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Human Luteinizing Hormone and Its Subunits. Physical and Chemical Characterization†

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ABSTRACT: Human luteinizing hormone, isolated and purified from human pituitary glands, has been separated into its constituent α and β subunits. The molecular weights of the native hormone and the subunits have been determined by the combined methods of density gradient centrifugation and analytical gel chromatography and compared to molecular weights derived from sedimentation equilibrium experiments. The agreement between the methods is good and furthermore the molecular weights obtained are consistent with the present knowledge of primary structure, amino acid composition, and carbohydrate content of the parent hormone and its subunits. The experimentally determined intrinsic viscosity, sedimentation coefficient, and partial specific volume of the native hormone are used to compute a molecular weight which agrees with those obtained by the other methods. The measured $\bar{V}$ agrees reasonably well with a $\bar{V}$ calculated from chemical composition. The viscometry indicates that the native hormone behaves as a relatively compact equivalent hydrodynamic particle of moderate asymmetry. The ultraviolet absorption spectra of native hormone and subunits have been determined and a difference spectrum derived from them. The difference spectrum is interpreted as indicating possible changes in environment of aromatic side chains when the α and β subunits combine to form the native hormone.

In the past few years, rapid progress has been made in elucidating the properties and primary structure of ovine and bovine LH[1] (Liu *et al.*, 1970, 1971; Papkoff *et al.*, 1971; Pierce *et al.*, 1971; Maghuin-Rogister *et al.*, 1971) and the related hormone, human chorionic gonadotropin (HCG) (Bahl *et al.*, 1972; Morgan and Canfield, 1972). All of these preparations consist of two nonidentical subunits, which are associated by noncovalent bonds.

Human LH has been shown to consist of noncovalently linked subunits (Ryan, 1968, 1969) and more recently these subunits have been separated and shown to differ in their amino acid and carbohydrate compositions (Hartree *et al.*, 1971; Closset *et al.*, 1972; Saxena and Rathman, 1971). The amino acid sequence of the α subunit has now been reported by Sairam *et al.* (1972) and the β subunit sequence by Closset *et al.* (1973) and by Shome and Parlow (1973). There has not been, however, a comprehensive study of the hydrodynamic properties of human LH and its subunits and an effort to compare them with chemical composition. This report is intended to provide some of these data.

At the present time, nothing is known concerning the mutual interactions responsible for the crucial fact that the biological activities of the intact native pituitary glycoprotein hormones are completely obliterated when the subunits are separated. With the further finding that one subunit (approximately one-half the mass of the native molecule) is constant or nearly so in primary structure through the series TSH, FSH, and LH, it appears that interesting information of a general sort might be gained concerning the various modes of noncovalent subunit–

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[1] Abbreviations used are: LH, luteinizing hormone; HCG, human chorionic gonadotropin; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; CMC, carboxymethylcellulose.

subunit interaction responsible for conferring biologic activity. We report here some observations for human LH concerned with this important problem.

Material and Methods

Preparation and Purification of Native Hormone. The starting material for this work was a dried glycoprotein extract from acetone dried human pituitary glands prepared by the method of Hartree (1966) and was a gift from the National Pituitary Agency. This extract was fractionated into its human FSH, TSH, and LH components by a combination of the procedures described by Hartree (1966) and Ryan (1968). The fraction called DEAE-I by Hartree, which is the purified native human LH, was carried through a final Sephadex G-150 filtration, dialyzed *vs.* water at 4° and then lyophilized.

Preparation of Subunits of the Native Hormone. The method used for subunit preparation was a modified version of the urea incubation described by Nureddin *et al.* (1971). Native hormone (182 mg) was dissolved in 6.0 ml of unbuffered 9.0 M urea and incubated at room temperature for 16 hr. The urea solution used for the incubation had been passed through a long column of the mixed ion exchanger Rexyn-300 (H–OH) (Fisher product) immediately before introduction of the protein.

A 2.5 cm × 12 cm carboxymethylcellulose column was brought to pH 5.5 and then equilibrated with 0.004 M ammonium acetate (pH 5.5) buffer. Next, it was equilibrated with a 0.004 M ammonium acetate (pH 5.5) buffer which was made 4.5 M in urea by the proper dilution of an unbuffered 9.0 M urea solution which had just been through the Rexyn column.

