A method has been developed for the purification of equine lutropin (eLH) and equine follitropin (eFSH) from horse pituitary glands which attains high yields of both hormones in contrast to previous methods that were devoted to one or the other with inferior recovery of the hormones. Two-pass chromatography over CM-Sephadex was used to separate eLH from eFSH. Subsequent steps employing QAE (quaternary amine-ethyl)-Sephadex chromatography and gel filtration on Sephacryl S-200 produced highly purified hormone preparations. Yields of purified eLH and eFSH were 110 and 60 mg/kg of frozen pituitaries, respectively. Subunits were prepared by dissociation in 8 M guanidine HCl followed by either gel filtration (eLH) or gel filtration followed by QAE-Sephadex chromatography (eFSH). The hormones and their subunits were characterized by sodium dodecyl sulfate-gel electrophoresis, amino acid analysis, NH$_2$-terminal analysis, and by LH and FSH radioligand receptor assays.

Purification of the equine pituitary gonadotropins has lagged behind purification of those from other species because of a relative scarcity of horse pituitaries and technical difficulties with the purification. Several laboratories have reported purification of either eLH or eFSH or of both gonadotropins (1-5). However, none has succeeded in obtaining high yields of both hormones. Braselton and McShan (1) were the first to prepare highly purified eLH and eFSH. They found the hormones to be difficult to separate and only succeeded in doing so by employing preparative electrophoresis. While this method did achieve separation of the two hormones, it also accounted for the greatest loss of LH activity. Final yields obtained were 16 mg of LH and 26 mg of FSH/kg of frozen pituitaries. Rathnam et al. (2) were able to obtain eFSH in high yield (67 mg/kg); however, they concentrated their efforts on FSH alone. Licht et al. (3) purified both eLH and eFSH, obtaining a high yield of eLH (54 mg/kg), but attended by a very low yield of eFSH (7 mg/kg). They also demonstrated that the rat was an inappropriate assay animal since eLH was found to have intrinsic FSH activity in binding assays and in the Steelman-Pohley assay. These results suggested that one of the problems of resolving LH and FSH activities from horse pituitary extracts was the use of rat-based assays. Combranous and Henge (4) reported a purification procedure for eFSH which produced 13.9 mg of eFSH/kg of pituitaries. Equine LH was not taken into consideration. However, they observed that eFSH readily dissociated into its subunits under slightly acidic conditions. Purification of both eLH and eFSH was later reported by Guillon and Combranous (5). The yield of eLH was 24.2 mg/kg, and the yield of eFSH had been improved over that of the earlier report to 26 mg/kg.

We have undertaken the purification of both eLH and eFSH attempting to develop efficient methods for the purification of both of these hormones in high yield. Our approach has avoided the use of low pH steps and has employed in vitro bioassays that can distinguish eLH from eFSH. In this report we describe procedures to obtain highly purified eLH and eFSH preparations in high yield.

EXPERIMENTAL PROCEDURES

Materials

Frozen horse pituitary glands (Lot S1011) were obtained from Armour. Sephadex and Sephacryl chromatography products were obtained from Pharmacia. Polybrene was obtained from Pierce Chemical Co. All other sequenator reagents were obtained from Beckman. Ovine LH and FSH reference preparations, NIH-LH-S19 and NIAMDD-oFSH-13, were obtained from the Hormone Distribution Officer, National Institute of Arthritis, Metabolism, and Digestive Diseases. All other reagents were reagent grade or the highest purity commercially available.

Methods

Extraction of Horse Pituitary Glands—Frozen horse pituitary glands were extracted by a modified version of the percolation extraction method used by Bates et al. (6) in the purification of human thyrotropin. The glands were extracted by suspension in the various solvents rather than by percolation, and a higher pH was maintained throughout.

All procedures were performed at 4 °C. Two kilograms of frozen glands were soaked overnight in 6.4 liters of alcohol. The glands were homogenized in 1-kg lots by four 15-s bursts at the high setting of a Waring Blender. Assuming a water content of 80% for the glands, the ethanol content of the resulting homogenate was 75%. The homogenate was immediately centrifuged at 6000 x g for 20 min in a Sorvall RC-3 centrifuge. The supernatant was discarded and the pellet resuspended in 4 liters of 75% ethanol, 25% 0.5 M sodium acetate, pH 6.0, and extracted for 2 h. Following centrifugation the supernatant was discarded and the pellet extracted for 2 h in 80% ethanol, 40% 0.5 M sodium acetate, pH 6. The supernatant was saved and the pellet extracted overnight with 4 liters of 50% ethanol containing 1 M NaCl and 0.5 M Tris-acetate, pH 7. This was followed...
by overnight extraction with 40% ethanol containing 1 M NaCl and 0.5 M Tris acetate, pH 7, and concluded with overnight extraction with 40% ethanol containing 1 M NaCl buffered with 0.5 M sodium acetate, pH 5. The supernatants of the 60, 50, and both 40% ethanol extracts were adjusted to 50% ethanol. The resulting precipitates were allowed to settle overnight and removed by centrifugation. The pellets were resuspended in 0.126 M ammonium bicarbonate, to which 2 mM phenylmethylsulfonyl fluoride was added, and dialyzed against distilled water. Following dialysis the insoluble fractions were removed by centrifugation, pooled, and lyophilized. The soluble fractions were lyophilized separately and assayed by rat testis radioligand assay.

