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Equine Luteinizing Hormone and Its Subunits. Isolation and Physicochemical Properties†

Thomas D. Landefeld*,‡ and W. H. McShan

ABSTRACT: Equine luteinizing hormone (LH) was dissociated into subunits by incubation with 8 M urea. These subunits were separated by chromatography on DEAE-Sephadex A-50 and isolated by gel filtration on Sephadex G-100. The subunits, α and β, proved dissimilar as evidenced by disc electrophoresis, molecular weights, amino acid, and carbohydrate contents. The molecular weight of the α subunit was 12,500 while that of the β subunit was 23,000. The α subunit was rich in threonine, glutamic acid, and half-cystine residues, whereas the β subunit showed high amounts of proline, alanine, and half-cystine. The α subunit was shown to be similar in amino acid content to human chorionic gonadotropin-α and human LH-α while the β subunits of these hormones showed differences. The carbohydrate analyses showed that the α subunit was high in mannose whereas galactose was the predominant sugar in the β. The hexosamines were distributed fairly equally between the two subunits. Sialic acid was found in nearly a two to one ratio between the β and α subunits. The β subunit contained 9.47% sialic acid, whereas α had only 4.97%. The electrophoretic patterns of the subunits differed although both indicated heterogeneity. The α subunit exhibited three distinct bands. The β subunit migrated much further indicating a greater negativity and appeared as a broader more diffuse zone than the α. The individual subunits exhibited very low biological activity. Upon recombination, the activity was increased significantly, showing a recovery of 50% of the original LH activity.

Luteinizing hormone (LH)[1] from several species has been shown to consist of two nonidentical subunits. These species are ovine (Liu *et al.*, 1972a,b), bovine (Reichert *et al.*, 1969), porcine (Hennen *et al.*, 1971) and human (Rathnam and Saxena, 1971; Bishop and Ryan, 1973). The hormones HCG (Swaminathan and Bahl, 1970), FSH (Saxena and Rathnam, 1971; Papkoff and Ekblad, 1970), PMSG (Gospodarowicz, 1972), and TSH (Pierce and Liao, 1970; Pierce, 1971) have also been shown to consist of subunits. The subunits of these gonadotropins have been compared and the α subunits were found to be similar. The β subunits have been shown to differ and possess the biological specificity of the hormone.

Equine LH, which has been previously characterized in this laboratory (Braselton and McShan, 1970; Landefeld *et al.*, 1972), is shown in this study to consist of two dissimilar subunits. These subunits have been characterized as to their physicochemical properties and these properties were compared to those of the intact hormone. A comparison of equine LH subunits is also made with human LH and HCG subunits. The similarities and differences are examined and studied in this report.

† From the Endocrinology-Reproductive Physiology Program, University of Wisconsin, Madison, Wisconsin 53706. *Received October 29, 1973.* Supported by the U. S. Public Health Service Training Grant 5-T01-HD-00104-09 from The National Institutes of Child Health and Human Development and The Ford Foundation Grant 630-0505A.

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[1] Abbreviations used are: LH, luteinizing hormone; HCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; PMSG, pregnant mare serum gonadotropin; TSH, thyroid-stimulating hormone.

