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Ovine luteinizing hormone

V. Significance of flow-through peaks observed during chromatofocusing as revealed by various methods of sample preparation and application

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ABSTRACT

In a previous study [Keel *et al.*, *Biol. Reprod.*, 36 (1987) 1102] the ovine luteinizing hormone (oLH) in pituitary extracts was chromatofocused on pH 10.5–7 gradients after equilibration in 25 m*M* triethylamine–HCl, pH 11.0, by gel permeation. Under these conditions, some immunoreactive oLH flowed through the columns unrestricted and this was interpreted to represent extremely basic isoforms. However, when selected flow-through peaks were re-chromatofocused, each was contaminated with other isoforms of oLH. In order to clarify this dilemma, various methods of sample preparation and application were systematically compared. Consistent with previous observations, variable amounts of the immunoreactive oLH in pituitary extracts equilibrated in triethylamine by gel permeation, dialysis, flow dialysis or ion-retardation chromatography eluted as flow-through peaks when chromatofocused. In contrast, when the ionic components in the pituitary homogenization buffer were removed by these methods as well as ultrafiltration and the proteins were applied to the resin in the elution buffer (1:45 Pharmalyte 8-10.5–HCl, pH 7.0), none of the immunoreactive oLH in pituitary extracts eluted as a flow-through peak. Thus, it appears that oLH eluting as a flow-through peak results from incomplete binding of the hormone to the chromatofocusing resin when applied in triethylamine.

INTRODUCTION

The heterogeneity of the glycoprotein hormones is abundantly documented in the scientific literature with many, if not all, exhibiting multiple-charge isomers (for examples, see Keel and Grotjan [1]). One method which has been utilized to examine the charge heterogeneity of the glycoprotein hormones is chromatofocusing [1–5]. In a previous study [2], the conditions utilized for chromatofocusing were essentially those recommended in the directions supplied with the reagents [6]. Accordingly,

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samples were applied to the resin in chromatofocusing "start" buffer (25 m*M* triethylamine–HCl, pH 11.0) after gel permeation on small columns of Sephadex G-25 Superfine [2]. Under these conditions, some of the ovine luteinizing hormone (oLH) in pituitary extracts flowed through the columns unrestricted when chromatofocused on pH 10.5–7 gradients. Furthermore, the percentage of oLH eluting as flow-through peaks was increased in the pituitaries of rams or wethers treated with androgens [2]. However, when selected flow-through peaks (designated as peak A in Keel *et al.* [2]) from several chromatofocusing profiles were dialyzed against water and re-chromatofocused (as decribed in detail in the subsequent paper [5]), it became apparent that each was contaminated with other isoforms of oLH [7]. In order to resolve this dilemma, various methods of sample preparation and application were systemically compared. During these experiments it became apparent that the buffer in which the pituitary extract was applied to the chromatofocusing column affected the percentage of oLH flowing through the columns unrestricted. When samples were applied to the resin in the elution buffer (1:45 Pharmalyte 8-10.5–HCl, pH 7.0), none of the oLH in pituitary extracts eluted as a flow-through peak.

MATERIALS AND METHODS

Purified oLH and pituitary extracts

Highly purified oLH was obtained from Dr. David Sherwood, University of Illinois, Urbana, IL, USA [8]. Ovine or bovine pituitaries were homogenized in 150 m*M* NaCl, 50 m*M* Tris (pH 7.4) containing 1% (v/v) Triton X-100 and a series of protease inhibitors (5 m*M* Na_2EDTA, 1 m*M* phenylmethylsulphonyl fluoride and 200 U/ml aprotinin), centrifuged at 100 000 *g* for 1 h and frozen at −70°C as decribed previously [2]. Selected pituitary extracts from Keel *et al.* [2] were also utilized. Purified oLH was supplemented with 2 mg cytochrome *c*, 3 mg myoglobin and 4 mg ovalbumin prior to chromatofocusing (see Figs. 4 and 5) while aliquots of pituitary extracts were supplemented with 2 mg cytochrome *c* and either 5 mg ovalbumin (see Figs. 1–3) or 3 mg myoglobin (see Figs. 6–8) prior to preparation for chromatofocusing by one of the methods subsequently described. All sample preparation and chromatographic separations were performed at 4°C.

Gel permeation

A 0.5- or 1.0-ml aliquot of a pituitary extract was equilibrated in either 25 m*M* triethylamine–HCl, pH 11.0, or 1:45 Pharmalyte 8-10.5–HCl, pH 7.0 (Pharmacia/LKB, Piscataway, NJ, USA), by gel permeation on 1.0 × 15 cm columns of Sephadex G-25 Superfine (Pharmacia/LKB). The columns were eluted at 4 ml/h and 0.5 ml fractions were collected. Columns eluted with Pharmalyte were washed with water between samples and re-equilibrated with 2–4 column volumes of Pharmalyte prior to sample application. The recovery of immunoreactive oLH from pituitary extracts prepared by gel permeation was typically >75%.