**Purification of Equine LH and FSH**—The fractions extracted at pH 7 with 50% ethanol and with 40% ethanol from three 2-kg batches of horse pituitaries were pooled. The 26.7 g of extract was applied to a column (15 x 11 cm) of CM-Sephadex (C-50) equilibrated with 0.605 M sodium phosphate buffer, pH 6.0 (Buffer A). The column was developed with the discontinuous buffer system of Ward et al. (7) at a flow rate of 3.1 ml/h/cm². The unabsorbed fraction, CMS-1A, contained a significant amount of LH and FSH activity. Two inactive fractions were eluted with 0.01 M sodium phosphate, pH 7.0 (Buffer B), and with 0.04 M sodium borate, pH 8.3 (Buffer C). The crude gonadotropin fraction (CMS-1D) was eluted with Buffer D (0.1 M sodium chloride in 0.04 M sodium borate, pH 8.3), and a crude eFSH fraction, CMS-1E, was eluted with Buffer E (1 M sodium chloride in 0.04 M sodium borate, pH 8.3). CMS-1A was lyophilized directly. Both CMS-1D and CMS-1E were concentrated and dialyzed against ultrapure water to a protein concentration of 0.1 M ammonium bicarbonate in 0.1 M Tris-HCL, pH 9.2 at a flow rate of 2.6 ml/h/cm².

Crude eLH (CMS-2D) was eluted with Buffer D while Buffer F eluted a crude eFSH fraction (CMS-2F). CMS-2D was rechromatographed on a CM-Sephadex column (5 x 28 cm) which was developed at a flow rate of 3.1 ml/h/cm² with Buffers A-E. The eLH fraction, CMS-2D, which was eluted with Buffer D, was desalted over a Sephadex G-100 column equilibrated with 0.126 M sodium phosphate buffer, pH 6.0. A portion of the desalted eLH fraction was designated eLH-A, which was eluted with Buffer D, was desalted over a Sephadex G-100 column equilibrated with 0.01 M sodium bicarbonate, to which 2 M phenylmethylsulfonyl fluoride was added, and dialyzed against distilled water. Following dialysis the insoluble fractions were removed by centrifugation, pooled, and lyophilized. The soluble fractions were lyophilized separately and assayed by rat testis radioligand assay.

**Purity of the Initial crude eLH and eFSH fractions determined the complexity of subsequent purification procedures. CMS-2F was applied to a column (5 x 3.9 cm) of QAE-Sephadex (A-50) equilibrated and developed with the ammonium bicarbonate Buffers I-IV used above in the purification of eLH. The fraction eluted with Buffer III yielded 378 mg of highly purified eLH, designated eLH-C. CMS-3E was purified by chromatography on a QAE-Sephadex column (2.5 x 6.5 cm). The Buffer III eluate (QAE-FSH) was chromatographed on the S-200 column (2.5 x 158 cm) yielding 94 mg of purified eFSH-B.

For the crudest eFSH fraction, CMS-1E (see Fig. 1, gel inset), a special procedure was required to remove many contaminants which could not be eliminated by QAE-Sephadex chromatography alone. A column (5 x 5 cm) of the cation exchanger SE-Sephadex (C-50) equilibrated with 0.05 M ammonium bicarbonate was coupled to a column (2.5 x 158 cm) of Sephacyr S-200 equilibrated with 0.126 M ammonium bicarbonate. Two inactive fractions were eluted separately and assayed by rat testis homogenate and crude horse testis membranes (11) or chicken testis homogenate (12) as receptor preparations. Relative potencies were calculated from the ID₅₀ values determined from the inhibition curves (13).

**Analytical Procedures**—Amino acid compositions of equine gonadotropin subunits were determined on samples hydrolyzed in 6 N HCl containing 0.1% phenol for 24 and 72 h. Half-cystine values were determined on performic acid-oxidized or reduced and carboxymethylated samples. The hydrolysates were analyzed on an LKB model 4000 amino acid analyzer equipped with a Shimadzu model C-RIB recording integrator (570-nm channel) and with a Hewlett-Packard model 3390 integrator (440-nm channel). Tryptophan was determined by the method of De Traglia et al. (14). Sialic acid determinations and NH₂-terminal analyses by the dansyl method were carried out as described by Moore and Ward (9). In addition, NH₂-terminal amino acids were determined by automated amino acid sequencer of intact hormone samples or on isolated subunits. 1 mg of intact hormone or 500 μg of subunit were dissolved in 300 μl of distilled water containing 3 mg of Polybrene. The mixture was loaded into the spinning cup of an updated Beckman 800B Sequencer equipped with a liquid nitrogen trap, Sequemat P-6 auto-converter, and Sequemat SC-510 programmer. After drying the sample under
Equine Gonadotropin Purification

Vacuum, three or more sequencer cycles were run using the 1 M Quadrol program 062275, provided by Beckman. PTH derivatives were analyzed by high performance liquid chromatography on a Glenco high performance liquid chromatograph equipped initially with a Hewlett-Packard model 3370A integrator and later with a Hewlett-Packard model 3390 integrator. The isocratic solvent system of Tarr (15% 38% acetonitrile/62% 0.065 M sodium acetate, pH 4.5) was employed on an Altex Ultrasphere ODS column at 55 °C using a flow rate of 0.55 ml/min.

RESULTS

**Extraction of eLH and eFSH from Frozen Horse Pituitaries**

The results of the extraction of frozen horse pituitary glands are summarized in Table I. Preliminary results indicated that only fractions extracted with less than 75% ethanol contained gonadotropin activity. Therefore, only the 60, 50, and both 40% ethanol extracts were tested in rat testis LH and FSH radioligand assays. As can be seen from the table, over 90% of the LH and FSH activities were recovered in the fractions extracted with 50 or 40% ethanol at pH 7. These fractions from extractions of three batches of glands were pooled for CM-Sephadex chromatography.

**Purification of eLH and eFSH**

The overall scheme for the purification of eLH and eFSH is shown in Fig. 1. The procedure consisted of: 1) separation of LH and FSH activities by ion exchange chromatography on CM-Sephadex; 2) removal of contaminants by QAE-Sephadex chromatography followed with 3) final purification by gel filtration (if required).

