous step 2 Ep was chromatographed on this column, the bulk of the activity eluted with 100 mmol/L of Na/K phosphate (pH 6.8) (Fig 4A), and resulted in a threefold increase in specific activity and a 50% recovery. However, when a typical step 2 Ep preparation was chromatographed, most of the activity eluted between 0 and 10 mmol/L of Na/K phosphate (pH 6.8) (Fig 4B).

Reverse-phase HPLC. Following wheat germ or hydroxylapatite chromatography, the main bulk of Ep activity was concentrated to ~1.5 mL, using an Amicon ultrafiltration unit with a PM 10 membrane, and was loaded onto a 3.9-mm x 30-cm μBondapak phenyl HPLC column equilibrated with 0.1 mol/L of K phosphate (pH 7.0). Samples up to 5 mL, using multiple injections and a 200-μL loop, could be applied with no deleterious effect on subsequent resolution. A biphasic linear 0% to 40% gradient of n-propanol in 0.1 mol/L of K phosphate (pH 7.0) was used, and 1-mL fractions were collected into tubes containing a final concentration of either 0.1% SDS or 0.02% Tween 20. Ep eluted as a single peak with ~34% n-propanol (Fig 5). Anomalous preparations required slightly more n-propanol for elution. This step gave a fivefold increase in purity to ~21,000 U/mg in 70% yield. The active fractions were pooled, dialyzed against 0.01% SDS at 23 °C for six hours, and lyophilized to dryness in preparation for SDS-PAGE.

SDS-PAGE. Ep samples were resuspended in ~2 mL of 62.5 mmol/L of Tris-Cl (pH 6.8), 10% glycerol, and sufficient SDS to give a final concentration of 2%. They were then applied directly to 1.8-mm thick 10% Laemmli gels.11 The samples were neither heated nor reduced because this diminished subsequent recoveries. Following electrophoresis, 0.2-cm slices above and below the expected Ep region, (assuming an Rf for Ep of ~0.64 compared with the bromphenol blue dye front) were shaken overnight at 4 °C with 0.5 mL of 0.02% Tween 20 in PBS. Centrifuged sample supernatants were assayed for bioactivity as described in the Materials and Methods section, and those fractions containing activity were pooled. Gel slices that contained Ep activity were re-shaken twice with 0.5 mL per slice of 0.02% Tween 20 in PBS, and the three eluates were pooled. Typically, the first extraction released 90% of the recoverable Ep activity, the second released 8%, and the third released 2%. There was total recovery of 85% using this step, and the specific activity was increased ~3.5-fold to 80,000 U/mg.

The five-step purification scheme for Ep from human urine is summarized in Table 1. Overall recovery using this procedure was ~25% as determined by the [3H]-thymidine Ep microassay method. Identical results were obtained using
a standard Ep assay based on in vitro $^{59}$Fe incorporation. Moreover, the step 5 material was also tested in the in vivo polycythemic mouse assay; the results indicated a potency of 80,000 U/mg, very similar to that obtained with the in vitro assay procedures.

**Determination of Purity**

Aliquots at each stage of the purification procedure were assessed for purity by analytical SDS-PAGE. For this purpose, samples were reduced with 5% 2-mercaptoethanol and were heated to 100 °C for two minutes. Following electrophoresis, most of the lanes were silver-stained by a modified Ohswara and Ebata method. The major modification in the silver-staining procedure involved use of 10% glutaraldehyde prior to gel shrinkage with PEG 2000. This was found to make the silver-staining intensity of various test proteins more quantitative, ie, the staining intensity more closely reflected the amount of protein applied to the gels. (G. Krystal and S. Ashwell, unpublished observations) As shown in Fig 6, the final Ep preparation (step 5) appeared as a single broad band on analytical SDS-PAGE; this band coelectrophoresed with Ep bioactivity. Moreover, the average mol wt of this broad band was 38.5 kd, in close agreement with the mol wt assignment reported by Miyake et al. However, the mol wt varied slightly depending on the percentage of acrylamide in the SDS gels. For example, step 5 material appeared to have a mol wt of 39 kd on an 8% gel and 37 kd on a 12% gel. These results are consistent with Ep being a glycosylated protein. One of the anomalous Ep preparations, discussed above, which did not bind to wheat germ and was purified to homogeneity using hydroxylapatite followed by HPLC and preparative SDS-PAGE, appeared to have a mol wt of 32 kd on a 10% acrylamide gel. This is consistent with its being substantially less sialated than most of the Ep preparations which we have purified.