The protein–urea incubation mixture was applied to the column followed by the ammonium acetate (4.5 M)–urea solution and the elution pattern was monitored spectrophotometrically at 280 nm (Figure 1a). An unretarded fraction, CMC-U-I was collected, frozen, and lyophilized. The urea containing running buffer was washed out of the column with 0.004 M ammonium acetate (pH 5.5) buffer. No 280-nm absorbing material was eluted from the column. A 0.004 M ammonium acetate (pH 5.5)–0.15 M NaCl solution was then started and a second peak, CMC-U-II, was obtained and also freeze-dried. No further 280-nm absorbing material was eluted from the column when the NaCl concentration in the running buffer was raised to 1.0 M.

The freeze-dried fraction CMC-U-I was redissolved in 0.10 M ammonium bicarbonate and placed on a 2.5 cm × 35 cm Bio-Gel P-30 column and the column was developed with 0.10 M ammonium bicarbonate solution. The early, protein-containing peak was well separated from the urea- and acetate-containing peak and was lyophilized directly from the bicarbonate solution. The yield from the CMC-U-I fraction was 35 mg and was assumed to be the α subunit.

Fraction CMC-U-II was dissolved in a 0.10 M ammonium bicarbonate–5% ethanol solution and placed on a 2.6 cm × 92 cm Sephadex G-100 column which was run in the bicarbonate–ethanol solvent. The protein-containing fractions from this calibrated column were clearly two overlapping peaks of native and subunit sized material. The appropriate fractions from this column were combined with the aim of depleting the subunit peak of the contaminating native hormone. The fractions were freeze-dried and the subunit fraction redissolved in the bicarbonate–ethanol solution and allowed to stand at room temperature for 2 hr before rerunning it through the G-100 column. On this second run the native peak was well reduced relative to the subunit peak and again the appropriate

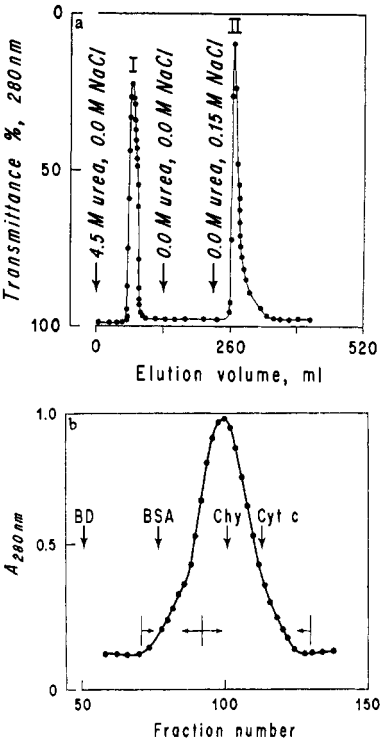

FIGURE 1: Preparation of subunits of human LH. (a) Elution profile of urea-treated hLH run on carboxymethylcellulose at room temperature. Buffer was 0.004 M ammonium acetate (pH 5.50) and other constituents added to this as indicated in the figure. Fractions CMC-UI and CMC-UII were each frozen and lyophilized. (b) Elution profile of the third G-100 column (2.5 × 92 cm) of CMC-UII. The jacketed column operated at 8° and the fraction size was 3.2 ml. The solvent composition was 5% ethanol in 0.10 M ammonium bicarbonate. Horizontal arrows show fractions combined for final lyophilization. Vertical arrows are elution positions of standard markers: Blue Dextran (BD), bovine serum albumin (BSA), chymotrypsinogen (Chy), and cytochrome *c* (CytC).

cuts were made and the fractions lyophilized. The subunit-containing fraction was redissolved and incubated once more in the bicarbonate–ethanol solution before rechromatographing it on the G-100 column. The results of this third G-100 column are shown in Figure 1b where the native hormone appears as a small shoulder on the subunit peak. The indicated fractions were combined and lyophilized. The combined side fractions from the G-100 runs totaled 80 mg and the final subunit-sized fraction yield was 12 mg and was assumed to be the β subunit. Therefore, the total mass recovery from the method described was 70% with about 40% of the theoretical α and about 13% of the theoretical β yields obtained.