**Separation of eFSH from eLH by Ion Exchange Chromatography on CM-Sephadex**

The elution profiles and activity recoveries illustrating the separation of LH and FSH activities of pituitary extracts and crude gonadotropin fractions on CM-Sephadex columns are found in Fig. 2. The relative purity of these fractions can be judged by the gel pattern insets in Fig. 1. Table II lists the material and activity recoveries at all stages of the purification. LH activity was determined in the horse testis RLA and FSH activity was determined in the chicken testis RLA.

Fig. 2A shows the results from a chromatograph of 26.66 g of horse pituitary extract on a column (15 x 11 cm) of CM-Sephadex. Over 70% of the LH activity but only half of the FSH activity were retained. The bound activities were recovered in two fractions. CMS-1D was 2.74 g of crude gonadotropin which contained all of the bound LH activity and half of the bound FSH activity. A 5-fold purification of LH and a 2.6-fold purification of FSH were obtained at this step. CMS-1E was 741 mg of protein which contained the remaining FSH activity, enriched 8-fold over the starting material. Similar results were obtained when a second batch of pituitary extract was chromatographed on CM-Sephadex.

The unabsorbed fractions from these two columns had about the same specific activities in LH and FSH as the fractions extracted with 40% ethanol at pH 5 (rat testis radioligand assay). Therefore, these fractions were combined (431 g) and fractionated by ammonium sulfate precipitation. All of the LH and over 90% of the FSH activities were found in the 18.5 g of protein recovered in the 0.55 saturated ammonium sulfate fraction (0.55 SAS). Fig. 2B shows the results of rechromatography of 0.55 SAS on CM-Sephadex (CMS-2). In this case over 90% of the gonadotropic activities were absorbed by the resin and eLH was separated from eFSH. Buffer D eluted 1.08 g of crude eLH (CMS-2D) enriched 8.5-fold for LH activity while having the same FSH specific activity as the starting material (Table II). Equine FSH slowly bled off the column as development continued with Buffer D. Only a small peak of material was eluted with Buffer F. These were pooled and 694 mg of protein recovered. The fraction, CMS-2F, was enriched 15-fold in FSH activity over the starting material.

Fig. 2C shows the elution profile for the rechromatography of CMS-1D on CM-Sephadex (CMS-3). Buffer D eluted 1.98 g of crude eLH (CMS-2D) enriched 8.5-fold for LH activity while having the same FSH specific activity as the starting material (Table II). Equine FSH slowly bled off the column as development continued with Buffer D. Only a small peak of material was eluted with Buffer E. This fraction (CMS-3E) consisted of 269 mg of crude eFSH that was virtually devoid of LH activity and contained 88% of the FSH activity. A 7-fold enrichment over starting material was obtained.

Fig. 2D shows the elution profile for the rechromatography of CMS-2D on CM-Sephadex. Virtually all of gonadotropic activity was recovered in 710 mg of CMS-4D eluted with Buffer D. A 1.4-fold purification of eLH was obtained. There was no change in the FSH specific activity in this fraction. In addition, no FSH was eluted by subsequent development with Buffer D.

**Chromatography of Partially Purified eLH on QAE-Sephadex**

When crude eLH (CMS-3D) was chromatographed on QAE-Sephadex (Fig. 3), LH activity was found in all fractions. About 9% was found in 136 mg of unabsorbed or weakly absorbed protein which bled off as the column was developed with Buffer I. Buffer II eluted 949 mg of crude eLH (QAE-II-LH) containing 70% of the LH activity. The 308 mg of crude eLH (QAE-III-LH) recovered from the peak eluted with Buffer III contained 12%. An additional 2% was found in 252 mg of protein eluted with Buffer IV. Equine LH from fractions I (9%) and IV (2%) was difficult to purify. They will, therefore, not be considered further.

**Gel Filtration of eLH on Sephacryl S-200 and Sephadex G-100**

Fig. 4, top, shows the elution profile for 947 mg of QAE-II-LH on Sephacryl S-200. The cross-hatched area indicates fractions that were pooled to obtain 159 mg of purified eLH (eLH-B). The 255 mg of S200-LH recovered from the shaded area of the chromatogram was 89% eLH. Contaminants from the lower M, peak were readily removed from S200-LH by gel filtration on Sephadex G-100, as can be seen in Fig. 4, bottom. This additional gel filtration step produced 228 mg of eLH-A.

The potencies of eLH-A and eLH-B in horse testis LH radioligand assay were 6.3 and 5.62 times NIH-LH-S19, respectively. Gel filtration of QAE-III-LH on S-200 yielded a...
1914

Equine Gonadotropin Purification

FIG. 1. Summary of the purification of eLH and eFSH. Frozen pituitary glands were extracted and the extracts fractionated by two-pass CM-Sephadex chromatography, supplemented by QAE-Sephadex or SE-Sephadex chromatography followed by gel filtration. For details see under "Methods." The gel insets show selected fractions which were electrophoresed on 12% SDS slab gels.

less active eLH preparation (3.84 x NIH-LH-S19 in horse testis RLA) which from gel scans was estimated to contain about 12% of the 40,000 and 12,000 bands, although an apparent 2.4-fold purification had been obtained.