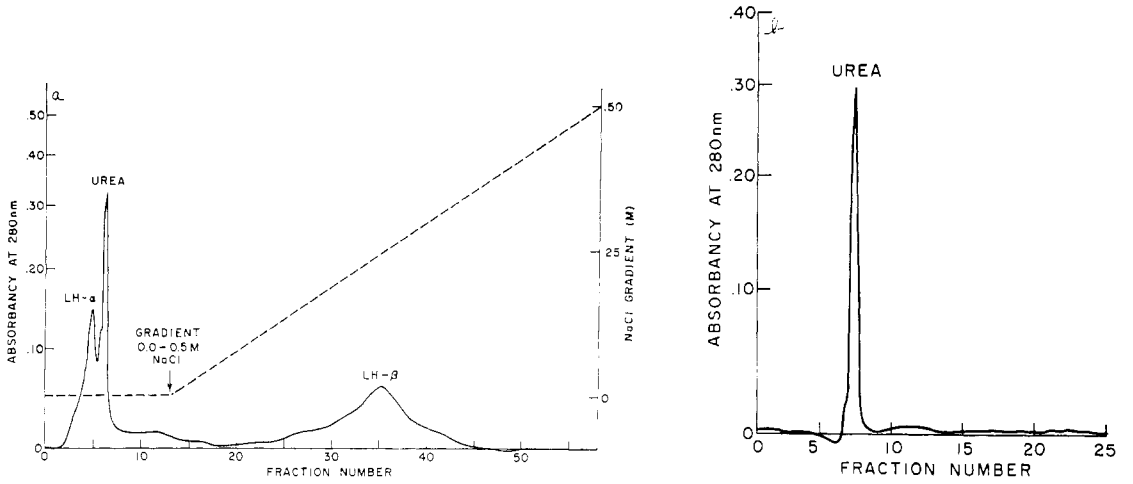

FIGURE 1: Ion-exchange chromatography on DEAE-Sephadex A-50: (a) of 12 mg of urea-treated LH; (b) of 8 M urea. A linear salt gradient from 0.0 to 0.5 M NaCl (100-ml total) was employed at a flow rate of 12 ml/hr.

Materials and Methods

Isolation of Native Hormone. The equine LH was prepared as described by Braselton and McShan (1970) with the minor modifications as mentioned by Landefeld *et al.* (1972). The preparations were analyzed on the basis of dry weight.

Isolation of LH Subunits. The equine LH was incubated in 0.04 M Tris–phosphoric acid buffer (pH 7.5) containing 8 M urea at 37° for 20 hr. The urea-treated LH was then chromatographed on a 1.1 × 15 cm column of DEAE-Sephadex A-50 equilibrated with 0.04 M Tris–phosphoric acid buffer (pH 7.5). The column was eluted with the same Tris–phosphate buffer using a linear gradient from 0.0 to 0.5 M NaCl. A flow rate of 12 ml/hr was employed and four tubes were collected per hour. The tubes for the individual subunits were pooled. Each subunit was subsequently purified on a 1.4 × 100 cm column of Sephadex G-100 equilibrated with 0.05 M ammonium acetate (pH 6.9). Owing to the high concentration of salt in the β subunit, it was partially desalted by ultrafiltration using a Diaflo UM-2 membrane (Amicon, Lexington, Mass.) before application to the G-100 column. A flow rate of 12 ml/hr was used and fractions were collected at 15-min intervals.

Amino Acid Analysis. Samples (100 μg) of equine LH and its subunits were dissolved in 0.5 ml of 6 N HCl, sealed under vacuum in Pyrex glass tubes, and hydrolyzed at 110° for 22, 48, and 72 hr. The hydrolysates were dried *in vacuo* over concentrated H_2SO_4 and KOH at 0°. They were dissolved in a small volume of water and lyophilized to remove residual HCl. The analysis was performed according to Spackman *et al.* (1958) on a Beckman 120C amino acid analyzer equipped with the expanded range bridge. Amino acid decomposition during hydrolysis was accounted for by extrapolations to zero-time values as described by Hirs *et al.* (1954).

Molecular Weight Determination. The method of Yphantis (1964) was used for the ultracentrifugation determinations. The runs were made on a Beckman-Spinco Model E analytical centrifuge at 4° for the intact hormone and 20° for the subunits. The samples were dissolved in 0.1 ionic strength, pH 5.0 sodium phosphate buffer and centrifuged at 28,000 rpm for the native hormone and 48,000 and 44,000 rpm for the α and β subunits, respectively. Photographs were taken after 20 hr using a 12-mm Rayleigh interference cell with sapphire windows and analyzed with a Gaertner microcomparator. The partial specific volume of native LH was calculated to be 0.699 ml/g. The subunits, α and β, had values of 0.705 and 0.693, respectively.

Analytical Disc Electrophoresis. Acrylamide disc gel electrophoresis of 200–300 μg of the native hormone, the subunits, and the urea-treated LH was performed at pH 8.9 in 0.04 M Tris–glycine buffer (Davis, 1964). These samples were run at 4 mA/tube. The staining was done with Buffalo Black; destaining by electrophoresis at 2 mA/gel.