Ion-retardation chromatography

A 1.0 × 40 cm column of AG11A8 ion-retardation resin (Bio-Rad, Richmond, CA, USA) was regenerated with 1 *M* NH_4Cl and re-equilibrated in water. A 1.0-ml aliquot of a pituitary extract was loaded and eluted at 14 ml/h with water. Fractions

of 1.0 ml were collected. The void-volume material was pooled, lyophilized and dissolved in triethylamine. Recovery of immunoreactive oLH was 87%.

Dialysis and flow dialysis

A 0.5- or 1.0-ml aliquot of a pituitary extract was placed in SpectraPor1 dialysis tubing (6000–8000 mol.wt. cut off; Spectrum Medical Industries, Los Angeles, CA, USA) and dialyzed against 1–2 l water overnight. Aliquots of extracts prepared by flow dialysis were placed on one side of a SpectraPor1 membrane in a 2.5-ml Spectrum flow-dialysis apparatus. Approximately 3 l water were allowed to flow through the opposite side overnight. In both cases, the conductivity of the dialyzed sample was checked with a hand-held conductivity meter (Model CDH-53, Omega, Stamford, CT, USA) and the sample was then supplemented with a concentrated (10 ×) solution of triethylamine or Pharmalyte. Prior to chromatofocusing, samples prepared by these methods were centrifuged at 12 800 *g* for 5 min to remove any particulate matter resulting from the reduction in ionic strength and/or removal of detergent. The recovery of immunoreactive oLH from samples prepared in this manner was typically >85%.

Ultrafiltration

A 0.5- or 1.0-ml aliquot of a pituitary extract was diluted with an equal volume of water, placed in an Amicon Centricon 10 ultrafiltration device (Amicon, Beverly, MA, USA) and centrifuged three times at 5000 *g* for 30 min. Between each centrifugation a volume of water equal to the volume of the diluted extract was added. The retentate was recovered by adding 0.5 ml water, inverting the device and centrifugation at 1000 *g* for 3 min. Conductivity of the sample was assessed as described above and each was supplemented with a concentrated Pharmalyte solution prior to chromatofocusing. Samples prepared in this manner yielded immunoreactive oLH recoveries of >83%. Typically, no more than 7% of the immunoreactive oLH was present in the filtrate.

Chromatofocusing

Chromatofocusing on pH 10.5–7 gradients was performed essentially as described by Keel *et al.* [2]. Columns (20 ml, 25 × 1.0 cm I.D.) of PBE 118 resin (Pharmacia/LKB) were equilibrated in 25 m*M* triethylamine–HCl, pH 11.0. Samples prepared in triethylamine were loaded to start the run while samples prepared in Pharmalyte were loaded after 3.0 ml of the elution buffer had been applied to the column. In both cases, the pH gradient was developed with 1:45 Pharmalyte 8-10.5–HCl, pH 7.0, which was eluted at 10 ml/h and collected as seventy or eighty 3.0-ml fractions. The pH gradient was monitored with a Pharmacia pH monitor. After the lower-limiting pH had been reached, materials bound to the column were eluted with 1 *M* NaCl which was collected as an additional twenty 3.0-ml fractions. Each chromatofocusing fraction was subsequently neutralized by the addition of 0.3 ml 1.1 *M* Tris-HCl, pH 7.0. When purified oLH was analyzed, chromatofocusing buffers were supplemented with 1% (v/v) glycerol. When pituitary extracts were analyzed, chromatofocusing buffers were supplemented with 0.1% (v/v) glycerol. The addition of extra proteins and glycerol enhances the recovery of oLH [9].

Chromatofocusing on pH 9–6 gradients was performed on 20 ml PBE 94 resin

(Pharmacia/LKB) equilibrated in 25 m*M* ethanolamine–acetate, pH 9.4. An aliquot of a bovine pituitary extract was de-salted by flow dialysis against water and supplemented with a concentrated solution of Polybuffer 96–acetate, pH 6.0 (Pharmacia/LKB). After sample application, the pH gradient was developed at 10 ml/h with 1:40 Polybuffer 96–acetate, pH 6.0, and collected as eighty 3.0-ml fractions. Materials bound at the lower-limiting pH were eluted with 1 *M* NaCl which was collected as an additional twenty 3.0-ml fractions. Each fraction was neutralized with Tris as described above. The recovery of purified oLH and LH in pituitary extract from the chromatofocusing columns was 53 ± 4 and 75 ± 5%, respectively.