Scavenging from the pooled unabsorbed fractions and the 40% ethanol, pH 5, extracts as above produced a relatively crude eLH suitably purified for further purification (Figs. 1 and 2) and essentially devoid of FSH activity following re-chromatography on CM-Sephadex. Therefore, the QAE-Sephadex chromatography step was omitted, and 710 mg of
The separation of eLH into its subunits by gel filtration on Sephacryl S-200 following dissociation in guanidine HCl is shown in Fig. 7. Two peaks emerged from the column. SDS-polyacrylamide gel electrophoresis (see Fig. 7, inset) identified the first peak that eluted with a \( V_r/V_o \) of 1.48 as the \( \beta \) subunit and the second peak, which eluted with a \( V_r/V_o \) of 1.79 as the \( \alpha \) subunit. Equine LH and its subunits were compared in both LH and FSH rat testis radioligand receptor assays. The \( \alpha \) subunit was found to have 1.3% the LH activity and 2.7% the FSH activity of intact eLH. The \( \beta \) subunit was slightly more active, having 2.3% the LH and 3.7% the FSH activity of eLH.

**Isolation of eLH Subunits**

Gel filtration of eFSH after overnight incubation in guanidine HCl resulted in a single peak eluting with a \( V_r/V_o \) of 1.67, midway between the positions of the subunits of eLH (see Fig. 7, dashed profile). Fig. 8 shows the elution profile when the subunit fraction was applied to a column of QAE-Sephadex equilibrated with 0.01 M ammonium acetate, pH 5.5. The unabsorbed fraction was identified as the \( \beta \) subunit by SDS-gel electrophoresis (Fig. 8, inset) while the fraction eluted with 0.4 M ammonium acetate was the \( \alpha \) subunit. When eFSH and its subunits were tested in rat testis radioligand assays, the subunits were found to have no detectable LH activity. In the FSH assay, eFSHa was 0.7% as active and eFSH\( \beta \) was 0.2% as active as intact eFSH.

**Activities of Purified Equine Gonadotropins in Radioligand Assays**

The four purified eLH preparations were found to have potencies ranging from 3.84 to 6.3 times NIH-LH-S19 in horse testis radioligand assays (Table II). In the chicken testis radioligand assay using \(^{125}\text{I}-\text{eFSH}\) as radioligand, the eLH preparations' potencies ranged from 1.5 to 6.7 times NIAMDD-oFSH-13. The eFSH preparations' potencies ranged from 116 to 156 times NIAMDD-oFSH-13 in this same assay. In the horse testis LH radioligand assays the eFSH preparations were found to have potencies ranging from <0.004 to 0.05 times NIH-LH-S19.

**Amino Acid Composition**

Preliminary results comparing 24-h hydrolysates of samples from each gonadotropin preparation indicated that the amino acid compositions of the eLH preparations were identical and that those of the eFSH preparations were also identical to each other. Only one preparation of eLH, eFSH, and their subunits was used for more detailed amino acid analysis. These results along with those reported by other workers can be found in Tables III–VII. Our results are very similar to those of other workers. The most frequent differences are seen in the values for threonine, serine, proline, and half-cystine which are difficult amino acids to quantitate. Tryptophan determinations were performed on eFSH, eFSHa, eFSH\( \beta \), and eLH\( \beta \). The absence of tryptophan in eLH\( \beta \) and
Equine Gonadotropin Purification

TABLE I

Recoveries of material and gonadotropin activity per kg of frozen pituitaries

| Fraction* | Weight (mg) | LH potency (× NIH-LH-S19) | LH units | Recovery % | FSH potency (× NIAMDD-oFSH-13) | FSH units | Recovery % |
|-----------|-------------|---------------------------|----------|------------|---------------------------------|-----------|------------|
| Extract   | 4,440       | 0.31                      | 1,380    | 100        | 6.2                             | 27,500    | 100        |
| CMS-1A    | 2,780       | 0.10                      | 278      | 20.2       | 4.8                             | 13,360    | 48.5       |
| CMS-1D    | 457.0       | 1.68                      | 767.8    | 55.7       | 16.4                            | 7,494.8   | 27.2       |
| CMS-1E    | 125.6       | 0.12                      | 14.8     | 1.1        | 49.2                            | 6,061.1   | 22.1       |
| CMS-3D    | 331.3       | 2.06                      | 682.5    | 49.6       | 2.2                             | 728.9     | 2.7        |
| CMS-3E    | 44.8        | 0.04                      | 1.8      | 0.1        | 120.0                           | 5,376.0   | 19.5       |
| 0.55 SAS  | 1,540       | 0.22                      | 339      | 24.6       | 6.4                             | 9,870     | 35.8       |
| CMS-2D    | 131.5       | 1.87                      | 245.9    | 17.9       | 6.4                             | 841.6     | 3.1        |
| CMS-2E    | 57.8        | 0.06                      | 3.5      | 0.3        | 96.0                            | 5,548.8   | 20.1       |
| QAE-II-LH | 158.3       | 2.88                      | 455.9    | 33.1       | 2.4                             | 379.9     | 1.4        |
| QAE-III-LH| 51.3        | 1.63                      | 83.6     | 6.1        | 2.2                             | 112.9     | 0.4        |
| S-200-LH  | 42.5        | 5.47                      | 232.5    | 16.9       | 5.3                             | 225.3     | 0.8        |
| eLH-A     | 38.0        | 6.30                      | 239.4    | 17.4       | 1.5                             | 57.0      | 0.2        |
| eLH-B     | 26.5        | 5.62                      | 148.9    | 10.8       | 5.4                             | 143.1     | 0.5        |
| eLH-C     | 17.8        | 3.84                      | 68.4     | 5.0        | 4.8                             | 85.4      | 0.3        |
| CMS-4D    | 59.2        | 2.88                      | 170.5    | 12.4       | 6.7                             | 396.6     | 1.4        |
| eLH-D     | 28.9        | 4.90                      | 141.6    | 10.3       | 4.6                             | 132.9     | 0.5        |
| SQ-FSH    | 19.4        | 0.05                      | 1.0      | 0.07       | 136.8                           | 2,653.9   | 9.6        |
| QAE-FSH   | 20.5        | 0.01                      | 0.2      | 0.01       | 141.6                           | 2,902.8   | 10.5       |
| eFSH-C    | 31.5        | 0.05                      | 1.6      | 0.1        | 116.4                           | 3,666.8   | 13.3       |
| eFSH-A    | 15.6        | <0.004                    | <0.00    | <0.005     | 156.0                           | 2,450.6   | 8.8        |
| eFSH-B    | 12.6        | 0.01                      | 0.01     | 0.01       | 146.4                           | 1,844.6   | 6.7        |

* Fractions designated are depicted in the flow chart shown in Fig. 1, and the subsequent Figs. 2–6 provide the specific fraction label.

