EXHIBIT A

D10

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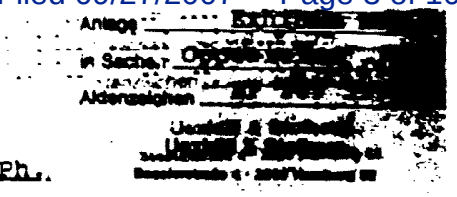

DECLARATION OF THOMAS HECKLER, Ph.

I, Thomas Heckler, Ph.D., hereby declare that:

1. I presently hold the position of Group Leader in the Biotechnology Division of the R.W. Johnson Pharmaceutical Research Institute Division of Ortho Pharmaceutical Corporation (RWJPRI) and I have held this position since February 1990. In this capacity, I am responsible for Bioseparations Engineering.

2. The work described in the attached report entitled, "r-HuEPO purification from CDM by Miyake Process" was conducted under my direction by Paul V. Remsen and Somesh C. Nigam who were working in my laboratories at RWJPRI at the time. The Concentrated Diafiltered Medium (CDM) used for this work was obtained from CHO cells which are the progeny of CHO cells that were prepared in accordance with the general principles described in Example 10 of EPO 148 605. This work demonstrates that recombinant human erythropoietin (EPO), when purified by the Miyake Process alone, yields a homogeneous EPO preparation characterized by: a potency of greater than 200,000 units/A_{280}, a molecular weight of about 34,000 daltons on SDS PAGE and movement as a single peak on reverse phase high performance liquid chromatography.

SIGNED:

THOMAS HECKLER, Ph.D.

May 19, 1992

R. W. JOHNSON PHARMACEUTICAL RESEARCH INSTITUTE
BIOTECHNOLOGY DIVISION - RARITAN
INTER-OFFICE MEMO

MAY 1 1992
RICHARD J. GROCHALA

TO: R. Grochala DATE: May 7, 1992

FROM: P. Remsen PVR
T. Heckler TH

CC: D. Omstead
J. Gardner

Subject: r-HuEPO Purification from CDM by Miyake Process

Summary

Recombinant Hu-EPO, derived from Chinese Hamster Ovary (CHO) Cell Conditioned Medium, was purified according to the procedure of Miyake, Kung, and Goldwasser (J. Biol. Chem. 252:5558 1977) to yield a preparation having a potency of greater than 200,000 units/A_{280} protein by the in vivo mouse bioassay. The Miyake procedure involves several purification steps including phenol extraction, fractional ethanol precipitation, DEAE-Agarose chromatography, SP-Sephadex chromatography, Sephadex G-100 gel filtration, and Hydroxylapatite chromatography. A sequential description of the purification process as performed by Paul V. Remsen and Somesh C. Nigam is described below.

Source of crude r-HuEPO:

A frozen 590 mL sample of Concentrated Diafiltered Medium (CDM) from CHO cell r-HuEPO harvest supernatant was obtained from the Manati EPO production facility. The CDM was thawed and concentrated using a tangential flow cartridge having a 20Kd cut off membrane. The final volume of the sample after concentration was 146 mL; the final protein concentration was determined to be 14.0 mg/mL by Bradford Assay. A 42 mL aliquot of this material was used as the starting material for the phenol extraction step.

Phenol Extraction:

Phenol Extraction of the crude r-HuEPO containing medium was, as in the Miyake et al. procedure, carried out as described by Chiba, Kung, and Goldwasser (Biochemical and Biophysical Research Communications 47:1372-1377, 1972).

The 42 mL sample of crude concentrated CDM was adjusted to 0.284 M p-aminosalicylate (PAS) and 0.04 M sodium phosphate by addition of 72 mL of 0.475 M PAS and 4.7 mL of 1 M sodium

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phosphate buffer, pH 7.0. The sample was then mixed with an equal volume of liquified phenol (equilibrated with phosphate buffer and PAS as described by Chiba et al., 1972) and shaken vigorously. The sample was subsequently centrifuged at 3000 rpm for 20 minutes at 4°C, and the resulting phenol phase dialyzed against 30 liters of PBS for 72 hours at 4°C. After all of the phenol had been visibly exchanged for PBS, the 120 mL sample was made 90% in cold ethanol by addition of 1080 mL of cold absolute ethanol to precipitate the protein. The sample was held overnight at 4°C, then centrifuged at 21,000 g for 30 minutes at -15°C to collect the precipitate. The precipitate obtained from this step was dissolved in 150 mL of PBS in preparation for the next step, fractional ethanol precipitation.