Radioimmunoassays

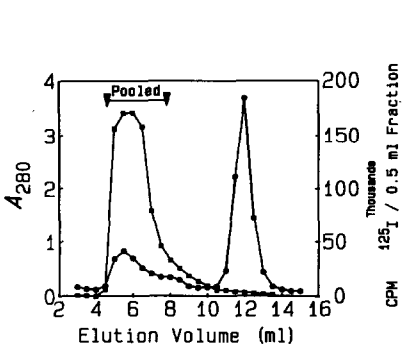
The oLH concentrations in chromatofocusing fractions were quantitated by radioimmunoassays using purified hormones obtained from Dr. D. N. Ward, Houston, TX, USA, and Dr. L. E. Reichert, Jr., Albany, NY, USA, and a polyclonal antiserum developed in rabbits against a human chorionic gonadotropin (hCG)α–oLHβ heterodimer [10]. Highly purified oLH (oLH-LER-1056-C2 or oLH-LER-1374A) was iodinated in 0.5 *M* Na_2HPO_4, pH 8.2, using a ^{125}I/oLH molar ratio of two and one Iodobead (Pierce, Rockford, IL, USA) for 15 min at room temperature. Unincorporated ^{125}I was removed by gel permeation. The immunoassay buffer contained 150 m*M* NaCl, 50 m*M* Tris–HCl (pH 7.4), 5 m*M* Na_2EDTA, 1 g/l gelatin and 0.1% (w/v) sodium azide. Purified oLH (oLH-DNW-HSN-10-124, potency = 1.7 × NIH-LH-S1) was used as the reference preparation. The relative cross reactivities of various hormones on a molar basis were: oLH 100%, oLHβ 113%, oLHα 0.14%, ovine follicle-stimulating hormone (FSH) 3.4%, porcine FSHβ <0.03%, bovine thyroid-stimulating hormone (bTSH) 2.0%, bTSHβ 0.4%, bovine growth hormone 0.5%, bovine prolactin <0.04% and porcine adrenocorticotropin <0.01%. When buffered with Tris, up to 0.2-ml aliquots of chromatofocusing fractions could be included in an immunoassay tube without significant interference from the chromatofocusing reagents. Because the molecular structure of ovine and bovine (b) LH are extremely similar [1], the LH concentrations in chromatofocusing fractions derived from bovine pituitary extracts were quantitated in the same assay system utilizing the oLH standard noted above as the reference preparation.

Titration of PBE 118 chromatofocusing resin

A volume of 10 ml of PBE 118 Resin (Lot MF 02136 with a stated capacity of 51 μmol pH unit^{-1} ml^{-1}) was equilibrated in 1 *M* KCl by repeated centrifugation. The resin was diluted to a 10% (v/v) suspension in 1 *M* KCl and adjusted to a pH > 12 with 10 *M* NaOH. The resin was the titrated to a pH < 3 with 0.5 *M* HCl at room temperature. All pH measurements were obtained with a Corning Ross pH electrode and Corning pH meter.

RESULTS

In our previous study [2], ovine pituitary extracts were equilibrated in 25 m*M* triethylamine–HCl, pH 11.0 (chromatofocusing "start" buffer) by gel permeation on 15 × 1 cm I.D. columns of Sephadex G-25 Superfine. A clear separation of proteins and ionic components was achieved (Fig. 1). In order to determine if the method by



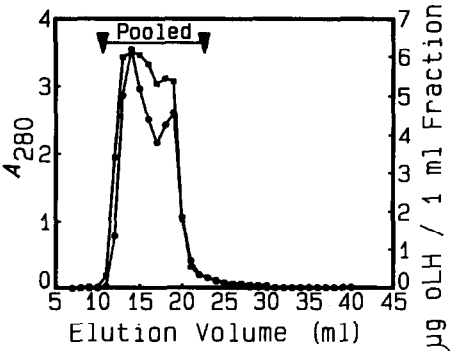


Fig. 1. Elution of proteins and ^{125}I during gel permeation on Sephadex G-25 Superfine. A 0.5-ml aliquot of an ovine pituitary extract (50 mg tissue equivalents) was supplemented with 2 mg cytochrome *c*, 5 mg ovalbumin and 520 000 cpm ^{125}I. The mixture was applied to a 15 × 1.0 cm I.D. column and eluted at 4 ml/h with 25 m*M* triethylamine–HCl, pH 11.0. Fractions (0.5 ml) were collected. (■) Elution of proteins; (●) radioactivity. When utilized to prepare pituitary extracts for chromatofocusing, the void-volume peak containing the proteins as judged by the absorbance at 280 nm was pooled (denoted by the bar).