Carbohydrate Analysis. Hexosamines were quantitated on a Beckman 120C amino acid analyzer after 4-hr hydrolysis in 4 N HCl at 100°. The sialic acid was determined by the thiobarbituric acid method of Warren (1959) following hydrolysis in 0.1 N H_2SO_4 at 80° for 1 hr. The hexoses and fucose were quantitated by gas–liquid chromatography (glc) following hydrolysis and derivatization by the method of Lehnhardt and Winzler (1968). A 1.83 m × 6.35 mm U-shaped column packed with 60–80 mesh Gas-Chrom Q precoated with 1% ECNSS-M was used (Niedermeier, 1971). The temperature was programmed from 135 to 175° at a rate of 2°/min.

Biological Assay. Luteinizing hormone activity was determined by the ovarian ascorbic acid depletion assay (Parlow, 1961). The results were analyzed by the parallel-line method (Bliss, 1952) adjusting ascorbic acid content to the mean ovarian weight for the entire assay using covariance analysis (Steel and Torrie, 1960). The data were expressed in terms of the NIH-LH-S1 standard.

Reassociation of the Subunits. The subunits were incubated in 0.01 M phosphate buffer (pH 7.0) for 20 hr at 37°. The ratio of α to β was about 1.5 to 1.0 (w/w), respectively. Following incubation, the reassociated hormone was tested for LH activity by the ovarian ascorbic acid depletion assay.

Results and Discussion

Native LH. The equine LH was characterized as to amino acid and carbohydrate contents, molecular weight, electrophoretic mobilities, and biological activity. These results are included in this report as a basis for comparison with the LH subunits.

Subunit Dissociation. As shown in Figure 1a, the α and β subunits of equine LH were successfully separated by chromatography on DEAE-Sephadex A-50. Figure 1b is included to provide an explanation for the "spike peak" which is associated with the α-subunit peak. This, as designated, was the peak obtained when 8 M urea, in the Tris–phosphate buffer, was applied to the column. Since urea normally does not ab-

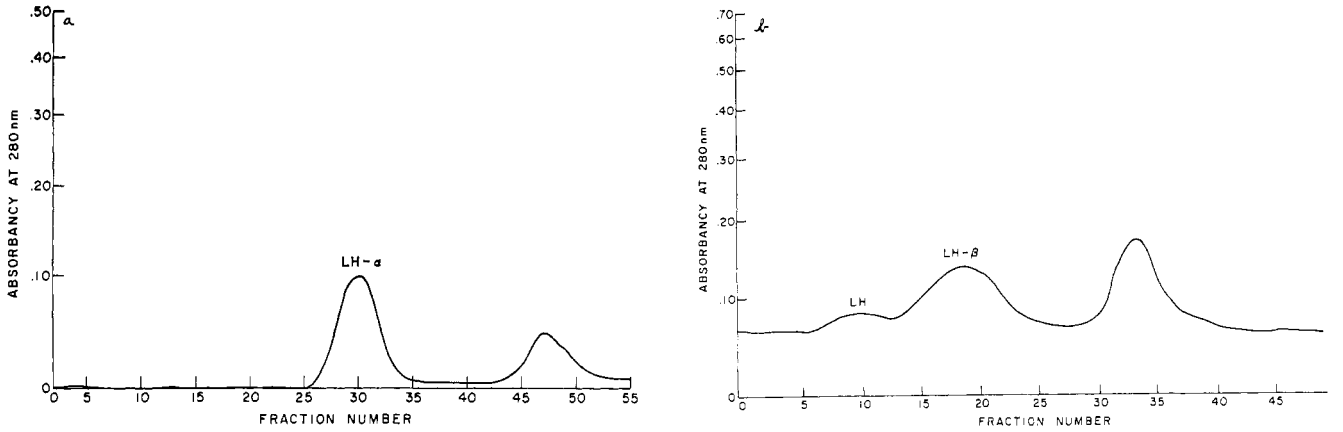

FIGURE 2: Gel filtration of equine LH subunits on Sephadex G-100: (a) LH-α on a 1.4 $\times$ 90 cm column with a void volume of 54 ml; (b) LH-β on a 1.4 $\times$ 85 cm column with a void volume of 54 ml. The flow rate was maintained at 12 ml/hr.

sorb significantly at 280 nm, this peak is believed to be due to the light-scattering effect of the urea in the cuvet. The fractions comprising each peak were pooled and lyophilized.