Fractional Ethanol Precipitation:

To the 150 mL sample derived from phenol extraction was added 37.5 mL of 10 M lithium chloride and 187.5 mL of cold absolute ethanol to achieve 50% ethanol saturation. The solution was stirred for 30 minutes, allowed to settle for 15 minutes, and then centrifuged at 21,000 x g for 10 minutes at -15°C. The resulting supernatant was decanted and the pellet rinsed with 20 mL of 50% ethanol/1 M LiCl. The rinse buffer was subsequently added to the supernatant; the pellet was discarded.

The resulting 380 mL sample was made 65% in ethanol by addition of 164 mL of cold absolute ethanol. Following addition of ethanol, the sample was stirred at 4°C for 30 minutes, then held at 4°C for 15 minutes without stirring. A pellet was collected by centrifuging at 21,000 x g for 10 minutes at -15°C; the supernatant was decanted and the pellet was rinsed with 20 mL of 65% ethanol/0.7 M LiCL. The rinse buffer was subsequently added to the supernatant; the pellet was discarded.

To the 550 mL of sample derived from this step, 220 mL of cold absolute ethanol was added bringing the sample to 75% ethanol. The sample was stirred for 30 minutes, then incubated at 4°C for 14 hours. The supernatant was collected as before and the pellet rinsed with 10 mL of 75% ethanol/0.5 M LiCl. The rinse buffer was subsequently added to the supernatant; the pellet was discarded.

1161 mL of cold ethanol was added to the 770 mL of 75% ethanol supernatant to bring the sample to 90% ethanol. The sample was stirred for 30 minutes at 4°C, then incubated at -30° C for 52 hours. Following incubation, the sample was centrifuged at 21,000 x g for 10 minutes at -15°C. The resulting precipitate was collected and dissolved in 43 mL of PBS; the supernatant was discarded. The dissolved sample

was then filtered through a 0.22 μm filter and stored at 4°C overnight. The entire 43 mL sample was placed into dialysis tubing (12,000-14,000 dalton porosity) and dialyzed overnight against 4 liters of 10 mM Tris-HCl, pH 7.0. The resulting 45 mL sample was subsequently removed from dialysis, filtered using a 0.22 μm filter, and stored at -70°C prior to processing by DEAE-agarose chromatography.

DEAE Agarose Chromatography:

A 44 mL sample of ethanol fractionated material (50.8 A_{280} units total) was loaded onto an 8.4 mL column of DEAE BioGel A (Bio Rad) equilibrated in 10 mM Tris-HCl, pH 7.0 at 2.8 mL/min. The ratio of absorbance units (at 280 nm) loaded per mL column volume was 5.92.

Following loading, the column was washed at 2.8 mL/min with 46 mL of 10 mM Tris-HCl, pH 7.0 until the A_{280} of the effluent reached baseline. The column was then washed in order with: 425 mL of 5 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.0; 115 mL of 17 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.0; 133 mL of 30 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.0; and 47 mL of 0.1 M $CaCl_2$. Each wash was of sufficient duration to allow the A_{280} of the effluent to reach baseline before the next wash was begun.

The A_{280} profile for this step is shown in Figure 1. Of the 50.8 A_{280} units of total column input, 29.8 units were recovered in the 17 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.0 eluate; this material was concentrated by pressure ultrafiltration using a YM-10 (Amicon) membrane. The resulting 20.2 mL of concentrate was then placed into dialysis tubing (12,000-14,000 dalton porosity) and dialyzed overnight against 4 liters of 5 mM $CaAc_2$, pH 4.5. The resulting sample was frozen at -70°C prior to processing by sulfopropyl-Sephadex chromatography.

Sulfopropyl-Sephadex Chromatography:

The 20 mL sample resulting from the previous step was loaded at 0.8 mL/min onto a 10 mL column of Sulfopropyl-Sephadex (Pharmacia) equilibrated in 5 mM $CaAc_2$, pH 4.5. The ratio of A_{280} units loaded per mL column volume was 2.58.

Following loading, the column was washed at 0.8 mL/min with 36 mL of 5 mM $CaAc_2$, pH 4.5 until the A_{280} of the effluent reached baseline. The column was washed at 0.8 mL/min in order with 72 mL of 7.5 mM $CaAc_2$, pH 4.7; 376 mL of 12.5 mM $CaAc_2$, pH 5.25; 340 mL of 15 mM $CaAc_2$, pH 5.5; and 210 mL of 0.1 M $CaAc_2$, 0.01 M Tris, pH 7.25. Each wash was of sufficient duration to allow the effluent to reach baseline before the next wash was begun.