**0.55 saturated ammonium sulfate.**

**NH2-terminal Amino Acid Determination**

For eLH—Phenylalanine, serine, and traces of glycine were consistently observed in NH2-terminal analyses by the dansyl method. Automated sequence analysis of intact eLH showed phenylalanine and serine as the two major PTH derivatives in Cycle 1 (56% and 35% of the total, respectively). Small amounts of glycine (5%) and aspartic acid (4%) were also observed. For eLHβ, only serine was found in Cycle 1. However, for eLHα, three PTH derivatives were observed after the first Edman cycle. The principal amino acid was phenylalanine which comprised 80% of the amino acids detected while aspartic acid (9%) and glycine (11%) comprised the remainder. The same pattern was followed on each successive cycle (see Table VIII); one principal PTH derivative appeared which corresponded to the NH2-terminal amino acid sequence for the α subunit of equine glycoprotein hormones (16), along with two minor PTH derivatives consistent with two subpopulations of α subunits, one which began with the aspartic acid at position 3 and the other beginning with the glycine at position 4. This was particularly evident at Cycle 5 where both minor sequences should be threonine. After allowing for carry over from the preceding step, the only minor PTH derivative detected was threonine.

For eFSH—Phenylalanine and aspartic acid (indicating either aspartic acid or asparagine) were the most prominent amino acids detected in the NH2-terminal analysis by the dansyl method. Traces of threonine, glycine, and in some preparations, traces of lysine, were also detected. Automated sequence analysis was performed only on isolated subunits. For the β subunit, asparagine was found to be the NH2-terminal amino acid. For eFSHβ the NH2-terminal sequencing results were the same as those described above for eLHβ (Table VIII). Phenylalanine was the NH2 terminus for the bulk (83%) of the preparation. The same two subpopulations, one starting with the aspartic acid at position 3 and the other starting with the glycine at position 4, were observed. Significantly, there was no evidence for a subpopulation whose presence in eFSH has been demonstrated previously by a spectrophotometric method (16). The small amount of tryptophan detected in eLHβ and in eFSHβ (0.2 and 0.3 mol of Trp/mol of protein) by the radiometric method probably represents nonspecific incorporation of the labeled 3-diazonium-1,2,4-triazole. The tryptophan determinations for eFSH and eFSHβ resulted in estimates of 1.5 and 1.2 mol of Trp/mol of protein, respectively. The results are consistent with the presence of 1 residue of tryptophan in eFSH, which is located in the β subunit as demonstrated by Fujiki et al. (17).
sequence began with the lysine at position 15, the NH₂ terminus proposed for eFSHα by Rathnam et al. (2). We have previously noted our inability to confirm their reported sequence (18).

Sialic Acid Contents of Equine Gonadotropin Preparations

Equine LH preparations A, B, C, and D contained 5.4, 6.3, 5, and 6% sialic acid, respectively. All of the eFSH preparations contained about 3% sialic acid; range, 2.9–3.2%.

DISCUSSION

On the Extraction of Horse Pituitary Glands—Our first application of a pumped column percolation extraction method (19) gave 1.26 g of protein/kg of frozen glands with over 70% of the total LH and FSH activities. This material readily provided purified eLH and eFSH by ion exchange chromatography on CM-Sephadex followed by gel filtration on Sephacryl S-200. However, the procedure suffered from two major drawbacks; it was very time consuming and the
initial results could not be easily reproduced. Eventually it was found that efficiency of extraction could be increased by stirring the pituitary homogenate in the extraction buffers. This allowed greater activity recoveries but also extracted ~ 1918 procedure, requiring the introduction of additional chromatographic steps as in the present report. The highest yield of protein extracted with the Koenig and King procedure (20) applied to similar glands yet provides as good or better solubilization of activity. Consideration of the Separation of eLH from eFSH on CM-5.5 Sephadex—In our initial purification method (19) virtually all of the gonadotropic activities were absorbed to CM-Sephadex and all but a few per cent were eluted with Buffers D and E. The results of chromatography of the pituitary extracts prepared in this study differ in that significant amounts of LH and especially of FSH were not retained by the resin. In addition the LH fraction, CMS-1D, contained half of the bound eFSH. The crude FSH fraction, CMS-1E, was essentially devoid of LH activity as before. However, it was heavily contaminated with other proteins. All three problems were eventually solved. When the unabsorbed CMS-1A fraction was applied to a second CM-Sephadex column over 90% of the gonadotropic activities were absorbed. We presume absorption to impurities precluded binding on the first column. In addition the LH and FSH activities were well resolved. The LH was in the CMS-2D fraction eluted by Buffer D. The FSH (CMS-2F) began to emerge in the tail of