The specific activity of step 5 Ep was estimated by scanning lanes iv and v and comparing their staining intensities with protein standards (Fig 6). This was necessary because the concentration of protein in our eluted step 5 material was too low to determine protein content by more

**Fig 4.** Hydroxyapatite chromatography of (A) anomalous and (B) typical step 2 Ep. One ml of step 2 Ep (1.100 U) was dialyzed against distilled water containing 0.02% Tween 20 and was applied to a 0.5-ml gel bed of hydroxylapatite preequilibrated with distilled water containing 0.02% Tween 20. After administration of the sample, the column was eluted with 0.2% Tween 20 in water, and 1-ml fractions were collected at 4 °C and at 10 ml/h until the OD returned to baseline. A gradient of 0 to 10 mmol/L of Na/K phosphate was then initiated, using 10 ml of distilled water and 10 ml of 10 mmol/L Na/K phosphate (pH 6.8), both containing 0.02% Tween 20. This was followed by 10 mmol/L of Na/K phosphate until the OD returned to baseline. Finally, 100 mmol/L of Na/K phosphate (pH 6.8) containing 0.02% Tween 20 was used to elute the remaining activity.

**Fig 5.** Phenyl high-performance liquid chromatography (HPLC) of step 3 Ep. Approximately 10,120 U (2.4 mg) of step 3 Ep was applied directly, using multiple injections of 200 μl each (arrows indicate the last of these injections), to a 0.39 x 30 cm phenyl HPLC column preequilibrated with 0.1 mol/L of K phosphate (pH 7.0) (buffer A). Chromatography was carried out at 23 °C, at a flow rate of 0.5 ml/min, and 1-ml fractions were collected into tubes containing 10 μL of 10% SDS. For the first five minutes, elution was carried out with buffer A. A biphasic linear gradient was then initiated: at 20 minutes, the percentage of buffer B (40% n-propanol in 0.1 mol/L of K phosphate [pH 7.0]) eluting through the column was up to 75%. A shallower gradient was then initiated: at 120 minutes, the column reached 100% buffer B.
Fig 6. Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis of the Ep fractions from each step in the purification procedure. The gel concentration was 10%. All samples were in 2% SDS, 10% glycerol and 50 mmol/L of Tris·Cl (pH 6.8). Samples applied to lanes 1 through 9 were reduced with 5% 2-mercaptoethanol and were boiled for two minutes. Samples 10 through 12 were neither reduced nor heated. Lanes 1 and 2 contained 100 ng and 50 ng, respectively, of bovine serum albumin and transferrin; lane 3 contained BioRad low mol wt SDS-PAGE standards; lanes 4 through 8 contained Ep of steps 1 through 5, respectively; lane 9 contained reduced and heated SDS sample buffer; lane 10 contained unreduced, unheated SDS sample buffer; lane 11 contained buffer unreduced and unheated step 5 Ep. Lane 12, containing unreduced, unheated step 3 Ep was cut into 0.5-cm slices, shaken overnight in PBS containing 0.02% Tween 20, and bioassayed.

conventional Coomassie, OD_{280} or OD_{278} methods. Scanning of lane iv and measuring of the areas under the peaks demonstrated that 29% of the total silver-staining material was present in the Ep region. Assuming that pure Ep was between 70,000 and 80,000 U/mg suggested that the HPLC-purified preparation had a sp activity of 20,000 to 23,000 U/mg, consistent with the 21,000 U/mg obtained for this material using Bradford's protein determination method (see Table 1), and lending credence to the scanning of silver-stained gels (using the silver-staining method described above) in order to obtain quantitative data on the protein content of gel bands. Known amounts (50 ng and 100 ng) of BSA and transferrin were electrophoresed on the same gels to help quantitate the amount of protein in lane v (Fig 6); it was determined that, for the same amount of protein, transferrin yielded 75% to 80% of the staining intensity of BSA. Therefore, taking into account band breadth and the fact that 11 U of Ep were applied to lane v, the scanning data suggested that the step 5 Ep preparation was between 69,000 U/mg (based on transferrin) and 89,000 U/mg (based on BSA). This is in close agreement with the previously reported specific activities for pure Ep using the Bradford method. Reduced electrophoretically pure Ep migrated marginally slower than did unreduced Ep (lane 12, Fig 6). This is consistent with the presence of an intrachain disulfide bridge in the native molecule, and in agreement with a recent report by Jacobs et al in which they suggest that at least two of the four cysteines in Ep may be involved in a disulfide bond. Reduced Ep appeared on our SDS gels as a slightly more diffuse band than that of reduced Ep. This may suggest that not all Ep molecules have the same number of disulfide bridges in the native state.

