Use of Immobilized Lectins and Other Ligands for the Partial Purification of Erythropoietin

By J. L. Spivak, D. Small, J. H. Shaper, and M. D. Hollenberg

The ability of a variety of affinity adsorbents to isolate erythropoietin (Ep) from contaminating proteins in crude preparations of the hormone was examined. Of 13 lectin-agarose derivatives, 6 bound Ep but only 2, wheat germ agglutinin (WGA) and phytohemagglutinin (PHA), bound the hormone quantitatively. The extent to which PHA bound Ep depended on the isoelectin composition of the PHA. The leukoagglutinating form (L-PHA) failed to bind the hormone completely, while the erythroagglutinating form (E-PHA) had such a high affinity for Ep that it could be released only with 4 M guanidine hydrochloride (pH 7.0). PHA-P, which contains both the E and L isoelectins, bound Ep quantitatively, and the hormone could be partially released by either N-acetylgalactosamine or sialic acid. Ep bound to WGA-agarose could be partially released with N-acetylglucosamine or sialic acid; with N,N-diacetylchitobiose recovery was quantitative. Two adsorbents, Cibacron Blue F3GA and octylsuccinic anhydride, which have a high affinity for albumin, a major contaminant of crude Ep preparations, also bound Ep quantitatively. Agarose-bound antialbumin IgG, however, was effective in removing albumin from crude hormone preparations without adsorbing a significant quantity of Ep. Neither agarose-bound neuraminidase nor hydrophobic interaction chromatography employing agarose coated with substituted or unsubstituted hydrocarbon chains separated Ep from contaminating proteins in crude preparations of the hormone.

INVESTIGATION of the mechanism of action of erythropoietin (Ep) has been hindered by lack of suitably pure preparations of the hormone. Recently, Miyake et al. described a conventional multistep procedure for purification of Ep to homogeneity from human urine that requires large amounts of starting material.1 When adequate quantities of crude hormone become available, their method will serve as a model for isolation of Ep from urine. Until then, alternative methods must be sought for obtaining purified hormone from the relatively restricted sources of crude hormone currently on hand. Two laboratories have employed affinity chromatography for this purpose.2,3 The technique is attractive because it is well suited for the manipulation of small quantities of protein, because it requires no special equipment, and because the required reagents are all commercially available.

Since our initial report,3 we examined the efficacy of a variety of affinity adsorbents in addition to agarose-bound lectins for isolating Ep from small quantities of crude starting material. In addition, we explored further the conditions
under which Ep can be adsorbed and eluted from lectin-agarose columns. The results of these studies are described in the present paper.

MATERIALS AND METHODS

Erythropoietin. Human urinary Ep was obtained from the NHLBI and sheep plasma Ep (step III) from Connaught Laboratories, Willowdale, Ont. Bioassay of hormone preparations and column eluates was performed in vivo using exphypoxic polycythemic mice as previously described. The second International Reference Preparation of human urinary Ep obtained from the World Health Organization was used as the standard. The plasma clot culture assay in vitro for CFU-E was also employed on occasion to monitor column eluates for the presence of Ep because, unlike the assay in vivo, it responds to asialoerythropoietin.

Preparation of affinity adsorbents. Concanavalin A Sepharose 4B (con A) and phytohemagglutinin (PHA) (L form) were obtained from Pharmacia, Piscataway, N.J. Ricinus communis 120 (RCA 120), Lens culinaris A and B (LCA and LCB), and peanut agglutinin (PA) were obtained from Miles Laboratories, Elkhart, Ind. Anti-A and anti-H lectins were purchased from P-L Laboratories, Milwaukee, Wisc. PHA (P form) was obtained from Difco, Detroit, Mich. Wheat germ agglutinin (WGA) was purified by the method of Shaper et al., while PHA (E form) was isolated by affinity chromatography on agarose-bound thyroglobulin. Hemolymph from Limulus polyphemus (a gift from Dr. J. Levin) was used to obtain limulin according to the technique of Roche et al.

Individual lectins were coupled to beaded agarose (Sepharose 4B or CL-4B, Pharmacia) after cyanogen-bromide activation as described by March et al. Coupling (2-10 mg lectin/ml activated Sepharose) was allowed to proceed for 20 hr at 4°C, followed by the addition of 1 M glycine (pH 8.0) for 4 hr at 24°C in order to mask unreacted groups on the agarose. In general, coupling efficiency was at least 50%, for both Sepharose 4B and CL-4B.