| Amino acid | This study | Landefeld and McShan (23) | Licht et al. (3) |
|------------|------------|---------------------------|-----------------|
| Aspartic   | 4.6        | 5.6                       | 5.7             |
| Threonine  | 7.8        | 8.0                       | 8.9             |
| Serine     | 8.7        | 9.3                       | 8.6             |
| Glutamic   | 6.8        | 7.4                       | 7.7             |
| Proline    | 12.8       | 10.5                      | 10.6            |
| Glycine    | 4.9        | 6.2                       | 5.4             |
| Alanine    | 8.2        | 8.0                       | 8.2             |
| Half-cystine| 10.0       | 8.0                       | 8.7             |
| Valine     | 4.9        | 5.6                       | 5.6             |
| Methionine | 2.6        | 1.2                       | 0.5             |
| Isoleucine | 5.4        | 4.9                       | 5.3             |
| Leucine    | 5.7        | 6.2                       | 6.3             |
| Tyrosine   | 2.9        | 1.9                       | 2.6             |
| Phenylalanine | 3.5    | 4.3                       | 4.4             |
| Lysine     | 6.6        | 5.6                       | 5.0             |
| Histidine  | 2.4        | 1.9                       | 2.0             |
| Arginine   | 5.9        | 5.6                       | 4.4             |

| Amino acid | This study | Landefeld and McShan (23) | Licht et al. (3) |
|------------|------------|---------------------------|-----------------|
| Aspartic   | 3.9        | 4.2                       | 4.2             |
| Threonine  | 6.8        | 6.8                       | 8.1             |
| Serine     | 9.0        | 9.0                       | 9.7             |
| Glutamic   | 6.1        | 6.1                       | 6.6             |
| Proline    | 16.0       | 14.9                      | 14.9            |
| Glycine    | 5.0        | 5.0                       | 5.5             |
| Alanine    | 9.2        | 9.2                       | 10.4            |
| Half-cystine| 8.0        | 8.9                       | 8.9             |
| Valine     | 5.6        | 5.2                       | 5.2             |
| Methionine | 2.1        | 2.3                       | 1.3             |
| Isoleucine | 5.2        | 5.1                       | 5.1             |
| Leucine    | 7.1        | 6.9                       | 6.9             |
| Tyrosine   | 1.7        | 5.1                       | 5.1             |
| Phenylalanine | 3.1    | 2.9                       | 2.9             |
| Lysine     | 3.0        | 2.7                       | 2.7             |
| Histidine  | 1.4        | 1.5                       | 1.5             |
| Arginine   | 6.8        | 4.7                       | 4.7             |

| Amino acid | This study | Landefeld and McShan (23) |
|------------|------------|---------------------------|
| Aspartic   | 9.1        | 9.5                       |
| Threonine  | 11.0       | 11.2                      |
| Serine     | 5.5        | 7.2                       |
| Glutamic   | 6.6        | 7.3                       |
| Proline    | 6.2        | 5.8                       |
| Glycine    | 6.6        | 7.9                       |
| Alanine    | 6.8        | 6.8                       |
| Half-cystine| 8.9        | 8.6                       |
| Valine     | 6.2        | 6.5                       |
| Methionine | 1.7        | 0.8                       |
| Isoleucine | 4.5        | 4.3                       |
| Leucine    | 4.8        | 5.0                       |
| Tyrosine   | 6.2        | 5.8                       |
| Phenylalanine | 2.8    | 3.2                       |
| Lysine     | 6.2        | 5.9                       |
| Histidine  | 2.7        | 2.7                       |
| Arginine   | 3.2        | 2.5                       |
| Tryptophan | 1.1        | ND                        |

*ND, not determined.
* Determined by radiometric method of DeTraglia et al. (14).
this peak as well as in the following fraction eluted with Buffer F. Since most of the contaminants which plagued the purification of the gonadotropin fraction eluted from the first CM-Sephadex column were already removed, subsequent purification of eLH and eFSH from fractions from CMS-2 was much easier. Rec chromatography of CMS-2D may not have been necessary since separation of eLH from eFSH had already occurred. The FSH fraction CMS-2E was found to be relatively pure so that QAE-Sephadex chromatography was sufficient to complete the purification. In contrast, purification of the gonadotropin fractions eluted from CMS-1 was more difficult because of the contaminating proteins. Rechromatography of crude gonadotropin CMS-3D on a smaller column of CM-Sephadex achieved separation of eLH from eFSH. The LH was eluted as a sharp peak with Buffer D. When the peak began to tail off, FSH activity began to emerge. By changing to Buffer E, the remaining FSH was eluted as a sharp peak. At first the problem with incomplete gonadotropin retention seemed to be due to overloading CMS-1 since the ratio of protein to packed resin was 13.7 mg of protein/ml of packed resin compared to the ratio of 5 mg/ml used in the initial purification method and in subsequent CM-Sephadex chromatography steps during this purification procedure (CMS-2-CMS-4). However, when crude pituitary extract was applied to a CM-Sephadex column at the ratio of protein to resin of 5 mg/ml or 40 mg/ml activity breakthrough was still observed. Thus, this seems to be dependent on interactions between the gonadotropin and contaminating proteins in the extract.

On the QAE-Sephadex Chromatography of eLH—It is difficult to evaluate the utility of QAE-Sephadex in the purification of eLH. It may be useful to remove some contaminants of intermediate size that interfere with the gel filtration step. On the other hand, crude gonadotropin is not rec chromatographed on CM-Sephadex, but rather, is subjected to QAE-Sephadex chromatography directly, the LH fraction (eluted with 0.125 M ammonium bicarbonate) fails to resolve adequately on an S-200 column. The variable results with anion exchange resins appear to be due to sialic acid heterogeneity.

When purified preparations of eLH are fractionated on anion exchangers the fractions eluting with the lowest ionic strength buffers have the lowest sialic acid content while those eluted with higher ionic strength buffers have correspondingly higher sialic acid contents. Therefore, one disadvantage in using QAE-Sephadex has been the separation of eLH into several fractions which must be further purified separately. Another disadvantage is the fact that samples bleed slowly off the column rather than eluting as sharp peaks. For this reason and because better separation of eLH and eFSH could be obtained, volatile buffers were employed in order to simplify recovery of protein from the large volumes of column effluent that were generated.