Fig. 2. Utilization of ion-retardation chromatography to remove the ionic constituents from the proteins in a 1.0-ml aliquot of pituitary extract supplemented with 2 mg cytochrome *c* and 5 mg ovalbumin. The 40 × 1.0 cm I.D. column of AG11A8 (Bio-Rad) was eluted at 14 ml/h with water and 1.0-ml fractions were collected. All of the immunoreactive oLH (●) co-eluted with proteins as judged by the absorbance at 280 nm (■). The void-volume material was pooled, lyophilized, dissolved in triethylamine and chromatofocused (lower left-hand panel of Fig. 3). In a separate experiment performed under similar conditions, it was demonstrated that >97% of the ^{125}I was removed from the proteins in a pituitary extract to which ^{125}I was added.

which the ionic components in the pituitary homogenization buffer were separated from the proteins had any effect on the chromatofocusing elution profile of oLH, aliquots of a pool of pituitary extracts were equilibrated in triethylamine by various methods including gel permeation (Fig. 1), ion-retardation chromatography (Fig. 2), dialysis and flow dialysis. In each case, the proteins were eluted or reconstituted in 25 m*M* triethylamine and the removal of ionic components was documented by checking the conductivity. Irrespective of the method of sample preparation, a small percentage of the oLH in each extract flowed through the chromatofocusing columns unrestricted (Fig. 3). However, the percentage of immunoreactive oLH eluting in these flow-through peaks (designated as peak A in Fig. 3) was not consistent.

Because similar results, at least qualitatively, were obtained with multiple methods of sample preparation (Fig. 3) and because the proteins were applied in a buffer approximately equal to the ionic strength to the chromatofocusing reagents, at least two possible explanations existed: (1) there were indeed a small percentage of extramely basic forms of oLH in pituitary extracts; or (2) the triethylamine might not be the optimal buffer to load the oLH in a pituitary extract. The latter possibility is tantamount to stating that the chromatofocusing resin had been overloaded under the specific experimental conditions utilized. Thus, a series of experiments was conducted to determine if the columns had, in effect, been overloaded.

Highly purified oLH [8] previously demonstrated to be devoid of extremely basic forms was utilized in one series of experiments designed to examine this possibil-

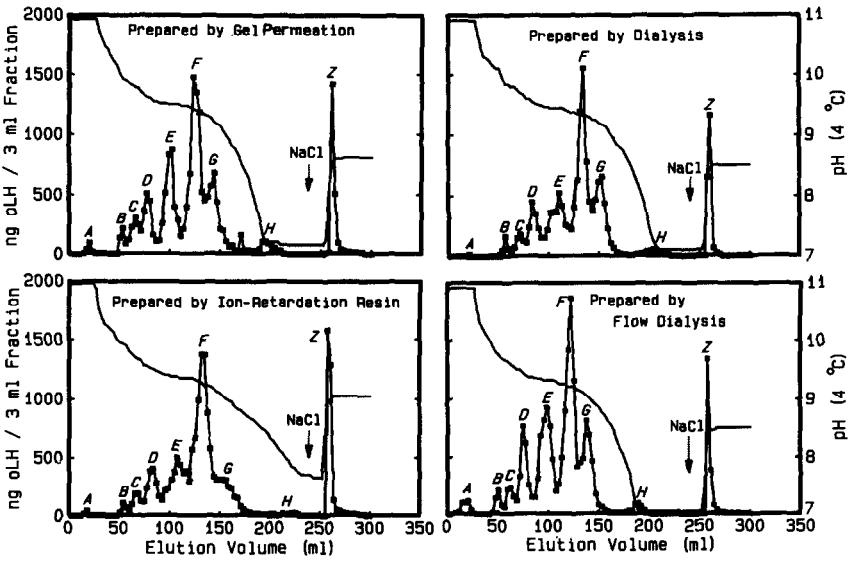


Fig. 3. Chromatofocusing elution profiles of the immunoreactive oLH in aliquots of a pool of pituitary extracts prepared by various methods. In each case, aliquots of a pool of pituitary extracts were supplemented with 2 mg cytochrome *c* and 5 mg ovalbumin. After removal of ionic components by the method indicated, samples were reconstituted in 25 m*M* triethylamine–HCl, pH 11.0, and chromatofocused on pH 10.5–7 gradients. The columns were eluted at 10 ml/h with 1:45 Pharmalyte 8-10.5–HCl, pH 7.0, and 3.0-ml fractions were collected. The oLH concentration of each fraction (■) was quantitated by radioimmunoassay and the pH gradient (solid line) was monitored with a flow-through pH monitor. Materials bound at the lower-limiting pH of 7.0 were eluted with 1 *M* NaCl and coded as peak Z. Other isoforms of oLH are coded with the letters A through H beginning with the most basic form [2,5]. See the text for additional experimental details.