Cibacron-Blue F3GA was obtained from Polysciences, Warrington, Pa. and was coupled to Sepharose CL-4B as described by Travis et al. Octylsuccinic anhydride was coupled to Sepharose 4B via a 1,6-diaminohexane spacer arm according to the method of Aslam et al. Alkyl agarose and α-aminoalkyl agarose derivatives were obtained from Miles. Goat anti-human albumin antibody, IgG fraction (Cappel Laboratories, Downingtown, Pa.) was coupled to Sepharose 4B by the method of Cuatrecasas. Agarose-bound neuraminidase was purchased from Sigma Chemicals, St. Louis, Mo.

The monosaccharides used for elution of Ep from agarose-bound lectins were obtained from Pfleisterlh., Milwaukee, Wisc.: N,N-diacetylchitobiose was obtained from Polysciences.

Chromatography. Hydrophobic interaction chromatography was performed at 25°C; otherwise chromatography was performed at 4°C. Disposable glass pipets 0.5 x 9 cm plugged with glass wool or 5-ml plastic syringes stoppered with sintered glass discs were employed as columns. The columns contained approximately 2 ml affinity adsorbent and were equilibrated with phosphate-buffered saline (PBS) pH 7.4 (NaCl 8 g, KH₂PO₄ 0.2 g, Na₂HPO₄ 1.13 g, in 1 liter distilled H₂O). For con A Sepharose the buffer contained 1 mM calcium chloride and 0.5 mM magnesium chloride. Buffer was applied to the columns by gravity, the flow rate was adjusted to 12 ml/hr, and 1-ml fractions were collected. Protein in the column effluent was monitored by absorption at 280 nm or by adding a trace quantity of ¹²⁵I-labeled crude Ep and counting aliquots of the column effluent in a gamma counter. The use of the radioactive tracer facilitated the monitoring of chromatographic procedures involving small amounts of protein. After application of the sample, usually in a volume of 1 ml, the column was washed with PBS until the effluent was free of protein. The agent chosen for elution of bound Ep was then applied. In the case of agarose-bound lectins, after the sugar specific for the lectin was applied, flow was stopped and the column was warmed by hand (22°C) for 5 min before resuming chromatography in order to facilitate the displacement of the bound hormone by the sugar. Column fractions were pooled and dialyzed against PBS prior to quantitation of protein and Ep activity.

With columns of the size described, containing WGA or PHA covalently bound to agarose at a concentration of 5 mg/ml, we applied up to 100 mg crude urine extract (54 mg protein) and up to 260 units Ep without a significant loss of hormone in the void volume.

Analytical procedures. Protein was determined by a micromodification of the Lowry pro
SDS-urea polyacrylamide gel electrophoresis was performed as previously described except that gel diameter was reduced from 7 to 2.5 mm to increase sensitivity.

RESULTS

Affinity chromatography with agarose-bound lectins. Since our initial report, the affinity of 7 additional lectins for Ep was examined. Because the sugar content of human urinary Ep has not been unequivocally established, the lectins were chosen for study on the basis of their affinity for the various monosaccharide and oligosaccharide moieties present in human glycoproteins. The results of these experiments are shown in Table 1 in conjunction with the data obtained with the 6 lectins initially studied. Only 5 of the 13 lectins (LCA, LCB, L-PHA, PHA-P, and WGA) bound hormone that could be eluted in appreciable quantities by a sugar specific for the particular lectin. Of the 5, PHA and WGA were of particular interest because they alone quantitatively bound Ep. For PHA, however, the extent to which Ep was bound varied with the form of the lectin employed. PHA-P (Difco) is a partially purified extract of Phaseolus vulgaris that contains both erythroagglutinating (E) and leukoagglutinating (L) isolecitins. Ep bound to PHA-P could be eluted in part by GalNAc and completely by 4 M guanidine hydrochloride (buffered in PBS to pH 7.0). E-PHA, however, had such a high affinity for Ep that the hormone could not be released from the lectin by the following monosaccharides: 0.5 M GalNAc, 0.5 M Gal or 0.5 M GlcNAc, a chaotropic agent, 0.2 M NaSCN, or 1 M NaCl. The hormone could be recovered by washing the column with 4 M guanidine hydrochloride (pH 7.0) (Table 2). In contrast to the E isolecitin, L-PHA failed to bind Ep quantitatively. This was not due to heterogeneity with respect to the Ep mole-