Subunit Isolation. The individual subunits were each applied to separate Sephadex G-100 columns for purification (Figure 2a,b). The α subunit eluted from the column with a $V_e:V_0$ of 1.80. The gel filtration pattern for the β-subunit purification showed three major peaks. The β subunit eluted with a $V_e:V_0$ of 1.49 with another peak eluting with a $V_e:V_0$ of 1.26 which probably represented either undissociated and/or reassociated equine LH. In both purifications, the final peak was representative of a low molecular weight fraction containing salt as indicated by conductivity readings. These peaks are probably attributable to the mixing and subsequent light-scattering effects of the "salts" in the cuvet, rather than actual absorbance at this wavelength. The $V_e:V_0$ of the salt peaks were 2.53 and 2.60 for the α- and β-subunit purifications, respectively. The fractions representing the subunit peaks were individually pooled, lyophilized, and weighed for analysis.

Amino Acid Analyses. The amino acid analysis of the native equine LH is shown in Table I. The equine LH consisted in part of high levels of proline and serine residues which is not unusual for LH molecules (Rathnam and Saxena, 1970). Threonine, alanine, and half-cystine were also present in substantial amounts.

TABLE I: Amino Acid Analyses of Equine LH and Its Subunits.

Amino Acid Residue	Residues/mol			
	LH-α	LH-β	Expected ($\alpha + \beta$)	LH
Lysine	5	2	7	9
Histidine	2	1	3	3
Arginine	4	5	9	9
Aspartic acid	5	4	9	9
Threonine	7	6	13	13
Serine	5	7	12	15
Glutamic acid	7	6	13	12
Proline	6	13	19	17
Glycine	4	4	8	10
Alanine	5	9	14	13
Half-cystine	7	8	15	13
Valine	4	5	9	9
Methionine	1	1	2	2
Isoleucine	4	4	8	8
Leucine	4	6	10	10
Tyrosine	4	1	5	3
Phenylalanine	4	3	7	7

The results of subunit analyses are also shown in Table I. The α subunit was especially high in threonine, glutamic acid, and half-cystine residues whereas the β subunit contained high amounts of proline, alanine, and half-cystine. Thus, Table I shows a composite summary of the amino acid analyses of equine LH and its subunits. The totals of the amino acid contents of the subunits are very similar to native equine LH. The results summarized in Table II compare the amino acid compositions of equine LH, human LH, HCG, and their subunits. The intact hormones show differences in their contents of aspartic acid, proline, alanine, tyrosine, and phenylalanine. The differences evident in the subunits of these hormones follow the well-established pattern of similarities among α subunits and disparities in the β subunit (Pierce, 1971; Rathnam and Saxena, 1971). In fact, the α subunits of HCG and human LH have been shown to be identical (Pierce, 1971), thus the differences observed in Table II probably represent variation between laboratories and not real differences. In this study, the differences in the β subunits are certainly not great since the three hormones are very similar in action, *i.e.*, HCG exhibits the LH activity of the other two. This then strengthens the concept that the β subunit is the hormone specific one.

Carbohydrate Analysis. The results of the carbohydrate analyses of equine LH and its subunits using glc are summarized in Table III. The values for the native hormone agree quite well with those reported in the earlier study (Landefeld *et al.*, 1972), although the initial results are slightly higher. The primary reason for this is most probably the difference in the methodology. Direct colorimetric analysis of an unhydrolyzed glycoprotein often gives high results (Cahill *et al.*, 1968). The α subunit contains a slightly higher amount of total hexoses and fucose than does the β. Mannose is present to a large extent in the α subunit whereas galactose is greater in the β subunit. The β contains about twice as much fucose as does the α subunit. The composition of the individual subunits is compared with the intact hormone (Table III). The results show a favorable comparison of the intact hormone with the totals of the subunits. The analyses of the hexosamines show that these were fairly equally distributed between the two subunits (Table III). The α subunit consisted of 7.32% hexosamines and the β 6.46%.