ity. Similar chromatofocusing elution profiles were noted when 20 μg of purified oLH were applied to the columns in 25 m*M* triethylamine–HCl, pH 11.0 (Fig. 4); 1:45 Pharmalyte 8-10.5–HCl, pH 7.0 (Fig. 4); or 25 m*M* Tris–HCl, pH 8.0 (data not illustrated). Furthermore, the chromatofocusing elution profile of purified oLH applied in 25 m*M* NaCl was also similar to that obtained when the oLH was loaded in one of the aforementioned buffers except for an extremely small flow-through peak (Fig. 5). However, when purified oLH was dissolved in the buffer used to extract pituitary tissue (150 m*M* NaCl, 50 m*M* Tris, etc.), all of the oLH flowed through the column unrestricted (data not illustrated), presumably because of the high ionic strength in which the sample was applied. This series of experiments suggested that the buffer in which *small amounts of purified* oLH is applied to the chromatofocusing resin has a minimal effect on the elution profile *if* it is of sufficiently low ionic strength.

In order to further test whether the buffer in which samples were applied to the chromatofocusing resin was an important consideration, aliquots of a single pituitary extract were prepared by gel permeation or flow dialysis and applied to the chromatofocusing columns in either triethylamine or Pharmalyte (Fig. 6). Removal of ionic constituents was again monitored by checking the conductivity of each sample. Irrespective of the method of sample preparation, a small flow-through peak (designated as A in Fig. 6) was observed when the proteins were applied to the chromatofocusing

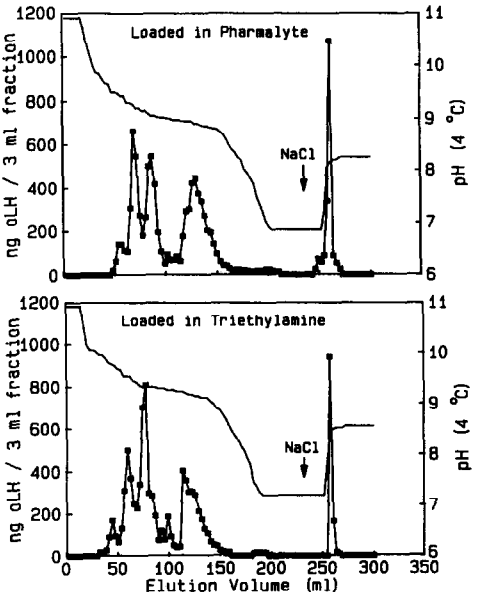


Fig. 4. Elution of 20 μg purified oLH [8] applied in 1:45 Pharmalyte 8-10.5–HCl, pH 7.0 (top panel) or 25 m*M* triethylamine–HCl, pH 11.0 (lower panel) on pH 10.5–7.0 chromatofocusing gradients. Each sample was supplemented with 2 mg cytochrome *c*, 3 mg myoglobin and 4 mg ovalbumin prior to application. Replicate samples dissolved in the above buffers as well as purified oLH dissolved in 25 m*M* Tris, pH 8.0, yielded similar elution profiles (data not illustrated). Additional experimental details are presented in the legend of Fig. 3 and the text.

columns in triethylamine. However, when the proteins in pituitary extracts were loaded in Pharmalyte, no flow-through peaks were observed. Likewise, no flow-through peaks were evident when pituitary extracts were prepared by ultrafiltration and loaded in Pharmalyte (data not illustrated). A small amount (< 1%) of the immunoreactive oLH eluted between the initial drop in the pH gradient and peak B. This peak is coded as peak A′ in Figs. 6–8 to distinguish it from peak A (exclusively used to denote

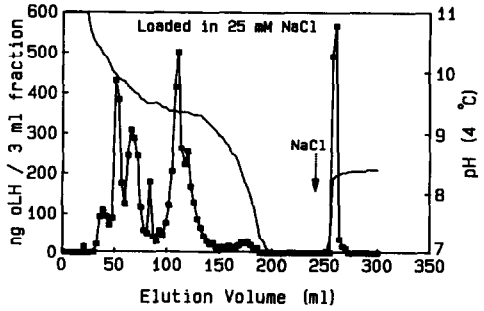


Fig. 5. Elution of 20 μg purified oLH applied in 25 m*M* NaCl on a pH 10.5–7.0 chromatofocusing gradient. See Fig. 4 and the text for remaining experimental details.