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Eluting Agent</th>
<th>Void Volume</th>
<th>Sugar Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limulin</td>
<td>NANA</td>
<td>16.0 ± 1.0</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Ricinus communis 120*</td>
<td>Gal</td>
<td>13.0 ± 2.0</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Peanut agglutinin</td>
<td>Gal</td>
<td>21.4 ± 7.7</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Soybean agglutinin*</td>
<td>GalNAc</td>
<td>21.0 ± 4.0</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Concanavalin A*</td>
<td>Man</td>
<td>15.0 ± 2.0</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>Anti-A lectin</td>
<td>GalNAc</td>
<td>22.0 ± 1.9</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>Anti-H lectin</td>
<td>Fuc</td>
<td>16.6 ± 4.6</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>Lens culinaris A</td>
<td>Man</td>
<td>11.3 ± 1.8</td>
<td>18.5 ± 2.1</td>
</tr>
<tr>
<td>Lens culinaris B</td>
<td>Man</td>
<td>22.9 ± 3.0</td>
<td>31.2 ± 3.4</td>
</tr>
<tr>
<td>Phytohemagglutinin</td>
<td>GalNAc</td>
<td>12.4 ± 2.1</td>
<td>14.0 ± 3.1</td>
</tr>
<tr>
<td>L form</td>
<td>GalNAc</td>
<td>1.0 ± 0.2</td>
<td>12.7 ± 3.3</td>
</tr>
<tr>
<td>P form*</td>
<td>GalNAc</td>
<td>2.2 ± 1.0</td>
<td>17.1 ± 1.9</td>
</tr>
<tr>
<td>E form</td>
<td>GlcNAc</td>
<td>1.0 ± 0.3</td>
<td>16.0 ± 2.0</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin*</td>
<td>GlcNAc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Approximately 5–15 units Ep were applied to each agarose-lectin column. The details of the chromatographic procedure are described in the text. Equal aliquots of the pooled fractions from the void volume and from the sugar eluate were assayed for Ep in vivo in exhypoxic polycythemic mice using incorporation of 59Fe into red cells as the index of erythropoietic activity. Values of 4% or less indicate no erythropoietic activity. NANA, N-acetyleuraminic acid; GalNAc, N-acetylgalactosamine; Gal, galactose; Man, a-methyl mannoside; GlcNAc, N-acetylglucosamine; Fuc fucose.

*a Lectins for which similar results were obtained with sheep plasma Ep.3,28
Table 2. Elution of Human Urinary Erythropoietin From Agarose-Bound PHA

<table>
<thead>
<tr>
<th>Column Fraction</th>
<th>$^{59}$Fe Incorporation Into Red Cells (Mean ± SEM)%</th>
<th>Recovery (Units)</th>
<th>Specific Activity (Units/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Void volume</td>
<td>12.3 ± 2.8</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>(34 µg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanidine HCl eluate</td>
<td>11.5 ± 1.9</td>
<td>160</td>
<td>1500</td>
</tr>
<tr>
<td>(0.1 µg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ep (260 units, 320 U/mg protein) was applied to a 0.5 x 9.0 cm column. The details of the chromatographic procedure are described in the text.

When the hormone that had passed through the column unretarded was reapplied to a fresh agarose-L-PHA column, part of the hormone was adsorbed to the column and part again passed through unretarded. Furthermore, when Ep was applied to a column containing an equivalent amount of E-PHA covalently coupled to agarose, all of the hormone was adsorbed by the lectin. This suggests that the affinity of the L-isolectin of PHA for Ep is less than that of the E-isolectin. Thus the behavior of Ep during chromatography on agarose-bound PHA will depend on the isolectin-lectin composition of the PHA employed.

From Table 1 it is also apparent that the established sugar specificity of a particular lectin does not predict the ability of that lectin to bind Ep. For example, limulin, which binds sialic acid and was itself purified by affinity chromatography on agarose-bound bovine submaxillary mucin,8 failed to bind Ep, which is known to contain sialic acid. Con A and LCA and LCB all bind mannose; however, only LCA and LCB bound Ep. The reason for the differences in the binding of Ep by different lectins having apparent specificities for the same sugar has not been established.