TABLE II: A Comparison of the Amino Acid Analysis of Equine LH, Human LH, HCG, and Their Subunits.

Amino Acid	g/100 g of Protein								
	HCG[a]	Equine LH	Human[b] LH	HCG-α	Equine LH-α	Human LH-α	HCG-β	Equine LH-β	Human LH-β
Lysine	5.2	6.3	4.4	7.8	7.3	5.3	3.5	3.2	3.9
Histidine	2.4	2.5	2.8	3.9	3.3	3.3	1.1	1.7	3.8
Arginine	9.9	6.6	7.0	4.2	5.6	5.3	10.7	6.8	8.0
Aspartic acid	8.5	5.7	6.5	7.3	6.4	8.1	9.7	4.5	6.8
Threonine	7.2	8.0	6.9	8.0	8.6	7.2	6.6	7.6	5.6
Serine	7.3	7.8	5.2	7.2	5.4	5.4	7.8	7.8	5.3
Glutamic acid	9.8	9.4	9.3	12.7	10.6	10.3	8.9	7.9	8.3
Proline	11.2	9.5	11.7	6.9	6.4	8.2	13.8	13.4	14.7
Glycine	2.9	3.6	3.0	2.5	2.8	2.7	3.3	2.9	2.6
Alanine	3.7	5.6	3.5	3.3	4.2	3.6	3.9	6.8	4.1
Half-cystine	8.3	7.5	5.7	9.4	7.9	7.6	7.4	8.4	7.9
Valine	6.9	5.2	5.3	7.0	5.3	8.2	6.6	4.8	5.8
Methionine	2.1	1.9	4.1	3.0	1.3	2.2	0.4	1.6	2.1
Isoleucine	2.3	5.1	5.2	0.9	5.7	4.9	2.9	5.3	5.2
Leucine	6.9	6.7	5.6	4.0	5.4	5.0	8.4	7.2	6.4
Tyrosine	4.3	2.8	5.2	5.9	6.2	6.0	3.0	2.2	3.3
Phenylalanine	3.6	5.8	4.0	5.8	7.3	5.3	2.2	3.9	4.3

[a] Values are from Swaminathan and Bahl (1970). [b] Values are from Saxena and Rathnam (1971).

The sialic acid determinations indicated that the β subunit contained the greater percentage, 9.47. The α subunit consisted of 4.97%, giving a total of 7.89% for the intact LH. This compares quite favorably with the determination of 7.70% sialic acid in the native LH (Table III). It is possible that this high content of sialic acid in the subunit may play a role in the hormone specificity exhibited by this subunit.

Molecular Weight Determination. The results of the sedimentation equilibrium ultracentrifugation analyses of two different preparations of equine LH were found to be 33,500 and 34,800. The apparent molecular weight of the α subunit was 12,500 whereas that of the β was 23,000. The average determined molecular weight of the native LH (34,000) agree favorably with the sum of the subunits (35,500). The values calculated from amino acid analysis differ considerably from the ultracentrifugation studies. The reason for this existing discrepancy may possibly be explained by the polymeric forms of the hormone. It has been previously shown that FSH and LH and their subunits do exist in more than one polymeric form (Saxena and Rathnam, 1971; Papkoff and Ekblad, 1970). Thus it is possible that the aggregated form exists in the ultracentrifugation studies due to the concentrations used (Saxena and Rathnam, 1971) while the monomeric form is present in the amino acid analysis. The determined values strongly support this possibility while more definitive proof will be provided in sequence studies.