Use of QAE-Sephadex in the Purification of eFSH—QAE-Sephadex proved to be very useful in the purification of eFSH, possibly because less sialic acid heterogeneity was observed in eFSH preparations. In one only of three columns was a significant amount of FSH activity found in any fraction other than that eluted with 0.2 M ammonium bicarbonate. Moreover, the $M_t = 28,000$ protein found in partially purified eFSH fractions (Fig. 1) which overlaps with eFSH on S-200 column can be removed by QAE-Sephadex.

Final Purification of eLH by Gel Filtration—The final step in the purification of eLH was gel filtration. The basic characteristics of most chromatograms are seen in Fig. 4, top. The eLH fraction emerged first followed by a second larger peak of inactive contaminants, which in some earlier preparations (extracted at pH 5) included eFSH subunits. The shape of the eLH peak depends on the sialic acid heterogeneity of the preparation. Equine LH-B which was recovered from the leading shoulder contained 6.3% sialic acid. $S_{200}$-LH, recovered from the main peak, contained 5.4% sialic acid. Sialic acid has been demonstrated to alter the apparent molecular weights of glycoproteins as determined by gel filtration experiments (21). $S_{200}$-LH contained 11% of a low $M_t$ contaminant which was readily removed by gel filtration on Sephadex G-100 (Fig. 4, bottom). This contaminant may be free $α$ subunit since its amino acid composition is very similar to those of purified eLH and eFSH $α$ subunits, and one of the principal amino acids observed in NH$_2$-terminal analysis was phenylalanine. Characterization at this point is inadequate to make a firm conclusion.

**Gel Filtration of eFSH on Sephacryl S-200—Following**
QAE-Sephadex chromatography the eFSH preparations were highly purified as indicated by SDS-gel electrophoresis (Fig. 1). The purity of these preparations was confirmed by gel filtration since the eFSH emerged as a single major peak, having only small amounts of contaminants emerging immediately before and after the eFSH fraction.

**Biological Assessment of Gonadotropin Purity**—We have used radioligand receptor assays to evaluate cross-contamination of our eLH and eFSH preparations. The potencies of eFSH compared with eLH in the horse testis LR/LRA were less than 1% as active as the eLH preparations. When the FSH activities of eLH preparations were determined with the chicken testis RLA they were found to be 3-4.5% as active as eFSH. This is not due to eFSH contamination but is intrinsic activity of eLH in this FSH assay. Radiocinunnoassay of some eLH preparations (19) indicated less than 1% contamination by eFSH. The chicken testis assay overestimates FSH contamination but is more specific than the rat testes assay and provides a convenient system to follow purification of equine gonadotropins. Chicken testes have several advantages over horse testes. They are easier to obtain, the receptor preparation is easier to prepare, and they have not been as employed.

**Equine Gonadotropin Purification**—Even though the NIH-FSH-S1 was used to separate eFSH subunits after gel filtration to the same size (Ref. 22 and Fig. 6), therefore, QAE-Sephadex chromatography the eFSH preparations were highly purified. Since intact hormone as well as intact hormone as well as our results to date show that eLHα and eFSHα are identical in amino acid sequence (18). Thus, the size differences must reflect differences in carbohydrate. It is interesting that both α subunits appear as relatively compact dark staining bands on SDS gels whereas both β subunit bands are broader and more diffuse, suggesting greater carbohydrate heterogeneity for the latter. The nature of the carbohydrate moieties of equine gonadotropins present an interesting topic for further study.

When the eLHα in the FSH/RLA is contrary to the results reported by Aggarwal et al. (24). According to these authors eLHα retains significant activity (12% that of intact eLH) in the porcine granulosa cell FSH-binding assay and is as active as eLH in inhibiting cyclic AMP production by seminiferous tubule cells from 18-day-old rats. However, the method by which eLH subunits were obtained was not reported and no chemical data on the purity of the subunits were presented. The residual binding activities of LH subunits have been demonstrated by immunological techniques to be due to the presence of intact hormone in the subunit preparation rather than to intrinsic activity of the subunits themselves (25). We have found that eLH remains intact under conditions of low pH that completely dissociate eFSH or eLH. It is entirely possible that the high biological activity of eLHα reported (24) was due to undissociated eLH in their subunit preparation.

**Studies on the NH2-terminal Amino Acids of Equine Gonadotropins**—Our results for the NH2-terminal amino acids of eLH are in agreement with the results of earlier studies (3, 26). We have extended these by determining phenylalanine to be the NH2 terminus for the α-subunit and serine to be the NH2 terminus of the β subunit, as is the case for several other lutropin species as well as for eCG (27). Our results for the NH2-terminal amino acids of eFSH agree with those of Nuti et al. (28). Licht et al. (3) reported phenylalanine as one NH2 terminus for their eFSH preparation, but also reported serine as the other, the same as they had found for eLH. Fujiki et al. (17) reported either aspartic acid or asparagine as the NH2 terminus of eFSHα. We determined that asparagine is the NH2-terminal amino acid of eFSHβ. The same NH2 terminus has been reported for the β-subunit of human, porcine, and ovine FSH (27, 29).