The A_{280} profile for the sulfopropyl-Sephadex column is shown in Figure 2. Of the 25.8 A_{280} units of total column input, 4.89 units were recovered in the 12.5 mM $CaAc_2$, pH 5.25 wash; this material was concentrated by pressure ultrafiltration using a YM-10 (Amicon) membrane. The resulting 3.8 mL of concentrate was stored at 4°C prior to processing by Sephadex G-100 chromatography.

Gel Filtration Chromatography:

The 3.8 mL sample from the previous step was loaded on to a 78 mL column of Sephadex G-100 (Pharmacia) equilibrated in 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 6.9. The mobile phase for the separation was 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 6.9. A 17.5 mL pool was collected which constituted the main peak of absorbance at 280 nm (as shown in Figure 3); this material was diafiltered against 120 mL of cold WFI initially and then 20 mL of 0.5 mM Sodium Phosphate. The 10.2 mL of material (4.17 A_{280} units) resulting from the diafiltration was frozen at -70°C prior to the next step, hydroxylapatite chromatography.

Hydroxylapatite Chromatography:

A 9.95 mL sample (4.07 A280 units) from the previous step was loaded at 0.1 mL/min on to a 2.0 mL column of BioRad HTP hydroxylapatite equilibrated in 0.5 mM sodium phosphate, pH 7.2. The ratio of A_{280} units loaded per mL of column volume was 2.03.

Following loading, the column was washed at 0.1 mL/min with 41 mL of 0.5 mM sodium phosphate buffer until the A_{280} of the effluent reached baseline. The column was then washed with 69.3 mL of 1.0 mM sodium phosphate, pH 7.2; 22.0 mL of 2.0 mM sodium phosphate, pH 7.2; 18.5 mL of 3.0 mM sodium phosphate, pH 7.1 and 96.0 mL of 100 mM sodium phosphate pH 6.8. Each wash was of sufficient volume to allow the A_{280} of the effluent to reach baseline before the next wash as begun.

The A_{280} profile for the hydroxylapatite column is presented in Figure 4. Material eluting with the 0.5 mM and 1.0 mM sodium phosphate were collected and concentrated separately by pressure ultrafiltration using an Amicon YM-10 membrane. Although in the procedure performed by Miyake et al., the majority of the total activity applied to the hydroxylapatite column was found in the 1.0 mM eluate, in the present procedure, the majority of the material absorbing at 280 nm was found in the 0.5 mM sodium phosphate eluate. As a result, both the 0.5 mM (2.84 A_{280} units) and 1.0 mM (0.67 A_{280} unit) eluates were subjected to further analysis.

Analysis of Purified r-EPO and Purification Intermediates

Samples taken at each step of the purification procedure were characterized by SDS polyacrylamide gel electrophoresis (SDS-PAGE), Isoelectric Focussing (IEF), and reversed phase high performance liquid chromatography (RP-HPLC). Samples from the final step were additionally characterized by determination of their in vivo biological activity.

In the SDS-PAGE analysis, an appropriate amount of protein from each step of the purification process was mixed with sample buffer containing SDS and reductant, heated for 5 minutes at 100°C, and loaded onto a 12.5% polyacrylamide gel. Following electrophoresis, the protein bands were visualized by Coomassie Blue R-250 staining. The results, presented in Figure 5, show that the pool from sulfopropyl-Sephadex exhibits a single band on the Coomassie stained polyacrylamide gel. Samples from subsequent steps also exhibit only a single band on SDS-PAGE. The molecular weight of the purified r-HuEPO band shown in Figure 5 was calculated by comparison of its migration to that of the protein standards and r-HuEPO reference standard. The r-HuEPO migrated identically to the reference standard (which had a molecular weight of 34,000 daltons) and appeared between the molecular weight markers of 32,000 and 43,000 daltons.

Samples from each step of the purification process were also analyzed by IEF. As shown in Figure 6, the IEF gel shows the reduction in the number of bands to the characteristic 5-band pattern characteristic of pure EPO as the purification proceeds. Hydroxyapatite purififed r-HuEPO compares very favorably with the 5-banded pattern exhibited by the r-HuEPO reference standard.