Analytical Disc Electrophoresis. The results from electrophoresis are shown in Figure 3. The native LH gave a broad

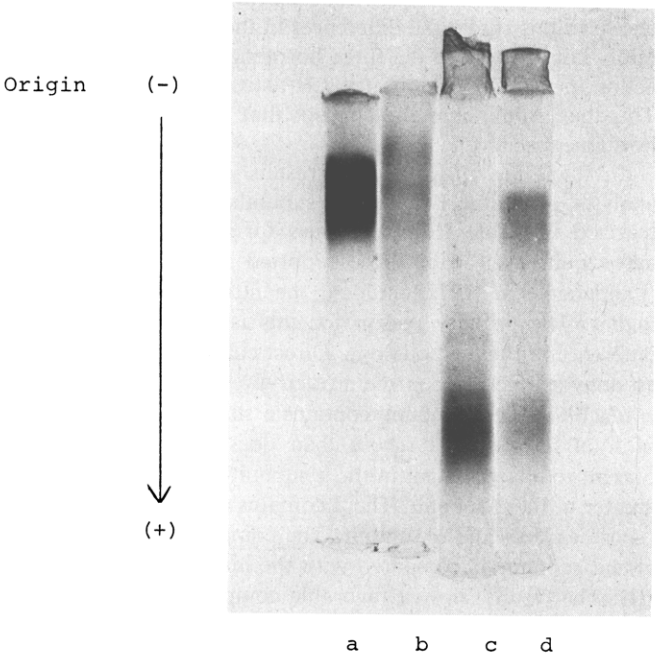

FIGURE 3: Analytical disc electrophoresis with polyacrylamide gel performed at 4 mA/tube in Tris–glycine buffer (pH 8.9): (a) LH; (b) LH-α; (c) LH-β; (d) urea-treated LH.

TABLE III: Comparison of Carbohydrate Analyses of Equine LH and Its Subunits.

Carbohydrate	g/100 g of Hormone			
	LH-α	LH-β	Expected ($\alpha + \beta$)	LH
Fucose	0.34[a]	0.67	0.55	0.60
Mannose	5.37	2.62	3.60	4.32
Galactose	1.75	3.65	3.00	3.31
		Totals	7.15	8.23
Glucosamine	5.73[b]	4.82	5.14	4.72
Galactosamine	1.59	1.64	1.62	2.90
		Totals	6.76	7.62
Sialic acid	4.97[b]	9.47	7.89	7.70

[a] Values represent triplicate determinations. [b] Values represent duplicate determinations.

zone (Figure 3a) which is comparable to that for equine LH reported by Braselton and McShan (1970).

The urea-treated LH showed a rather diffuse pattern in which one band was present at the top and a broader diffuse zone near the bottom (Figure 3d). This pattern became significant when observing the α and β subunits, individually. The α subunit appeared to correspond with the band near the top of the gel in the urea-treated LH (Figure 3b). It consisted of three separate zones. The diffuse pattern near the bottom corresponded to the β subunit (Figure 3c). The similarities of these four gels are evident. The recombined α and β subunits gave a pattern essentially indistinguishable from intact LH.

Biological Studies. The yields and biological activities of the native LH and its subunits were determined. From a total of 12.0 mg of LH used in one dissociation trial, 4.4 mg of α and 5.8 mg of β were recovered after the final purifications steps. The relative potency of the β subunit was higher than that of the α and this finding is in agreement with the results in the literature that show the β as the biologically active one (Papkoff and Gan, 1970; Rathnam and Saxena, 1971; Saxena and Rathnam, 1971; Papkoff and Ekblad, 1970; Pierce, 1971). But it must be noted that even though the β subunit is more active, its relative potency is only one-fifth that of NIH-LH-S1. This is relatively inactive when compared to the native LH activity of approximately 6.2X NIH-LH-S1. The α subunit had a relative potency of less than one-tenth NIH-LH-S1. (These subunits were assayed at levels in excess of 40 times that of the highest dose level of native LH.) When recombination of the subunits was done, 50% of the original activity was recovered.

In conclusion, the LH from equine pituitary glands was dissociated into two nonidentical subunits by treatment with 8 M urea. They were separated by chromatography on DEAE-Sephadex A-50 and further purified by gel filtration on Sephadex G-100. The dissimilarity between the subunits was observed in the amino acid and carbohydrate compositions, molecular weights, electrophoretic patterns, as well as the biological potencies. These results should provide a basis for future structural characterization studies on this hormone.

Acknowledgments

The authors express their gratitude to Mr. H. J. Grimek for his assistance in the carbohydrate analyses, to Dr. Jack E. Lilien and Mr. Donald O'Brien for their advice and help in the glc studies, and to Dr. James E. Blair for the molecular weight determinations.

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