The purity of step 5 Ep was firmly established by amino acid sequencing. The material was subjected to 18 cycles of NH2-terminal amino acid sequence analysis as described in the Materials and Methods section. Approximately 200 pmols of protein was analyzed by sequential stepwise Edman degradation yielding the following unique polypeptide sequence: Arg-Leu-Ile-Asp-Ser-Val-Leu-Glu-Tyr-Leu-Glu. The first two amino acids were not determinable because of contamination of the step 5 Ep with glycine and Tween 20 from the electrophoresis step. The sequence obtained is in total agreement with three previously reported Ep sequences. Consequently, we can explain the lack of scores at positions 10 and 14 by noting that the previously cited Ep sequences predict Arg residues at these positions, which give very low PTH yields and would be below the level of detection in our sample.

Biochemical Characterization of Step 5 Ep

Step 5 Ep was iodinated using [125I]-NaI and chloramine T and was then subjected to trypsin digestion as described in the Materials and Methods section. Reverse-phase HPLC...
using a C_{18} column and a linear gradient of ethanol from 0% to 62.5% revealed two well-defined peaks (Fig 7). This profile has been obtained by Smart and Farber (J.E. Smart and N.M. Farber, unpublished observations) with several homogeneous preparations of Ep, including one that they have sequenced extensively (48%) and that shows 100% agreement with the recently published sequence for Ep.31

**DISCUSSION**

In this report, a method for the purification of human urinary Ep to apparent homogeneity is described. The advantages of this method over that previously presented by Miyake et al are: first, fewer steps are used; second, far less starting urine is required to ensure reasonable final recoveries; and third, significantly higher final recoveries (25% v 15%) are obtainable. Thus, investigators who have been anxious for homogeneous preparations of Ep but who have been hindered by limited supplies of high Ep titer urine can now obtain ~1,250 U of pure Ep from only 5,000 U of starting urine.

The first two steps in this new procedure, namely CM Affi-Gel Blue chromatography and chromatofocusing, serve not only to purify but to concentrate as well. Thus, these two high-protein-capacity gels are well suited for the first two fractionation steps. The last four steps, ie, chromatofocusing, wheat germ lectin–Sepharose 6MB chromatography, reverse-phase HPLC and SDS-PAGE, all provide information concerning the amount of heterogeneity in the particular Ep preparation being processed. For example, both the anomalous Ep preparations and neuraminidase-treated typical Ep preparations displayed: (1) an increased Ep activity in the unbound flow through and the PB74 fractions following chromatofocusing; (2) less binding to wheat germ lectin beads; (3) a greater proportion of the eluted Ep activity, following hydroxylapatite, in the 100 mmol/L Na/K phosphate pool, and (4) an increased binding affinity to the phenyl-HPLC column, ie, such that elution required higher concentrations of n-propanol. This last phenomenon is compatible with these Ep preparations being less siallated, since removal of negative sialic acid residues would be expected to make Ep more hydrophobic and would thus require a more hydrophobic buffer for elution. Finally, some anomalous Ep preparations and neuraminidase-treated typical Ep preparations displayed mol wt as low as 32 kd, significantly lower than the 38.5 kd seen with untreated typical Ep preparations. This dramatic difference in mol wt is not inconsistent with simple desialation of native Ep, since Goldwasser has reported that there are between 16 and 18 sialic acid residues per molecule of native Ep.23 This ability to assess the degree of sialation during Ep purification is a great asset since it is well established that desialated Ep is without effect in vivo.24 Thus, for example, researchers who are interested in purifying Ep for therapeutic administration to patients can abort their purification procedure at the chromatofocusing stage if anomalous elution profiles are observed. This feature may also be of more general interest since desialated Ep has been reported to be less stable than the native hormone.24

Trypsinized pure 125I-labeled Ep yields two well-defined peaks of radioactivity upon Spherisorb 10 ODS (C_{18}) reverse-phase HPLC. This finding can therefore be used as a fast check of Ep purity since it requires far less time and material (ie, 100-fold less) than does gas-phase amino acid sequencing.

The recently published cDNA nucleotide (and therefore amino acid) sequence for Ep suggests that approximately one-half of the Ep molecule must be contributed by sugar side chains. The availability of both recombinant and human urine-derived Ep should now permit a more detailed examination of the contribution by these sugar moieties. It will also be of value to determine whether recombinant Ep that is glycyslated in non-human cells has a shorter half-life in the human circulatory system than does human urine-derived Ep.

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