Samples from each step of the purification process were analyzed by RP-HPLC using an analytical Vydac C$_4$ column. The chromatograms, monitored at 280 nm, are presented in Figures 7a-e. From these figures, it can be seen that the r-HuEPO when purified by the Miyake et al. procedure is homogenous and moves as a single peak on reversed phase high performance liquid chromatography.

The results of the mouse bioassay tests indicate that high activity r-HuEPO is eluted from the hydroxylapatite column. A total of two r-HuEPO rich fractions are eluted from this column. One of these fractions is eluted by 1.0 mM sodium phosphate buffer. Miyake, et al. found this fraction to contain the majority of r-HuEPO. In our experiment, we found this fraction to contain r-HuEPO with a potency of approximately 240,000 U/AU. A significant amount of r-HuEPO also eluted with the 0.5 mM sodium phosphate buffer. We found this fraction to have a potency of approximately 217,000 U/AU.

FIG. 1. DEAE-Bio Gel A Chromatography

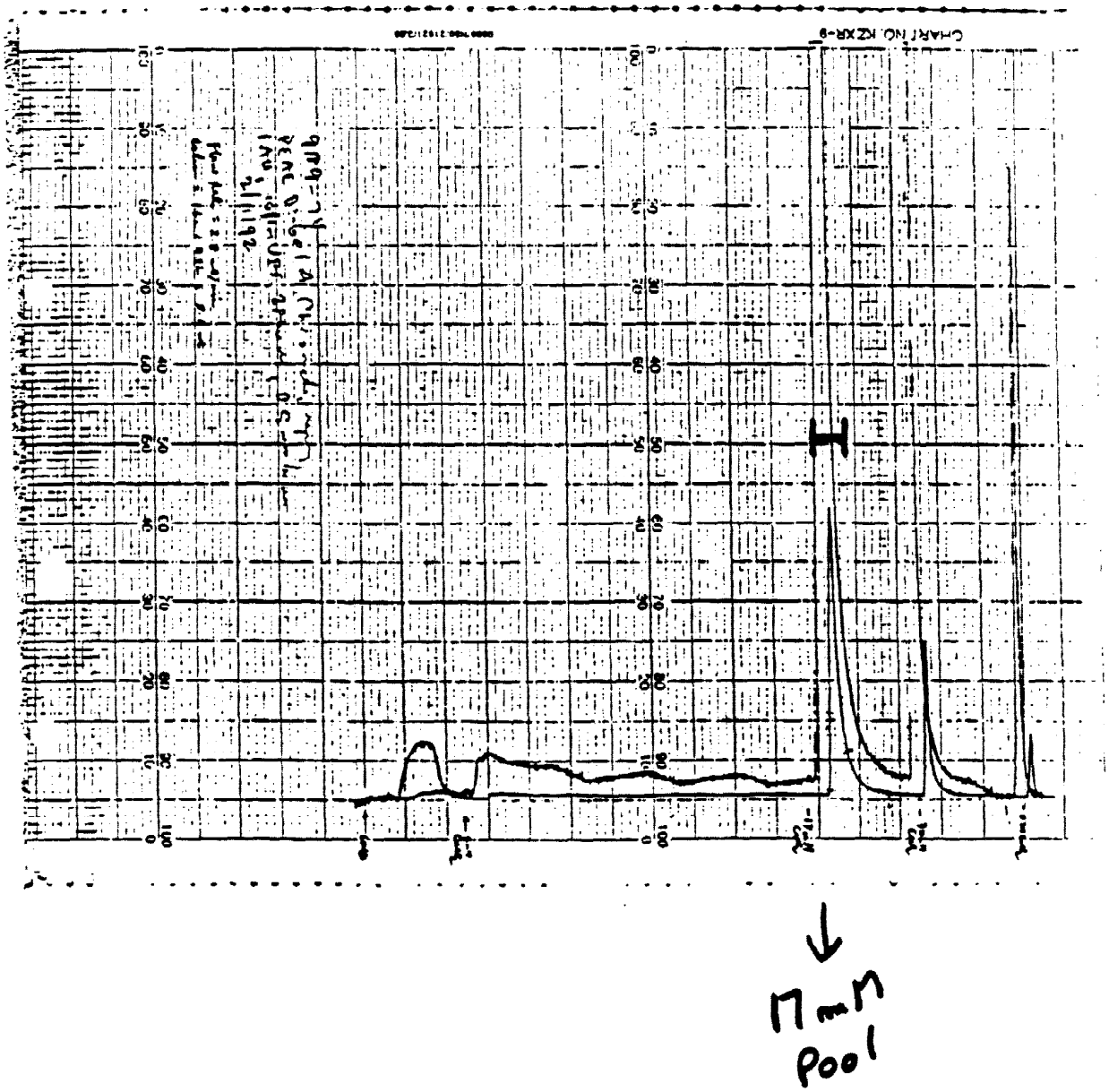

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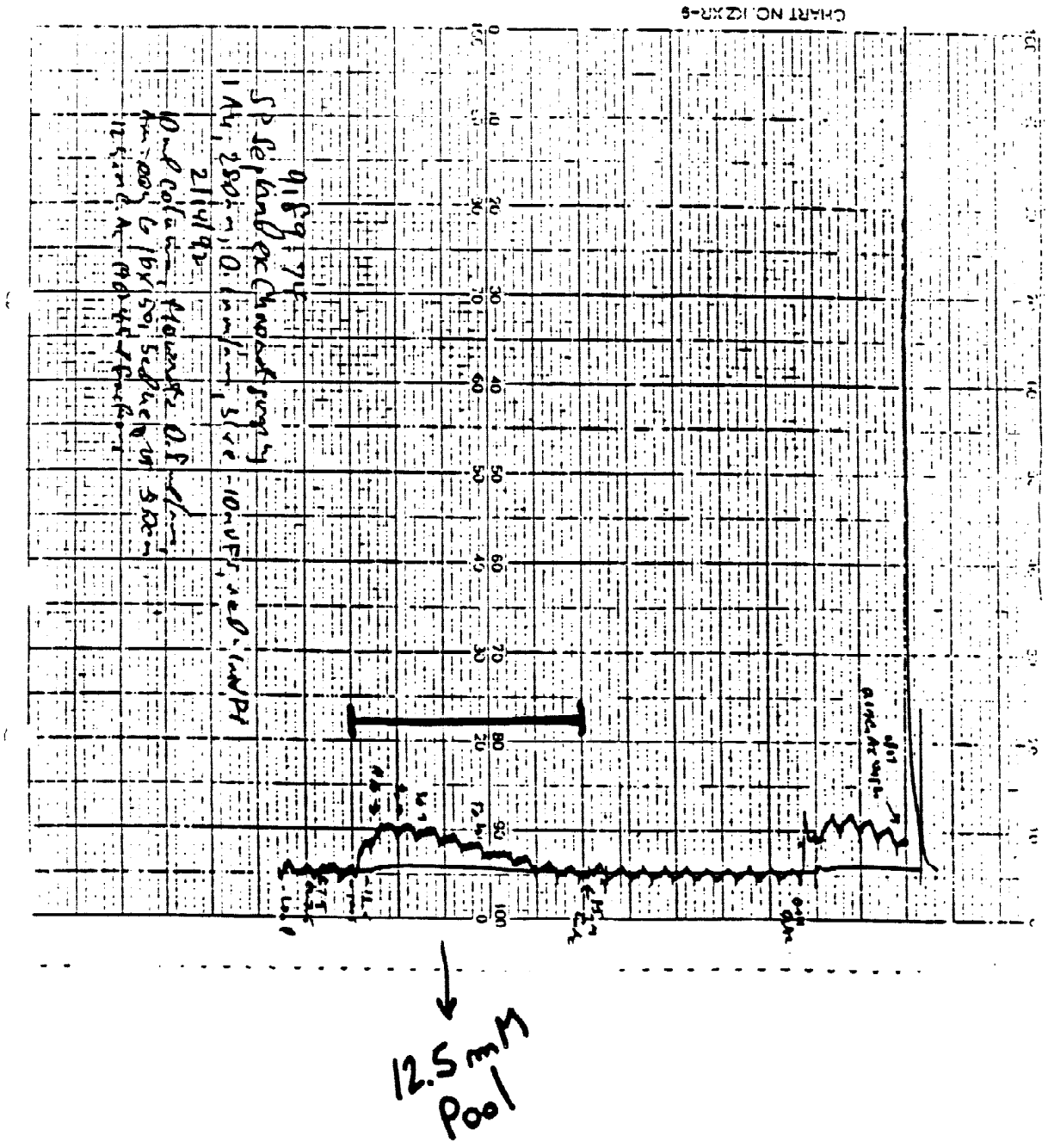
FIG. 2. Sulfopropyl-Sephadex Chromatography



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