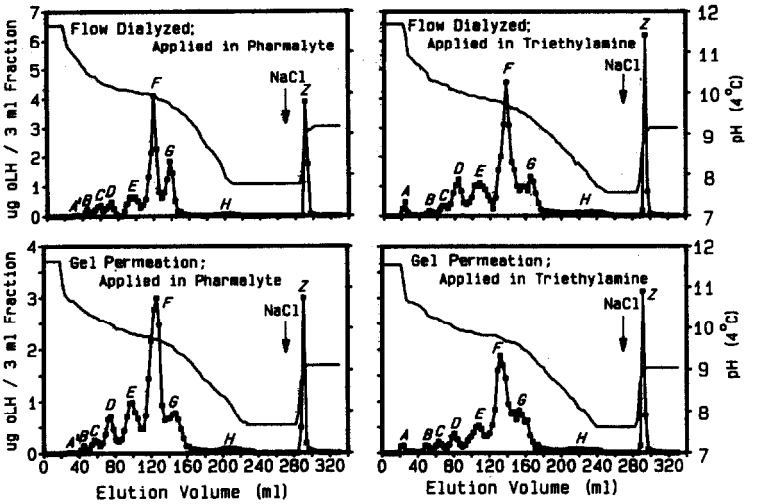


Fig. 6. Chromatofocusing elution profiles of the immunoreactive oLH in aliquots of a pituitary extract prepared by flow dialysis (upper panels) or gel permeation (lower panels) and applied to the resin in 1:45 Pharmalyte 8-10.5–HCl, pH 7.0 (left-hand panels) or 25 m*M* triethylamine–HCl, pH 11.0 (right-hand panels). Note that each sample loaded in triethylamine exhibited a small, but detectable, flow-through peak (denoted as A) but that samples applied in Pharmalyte did not. See Fig. 3 or the text for remaining experimental details.

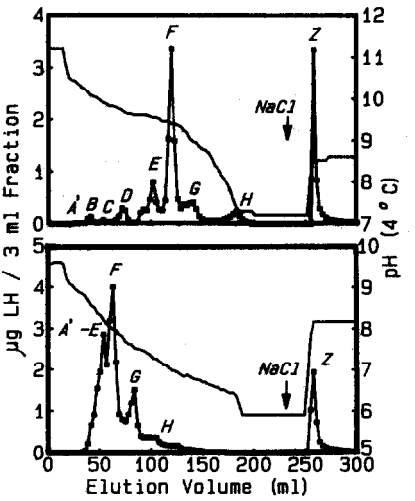


Fig. 7. Chromatofocusing elution profile of the immunoreactive LH in a bovine pituitary extract on pH 10.5–7 (top panel) and pH 9–6 (bottom panel) gradients. Aliquots (1 ml) of a pituitary extract from a steer were desalted by flow dialysis and applied in the corresponding elution buffer. Each peak of immunoreactive bLH was identified with letters beginning with the most basic isoform [2,5,10]. Note that the five most basic forms of bLH (peaks A′ to E) eluted as a shoulder on peak F when chromatofocused on a pH 9–6 gradient. See text for remaining experimental details.

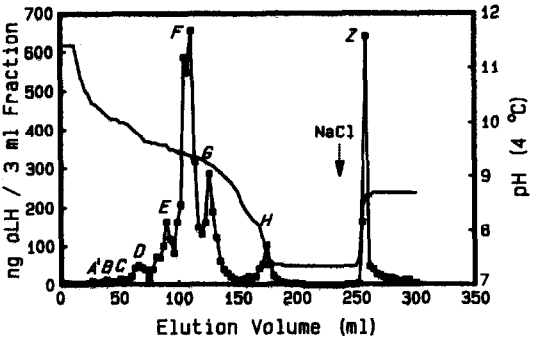


Fig. 8. Chromatofocusing elution profile of the immunoreactive oLH in the pituitary extract of a dihydrotestosterone-implanted wether [2]. Ionic components were removed by flow dialysis and the proteins were reconstituted in Pharmalyte 8-10.5–HCl, pH 7.0. Several other pituitary extracts from rams or androgen-implanted wethers [2] also were devoid of flow-through peaks when prepared by flow dialysis and applied in Pharmalyte (data not illustrated). See Fig. 3 and the text for remaining experimental details.

flow-through peaks). In addition small amounts (typically $\leqslant 3\%$) of the immunoreactive oLH eluted as the pH gradient approached its lower-limiting plateau. Forms eluting in this region are denoted as peak H in the figures. Thus, when pituitary extracts are prepared by dialysis, flow dialysis, ion-retardation chromatography, gel permeation or ultrafiltration and *applied to the chromatofocusing columns in Pharmalyte*, no immunoreactive oLH is observed as a flow-through peak. These results suggest *that the buffer in which the oLH in a pituitary extract* is applied to the chromatofocusing resin has a significant effect on the pattern of oLH isoforms observed. It also appears that the additional proteins in pituitary extracts, *versus* the 20 μg of purified oLH, contribute to the degree of binding even though both types of samples were supplemented with 7–9 mg of exogenous proteins.