In our preliminary studies3 only 40% of the human urinary Ep bound to agarose-WGA was subsequently recovered when GlcNAc was used as the eluting sugar. In order to determine the reason for this, we examined the ability of other sugars known to bind to WGA to elute the hormone from the lectin. The results of sequential elution of Ep from agarose-bound WGA with GlcNAc and NANA are shown in Fig. 1. Each sugar eluted some Ep from the lectin, but neither eluted the hormone completely. Thus Ep does not bind to WGA solely by GlcNAc or NANA residues. Since polymers of GlcNAc have a higher affinity for WGA than GlcNAc,14 N,N-diacyltchitobiose was employed to elute the hormone. When this disaccharide was used, virtually all of the hormone was recovered from the column (Table 3). As indicated by gel electrophoresis (Fig. 2), the removal of contaminating proteins after a single chromatographic step was substantial.

Since Ep was bound quantitatively by two different lectin adsorbents (PHA and WGA), it was of interest to determine if they bound the hormone in an identical fashion. From the data in Table 4 it is apparent that only NANA is capable of partially eluting hormone from each lectin adsorbent. Since the hormone could be released only from WGA-agarose but not from PHA-agarose by N,N-diacyltchitobiose, it appears that the lectins bind Ep through different sugar determinants. PHA has an affinity for oligosaccharides with a terminal sialic acid residue and a penultimate galactose.15 This carbohydrate...
sequence is found in Ep and may explain the affinity of PHA for the hormone.

Although 8 of the 13 lectins studied failed to bind Ep in a quantitative fashion, they did bind other proteins present in the crude starting material and thus are potentially useful in purifying the hormone. To examine this possibility, we applied crude human urinary Ep to a column containing a mixture of

<table>
<thead>
<tr>
<th>Column Fraction</th>
<th>59Fe Incorporation Into Red Cells (Mean ± SEM) (%)</th>
<th>Recovery (Units)</th>
<th>Specific Activity (Units/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Void volume</td>
<td>2.3 ± 1.1</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>N,N-diacetylchitobiose eluate</td>
<td>31.3 ± 3.7</td>
<td>40</td>
<td>181</td>
</tr>
<tr>
<td>Guanidine HCl eluate</td>
<td>7.0 ± 2.1</td>
<td>0.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Ep (20 units, 40 U/mg protein) was applied to a 0.5 x 9.0 cm column. The details of the chromatographic procedure are described in the text. Equal aliquots of each column fraction were assayed for Ep. The increase in recovery of Ep presumably represents the removal of inhibiting substances during chromatography.
Fig. 2. SDS-urea, 7.5% polyacrylamide gel electrophoresis of (A) of 35 μg crude human urinary Ep (40 U/mg protein), (B) 30 μg partially purified Ep (70 U/mg protein) obtained from N,N-diacetylglycosamine eluate after chromatography of 1 mg human urinary Ep on agarose-WGA, and (C) 25 μg partially purified Ep (100 U/mg protein) obtained from 4 M guanidine HCl (pH 7.0) eluate after chromatography of 1 mg human urinary Ep on agarose-PHA. Gel dimensions, 0.25 x 6.0 cm. Direction of electrophoresis indicated by arrow. TD, location of tracking dye, not visualized after destaining.

six lectins (con A, SBA, RCA 120, PA, anti-A, and anti-H) that failed to bind Ep in a sugar-specific manner: 50% of both protein and hormone activity applied to the column was recovered in the void volume (Fig. 3). The remainder bound to the column and could be removed in part with buffered 4 M guanidine HCl. Failure to quantitatively recover Ep from this column was probably the

Table 4. Competitive Elution of Human Urinary Erythropoietin From Agarose-Bound WGA and PHA

<table>
<thead>
<tr>
<th>Eluting Sugar</th>
<th>Agarose-bound Lectin</th>
<th>Activity Applied (Units)</th>
<th>Activity Recovered (Units)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylglucosamine (0.1 M)</td>
<td>WGA</td>
<td>50</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>PHA-P</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-acetylgalactosamine (0.1 M)</td>
<td>WGA</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PHA-P</td>
<td>8</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>N-acetyleneuraminic acid (0.1 M)</td>
<td>WGA</td>
<td>19</td>
<td>7.5</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>PHA-P</td>
<td>20</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>N,N-diacetylglycosamine (0.01 M)</td>
<td>WGA</td>
<td>40</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>PHA-P</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
result of nonspecific hydrophobic or electrostatic binding, since none of these lectins individually is capable of binding the hormone through interaction with a specific monosaccharide.