For eFSHα we found phenylalanine as the NH2 terminus; Rathnam et al. (2) reported lysine. Furthermore, their sequence lacks the first 14 amino acids, including two half-cystines, that have been reported in the amino acid sequence determination of eCGα (16). Their bioassay results indicate that they prepared highly active hormone. They interpreted this to mean that the NH2-terminal portion of the α subunit is not important for biological activity. However, their NH2-terminal determinations were performed on isolated subunits, not on intact hormone. It would have been informative to compare results of an NH2-terminal determination on their intact biologically active eFSH. Except for trace amounts of lysine observed in NH2-terminal analysis of the eFSH preparations, we have no evidence for a small subpopulation of α-subunit molecules having an NH2-terminal lysine. At cycle one during automated sequence analysis of the α-subunits of both eLH and eFSH no lysine was detected, nor at cycle 2 was there leucine detectable as would be required by their proposed sequence. In our equine gonadotropin α-subunit preparations we conclude there is no

---

*G. R. Bousfield and D. N. Ward, manuscript in preparation.*
detectable population of α-subunit molecules having an NH₂-terminal sequence as proposed by Rathnam et al. (2).

In conclusion, we have been able to extract and purify both LH and FSH from horse pituitary glands in high yield. Our method is more cumbersome than that we originally reported (19); however, the yield of highly purified eFSH compares very favorably with those of other laboratories, and the yield of eLH is the highest yet reported. After a two-pass chromatography on CM-Sephadex, separation of eLH from eFSH by methods described herein had little cross-contamination. This was probably due to less heterogeneity in the sialic acid content of eFSH. In contrast, QAE-Sephadex was very useful in the purification of eFSH. This was probably due to its tendency to separate LH into subfractions as we used it may be unnecessary for the purification of eFSH.

The hormones produced are highly purified according to SDS-polyacrylamide gel electrophoresis and NH₂-terminal amino acid sequence determination. The subunits prepared and are suitable for chemical analysis including amino acid sequence determination. Equine LH subunits had greater residual activity, which may reflect their resistance to disso-
ciation compared with that of eFSH.

Acknowledgments—We would like to thank Dr. W. T. Moore, Jr. for his help with the tryptophan determinations. The skilled technical assistance of Nancy S. Jones and Mei-Ying Liu is gratefully acknowledged.

REFERENCES
1. Braselton, W. E., Jr., and McShan, W. H. (1970) Arch. Biochem. Biophys. 139, 45–58
2. Rathnam, P., Fuji, Y., Landefeld, T. D., and Saxena, B. B. (1978) J. Biol. Chem. 253, 5355–5362
3. Licht, P., Gallo, A. B., Aggarwal, B. B., Farmer, S. W., Castelino, J. B., and Papkoff, H. (1979) J. Endocrinol. 83, 311–322
4. Combarbous, Y., and Heng, M.-H. (1981) J. Biol. Chem. 256, 9567–9572
5. Guilou, F., and Combarbous, Y. (1983) Biochim. Biophys. Acta 755, 229–236
6. Bates, R. W., Garrison, M. M., and Howard, T. B. (1959) Endocrinology 65, 7–17
7. Ward, D. N., McGregor, R. F., and Griffin, A. C. (1959) Biochim. Biophys. Acta 32, 305–314
8. Laemmli, U. K. (1970) Nature (Lond.) 227, 680–685
9. Moore, W. T., Jr., and Ward, D. N. (1980) J. Biol. Chem. 255, 6923–6929
10. Weber, K., and Osborn, M. (1965) J. Biol. Chem. 244, 4406–4412
11. Moore, W. T., Jr., and Ward, D. N. (1980) J. Biol. Chem. 255, 6930–6936
12. Glenn, S. D., and Ward, D. N. (1981) Biol. Reprod. 24, Suppl. 1, 117A
13. Liu, W.-K., Yang, K.-P., Nakagawa, Y., and Ward, D. N. (1974) J. Biol. Chem. 249, 5544–5550
14. De Traglia, M. C., Brand, J. S., and Tomesko, A. M. (1979) Anal. Biochem. 99, 464–473
15. Tarr, G. E. (1981) Anal. Biochem. 111, 27–33
16. Ward, D. N., Moore, W. T., Jr., and Burleigh, B. D. (1982) J. Protein Chem. 1, 263–266
17. Fuji, Y., Rathnam, P., and Saxena, B. B. (1978) J. Biol. Chem. 253, 5363–5368
18. Bousfield, G. R., and Ward, D. N. (1982) Program and Abstracts of the 64th Annual Meeting of the Endocrine Society, San Francisco, (Abstr. 197)
19. Teng, C. T., Teng, C.-S., Bousfield, G. R., Liu, W.-K., and Ward, D. N. (1982) Gen. Comp. Endocrinol. 48, 325–332
20. Koenig, V. L., and King, E. (1980) Arch. Biochem. Biophys. 26, 219–229
21. Alhadef, J. A. (1978) Biochim. J. 173, 315–319
22. Landefeld, T. D., and McShan, W. H. (1974) J. Biol. Chem. 249, 3527–3531
23. Landefeld, T. D., and McShan, W. H. (1974) Biochemistry 13, 1393–1393
24. Aggarwal, B. B., Papkoff, H., and Licht, P. (1981) Endocrinology 106, 2460–2469
25. Williams, J. F., Davies, T. F., Catt, K. J., and Pierce, J. G. (1980) Endocrinology 106, 1353–1359
26. Landefeld, T. D., Grimek, H. J., and McShan, W. H. (1972) Biochem. Biophys. Res. Commun. 46, 463–469
27. Ward, D. N., and Moore, W. T., Jr. (1978) in Animal Models for Research on Contraception and Fertility (Alexander, N. J., ed) pp. 151–161, Harper and Row, Hagerstown
28. Nuti, L. C., Grimek, H. J., Braselton, W. E., Jr., and McShan, W. H. (1972) Endocrinology 91, 1418–1422
29. Saxena, B. B., and Rathnam, P. (1976) J. Biol. Chem. 251, 993–1005