The results of these experiments suggested that the PBE 118 resin exhibited a reduced binding capacity when samples were applied in triethylamine. In order to obtain additional information about the characteristics of this resin, a titration curve was prepared (Fig. 9). In the pH 10–7 range, the observed exchange capacity of the

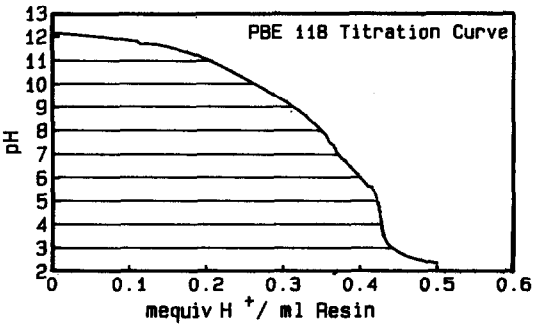


Fig. 9. Titration curve for PBE 118 chromatofocusing resin. A 10-ml volume of resin as a 10% (v/v) suspension in 1 *M* KCl was adjusted to a pH > 12 with 10 *M* NaOH and then titrated with 0.5 *M* HCl. The resulting pH was measured with a Ross pH electrode.

resin was similar to that reported by the manufacturer, *i.e.* $\approx$ 50 μmol pH unit^{-1} ml^{-1}. Nonetheless, the titration curve suggests that the amount of positive charge and hence effective binding capacity of the resin decreased as pH increased. Furthermore, above pH 11, this drop was rather rapid. Under the experimental conditions employed, the pH of the triethylamine was adjusted to 11.0 at room temperature using a Ross electrode. At 4°C, the temperature used for chromatofocusing, the pH of this buffer was approximately 11.4. Furthermore, the microenvironment of an anion exchanger may be one pH unit higher than the surrounding buffer [11]. These considerations plus the experimental observations suggest the PBE 118 chromatofocusing resin exhibits a somewhat limited binding capacity at high pH values. They also explain why proteins bound to the resin more effectively when applied in Pharmalyte at pH 7.0.

Because the above results suggested that ovine pituitary extracts contained lesser amounts of extremely basic forms of oLH than was previously thought, the possibility of substituting pH 9–6 gradients was examined (Fig. 7). Aliquots of a bovine pituitary extract, which exhibited a pattern of isohormones similar to those in ovine pituitary extracts [10,12], were utilized in this experiment. When chromatofocused on a pH 10.5–7 gradient, no bLH was observed as a flow-through peak. Similarly, no flow-through peak was observed on the pH 9–6 gradient. However, it did appear that the five most basic forms of bLH (peaks A′ through E) eluted as a shoulder on the major peak F when the pH 9–6 gradient was used. Thus, it appears that pH gradients starting at a relatively high pH are required to analyze the charge isomers of ovine and bovine LH by chromatofocusing.

In order to further clarify the presence or absence as well as the percentage of extremely basic forms of oLH in the pituitaries of rams and androgen-treated wethers [2], aliquots of samples which previously exhibited significant amounts of oLH in a flow-through peak were re-analyzed under conditions where the sample was loaded in Pharmalyte. A representative chromatofocusing profile is illustrated in Fig. 8. In each case, no immunoreactive oLH was observed as a flow-through peak (A) but a small percentage (typically $<2\%$) eluted as a peak (coded as peak A′) between the flow through and peak B. This suggests that the percentage of extremely basic forms of oLH was previously overestimated [2], in part, because of the specific experimental conditions utilized for sample preparation and chromatofocusing.

DISCUSSION

Chromatofocusing, the chromatographic analog of isoelectric focusing [13], has proven effective in analyzing the charge isomers of the glycoprotein hormones [1–5]. Both the theory and practical aspects of the technique have been described [6,13]. Nonetheless, it has been our experience that the general guidelines [6,13] do not universally apply to all pH gradients and that this is especially true for gradients starting at very basic pH values. The general instructions suggest that proteins may be applied to the chromatofocusing resin in either the "start" or elution buffer [6,13]. While true for most pH gradients [7], this study suggests that the sample application buffer is an important consideration for pH 10.5–7 gradients.