*Other affinity adsorbents.* Albumin is a major constituent of crude Ep-rich extracts of human urine. Consequently, several ligands known to bind albumin were employed to remove this protein from such extracts. Cibacron-Blue F3GA, the chromophore of Blue Dextran,\(^{10}\) quantitatively bound the albumin in the crude starting preparation, but it also bound more than 90% of the Ep, much of which could be subsequently recovered by washing the column with 0.5 \(M\) NaSCN. Alkylsuccinic anhydrides coupled to agarose also bind albumin efficiently.\(^{11}\) When crude Ep was applied to a column containing the octyl derivative, the hormone was quantitatively bound and could not be completely recovered from the adsorbent by elution with either 0.5 \(M\) NaCl or 0.3% SDS. An agarose-bound goat anti-human albumin IgG adsorbent was more effective. Chromatography of crude human urinary Ep resulted in recovery of 75% of the hormone and removal of 50% of the contaminating protein. A purification of 1.6-fold was obtained with this single chromatographic step.

Since Ep is a sialoglycoprotein, it was of interest to determine if neuraminidase immobilized on agarose could be used to separate the hormone from contaminating proteins. However, when crude Ep was applied to a column containing agarose-bound neuraminidase at 4\(^\circ\)C, all of the hormone and 75% of the protein appeared in the void volume. The Ep recovered was desialylated to such a degree that it could not be detected using the exhypoxic polycythemic mouse assay in vivo.

Based on the results of the experiments employing immobilized Cibacron-Blue F3GA and octylsuccinic anhydride coupled to 1,6-diaminohexyl agarose, we investigated further the use of hydrophobic interaction chromatography for the purification of urinary Ep. Crude extracts of the hormone were applied to columns containing agarose hydrocarbon chains of varying lengths, both unsubstituted and substituted with an amine group on the terminal carbon.\(^{18}\) With increasing hydrocarbon chain length, increasing amounts of protein and hormone were bound to the columns, but there was no appreciable purification of Ep with either the substituted or unsubstituted hydrocarbon chains (data not shown).
DISCUSSION

The Ep-rich crude extracts of human urine that are used extensively to study erythropoiesis in vivo and in vitro contain a variety of contaminating proteins. They include proteolytic enzymes such as urokinase, plasminogen, and pepsin, proteins that stimulate granulopoiesis and thrombopoiesis as well as erythropoiesis, and proteins that inhibit erythropoiesis. Crude urine extracts are also usually contaminated with bacterial products such as glycosidases and endotoxin that may either inhibit or potentiate the activity of Ep. In view of the heterogeneity in cell composition of hematopoietic tissues, many of the biochemical effects previously ascribed to Ep may well be due to the presence of contaminants when such crude extracts of the hormone are employed. Furthermore, even if specificity can be established, such as when erythroid colony formation is used as an endpoint, there is no assurance that Ep alone is responsible for the observed results. For example, Aye recently identified an agent associated with the adherent cell population of bone marrow that enhances the effects of Ep on the growth in vitro of erythroid cells.

Ep found in crude extracts of urine constitutes only a minor fraction of the proteins present. Albumin is a major constituent of the urinary proteins, and there are at least 25 glycoproteins, 12 of which have similar isoelectric points. It therefore is not surprising that conventional techniques for separating the hormone from other proteins on the basis of size and charge require large quantities of starting material. The quantities of crude Ep available to most investigators, however, are limited, and many of the conventional techniques required for purification are too cumbersome and inefficient to employ on a small scale. Affinity chromatography is an attractive alternative because it does not rely on physicochemical properties shared in common by many proteins but instead can be tailored to the specific properties of the protein of interest. In addition, since no special equipment is required and since the required reagents are available commercially in a ready-to-use form, immediate application of the technique is possible.

Affinity chromatography, however, is not free of problems, such as leakage of ligand and nonspecific adsorption of protein to the affinity adsorbent by either hydrophobic interaction or electrostatic attraction. We found the leakage of ligand to be negligible with the use of the simplified acetonitrile-cyanogen bromide activation procedure of March et al. However, we have not tested commercial agarose derivatives in this regard. Nonspecific leakage of protein from the columns is also minimal; crosslinked agarose (Sepharose CL-4B) is the most satisfactory in this respect. Small quantities of lectins such as WGA, PHA, and con A can affect the growth in vitro of erythroid cells. However, if present the lectins can easily be removed by a simple affinity procedure using an appropriate immobilized ligand. For example, agarose-bound GlcNAc binds WGA, agarose-bound thyroglobulin binds PHA, and Sephadex G-50 binds con A; none of these agents adsorb Ep, which can be recovered intact in the column void volume.

Owing to the nature of the process for activating the agarose, the beads acquire a cationic charge, which in conjunction with any charge on the ligand can result in nonspecific adsorption of proteins due to electrostatic attraction. This unavoidable problem can be minimized by using buffers of high ionic strength.
strength. Hydrophobic interactions are also inevitable when proteins are derivatized on agarose; from our experience and that of Goldwasser, it appears that Ep is a very hydrophobic protein. Hydrophobic interactions can be reduced by performing chromatography at 4°C at a pH above 7.0 and by avoiding a high degree of ligand substitution.32

In addition to effecting a substantial purification of Ep in a single step,3 there are other applications in Ep research for which affinity chromatography should be useful. Iscove et al. showed that colony-stimulating factor, which is present in crude preparations of urinary Ep, could be separated from the hormone by chromatography on agarose-bound con A.33 More recently, Aye identified an Ep-enhancing factor associated with the adherent marrow cell population that in contrast to Ep does not bind to WGA.34 Furthermore, evidence has been presented that colony-stimulating factor may block the interaction of Ep with its target cells.35 Separation of colony-stimulating factor and the Ep-enhancing factor from Ep by affinity chromatography should therefore facilitate identification of the specific biologic effects of each of these humoral agents.

A major problem in studying the behavior of erythroid cells in cultures in vitro is the presence of endogenous Ep in the fetal calf serum used in the culture media.5 One approach to the removal of this unwanted endogenous hormone has been to adsorb the fetal calf serum with an anti-Ep antibody that is itself then neutralized by a second heterologous antibody. Although this technique has been put to elegant use,6 it is cumbersome and requires a source of Ep antibody. Because the Ep used as the antigen for producing these antibodies is itself impure, the antibodies may have additional unidentified and unwanted effects. Since Ep is bound avidly by WGA and PHA, adsorption of reagents such as fetal calf serum with these immobilized lectins should remove any endogenous hormone present. We successfully used such a technique to lower the endogenous Ep level in fetal calf serum used for the growth of CFU-E.37

Additional uses for affinity chromatography include the isolation of Ep from unconcentrated urine2 and the purification of anti-Ep antibodies.38 Most important, of course, is the application of the technique to purification of the hormone on a preparative scale from crude urine extracts. It is apparent from our data and those of Sieber that it will be necessary to rely on other separation techniques in addition to affinity chromatography in order to obtain Ep purified to homogeneity. It remains at present to define the most effective combination of techniques for this purpose. The experience of Miyake et al.1 will be valuable in this regard. In the meantime, for routine investigative work, a single chromatographic procedure using either agarose-bound WGA or PHA alone or in combination with agarose-bound con A should remove the majority of the contaminants present in currently available Ep preparations. The degree of purification will vary depending on the source and initial specific activity of the crude hormone.1 For human urinary Ep (40 U/mg protein), we obtained a two-fold purification and an 80% recovery with chromatography on agarose-bound con A; with agarose-bound WGA or PHA, a two- to eightfold purification can be achieved depending on the agent chosen to release the hormone from the affinity adsorbent.23 Our data indicate that the disaccharide N,N-diacetyl-
chitobiose is more effective than the monosaccharide N-acetylgalactosamine in eluting Ep from agarose-WGA, while 4 M guanidine hydrochloride is an effective eluting agent when agarose-PHA is employed as the ligand. The use of these agents to release Ep from the affinity adsorbent will not compromise further studies in vivo or in vitro employing the hormone, since these agents are dialyzable. The use of the oligosaccharides or monosaccharides should also not interfere with determination of the carbohydrate composition of the hormone, since sugar molecules that adsorb to Ep during lectin-affinity chromatography can be separated by a subsequent chromatographic step under denaturing conditions with SDS or guanidine HCl.

ACKNOWLEDGMENT

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Use of immobilized lectins and other ligands for the partial purification of erythropoietin

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