When chromatofocusing was implemented in our laboratory, gel permeation was chosen as a means to equilibrate the proteins in the desired buffer [6]. Triethyla-

mine was chosen over the elution buffer (1:45 Pharmalyte 8-10.5–HCl, pH 7.0) simply because it would be extremely expensive to routinely use diluted Pharmalyte for gel permeation. A column volume of 20 ml, with a stated capacity to handle approximately 200 mg protein [6], was selected because this yielded a theoretical capacity approximately ten-fold larger than the amount of protein to be applied to the columns (including both the endogenous proteins in pituitary extracts and the exogenous proteins added prior to sample preparation). With this approach, some of oLH in pituitary extracts flowed through the columns unrestricted and this was previously interpreted to indicate the presence of extremely basic forms [2]. The results of the present studies suggest that these flow-through peaks *do not* represent extremely basic forms but primarily result from incomplete binding of the hormone to the resin when it is applied in triethylamine. The seemingly contrasting results obtained with purified oLH and oLH in pituitary extracts can be attributed to the quantity of hormone and other basic proteins applied to the columns. Rather than simply using the stated exchange capacity to calculate column volume, it would appear desirable to experimentally determine the binding capacity of the resin at the pH of sample application.

There are also simple alternatives to verify that the separation is being effected as anticipated. For example, the addition of the colored proteins cytochrome *c* and myoglobin, which have isoelectric points of $\approx$ 10.6 and $\approx$ 7.2, respectively, provide a readily visible check on binding of the proteins to the resin. When applied in either triethylamine or Pharmalyte, cytochrome *c* was retarded slightly and eluted a few fractions after the flow-through peak. However, it generally eluted as a broader peak when applied in triethylamine. When samples were applied in Pharmalyte, the myoglobin bound tightly to the top of the column as the sample was applied. However, when applied in triethylamine, the myoglobin frequently migrated a variable distance onto the column before it began to focus. Nonetheless, its elution position was similar in both cases. Interestingly, on pH 10.5–7 gradients myoglobin elutes approximately two pH units above its true isoelectric point [14]. During chromatofocusing proteins elute according to their surface charge characteristics but not necessarily at their isoelectric points [13]. Thus, cytochrome *c* and myoglobin serve primarily to verify binding and establish reproducibility. If oLH applied in triethylamine, similar to myoglobin, migrated variable distances through the column before binding, it is likely that variable amounts of oLH in pituitary extracts simply moved completely through the column without binding and were detected as flow-through peaks.

In subsequent experiments, Grotjan and Zalesky [9] have utilized column volumes as small as 3.0 ml and noted quantitative binding (*i.e.*, no LH observed as a flow-through peak) of the LH in a 0.5-ml aliquot of an ovine or bovine pituitary extract supplemented with 2 mg each of cytochrome *c*, myoglobin and ovalbumin if the sample is applied in Pharmalyte. These observations provide further experimental evidence consistent with the above hypothesis.

In order to further characterize the revised chromatofocusing protocol, the subsequent paper [5] examines the misclassification errors associated with individual peaks for a pituitary extract prepared by flow dialysis and applied in Pharmalyte. It demonstrates that chromatofocusing is an effective method for separating the charge isomers of oLH when utilized under the slightly revised sample preparation and application conditions developed and validated herein. In view of the present findings, were are currently re-assessing the effects of castration and androgen-replacement on the pattern of oLH isohormones.

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REFERENCES

1 B. A. Keel and H. E. Grotjan, Jr., *Microheterogeneity of Glycoprotein Hormones*, CRC Press, Boca Raton, FL, 1989.
2 B. A. Keel, B. D. Schanbacher and H. E. Grotjan, Jr., *Biol. Reprod.*, 36 (1987) 1102.
3 S. C. Chappel, C. L. Bethea and H. G. Spies, *Endocrinology*, 115 (1984) 452.
4 W. F. P. Blum, G. Riegelbauer and D. Gupta, *J. Endocrinol.*, 105 (1985) 17.
5 H. E. Grotjan and D. D. Zalesky, *J. Chromatogr.*, 549 (1991) 153.
6 *Chromatofocusing with Polybuffer and PBE*, Pharmacia/LKB Technical Bulletin, 1980.
7 H. E. Grotjan, unpublished results.
8 O. D. Sherwood, H. J. Grimek and W. H. McShan, *Biochim. Biophys. Acta*, 221 (1970) 87.
9 H. E. Grotjan and D. D. Zalesky, in preparation.
10 D. D. Zalesky and H. E. Grotjan, *Dom. Anim. Endocrinol.*, 8 (1991) 179.
11 R. K. Scopes, *Protein Purification: Principles and Practice*, Springer, New York, 1982.
12 D. D. Zalesky and H. E. Grotjan, *Biol. Reprod.*, 44 (1991) 1016.
13 T. W. Hutchens, in J.-C. Janson and L. Ryden (Editors), *Protein Purification: Principles, High Resolution Methods, and Applications*, VCH, New York, 1989, Ch. 5, p. 149.
14 H. E. Grotjan, S. E. DesJarlais and S. E. Rand, in W. W. Chin and I. Boime (Editors), *Glycoprotein Hormones*, Serono Symposia, USA, Norwell, MA, 1990, Ch. 4, p. 27.