A second batch of β subunit (DEAE-SU-I) was prepared by the method of Closset *et al.* (1972). LH (85 mg) was incubated at room temperature in 10 ml of 8.5 M urea (Rexyn-treated)–0.05 M HCl. After 24 hr the pH was adjusted to 9.5 with 1 M NaOH and the solution immediately diluted to 40 ml with 0.01 M glycine buffer (pH 9.5). This was applied to a 2.5 × 10 cm column of DEAE-Sephadex A-25 equilibrated and developed with the same glycine buffer. The unadsorbed fraction was collected, concentrated, and gel filtered through a Sephadex G-100 column developed with 0.1 M ammonium bicarbonate. A single symmetrical peak of subunit size was obtained. Fractions were pooled and lyophilized and yielded 8.3 mg of weight.

Assay Procedures. Biological activity was measured in an *in vitro* receptor assay relying upon competition between ^{125}I-

labeled human LH and unlabeled hormone for binding to a particulate fraction of homogenized pseudopregnant rat ovaries as described by Lee and Ryan (1972). The specificity of the assay and the lack of activity of subunit preparations were previously reported (Lee and Ryan, 1971a,b). Native LH was also assayed radioimmunologically using the assays of Faiman and Ryan (1967a,b).

Purification of subunits was monitored by specific radioimmunoassays. LHα was assayed using an antiserum (Sa 6) against HCGα prepared and donated to us by Dr. Judith Vaitukaitis of the National Institutes of Health. HCGα, a gift of Dr. Robert Canfield, Columbia University, was used for radioiodination. The specificity of this system has previously been reported by Vaitukaitis *et al.* (1971). The assay for LHβ utilized an antiserum against human LHβ prepared by a modification (Lee and Ryan, 1971b) of the countercurrent distribution system of Papkoff and Samy (1967). HCGβ (a gift of Dr. Robert Canfield) was used as the radioiodinated tracer and as the standard. The details of the human LHβ system will be reported separately (Prentice, L. G., and Ryan, R. J., unpublished data).

Radioiodination was done by the method of Greenwood *et al.* (1963).

Viscometry of Native Hormone. The procedures followed in these experiments were essentially those described by Schachman (1957). The outflow times of protein solutions of stepwise decreasing concentration were measured in a Cannon-Ubbelohde seimimicro dilution type viscometer. Weighed amounts of the purified hormone which had been stored at room temperature overnight in a vacuum chamber containing P_2O_5 were dissolved in volumes of 1.2–1.4 ml of a buffer solution whose composition was 0.20 M NaCl–0.01 M phosphate (pH 7.5). This buffer solution was also employed as the reference liquid in the viscometer. Immediately before introduction of each protein solution into the viscometer, the solution was passed through a Millipore filter of pore size 0.45 μ. A Beckman thermometer suspended in the constant temperature bath which held the viscometer indicated temperature control to within $\pm 0.015°$. As the outflow times of these solutions were above 120 sec, the kinetic energy corrections to the data were neglected. In the absence of systematic density data for the protein in this solvent, the procedure recommended by Schachman which assumes additivity of weights and volumes of protein and solvent and a density of 1.35 g/ml for dry protein was used to estimate the densities of the various protein solutions.

Partial Specific Volume of Native Hormone. A single density measurement, carried out using a 2-ml pycnometer with water as solvent, was performed at 25° in a constant temperature bath. Hoping to avoid the 110° drying temperature recommended by Schachman (1957) in the concentration determination, we adopted the less than ideal method of simply dissolving in a measured volume of water at 25° a carefully weighed sample of protein which had been stored in the weighing vessel at 25° over P_2O_5 *in vacuo* until constant weight was obtained.

Molecular Weights from Density Gradient Centrifugation and Gel Filtration. The technique given originally by Martin and Ames (1961), as modified by Ryan *et al.* (1971), was used to determine the sedimentation coefficients of native human LH and its subunits. A Spinco Model L2-65 ultracentrifuge with an SW-65 rotor was used to compare the rates of sedimentation of standard proteins and ^{125}I-labeled hormone or hormone subunits. Both proteins, standard and unknown, were layered together over a 5–27% sucrose density gradient and centrifuged for 12 hr at 60,000 rpm at 20°. The standards employed were bovine serum albumin, ovomucoid, and, in some cases, lysozyme. The buffer system used in these experiments was 0.05 M total phosphate (pH 7.5).

A 2.5 cm $\times$ 92 cm column of Sephadex G-100 was calibrated with Blue Dextran 2000, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochome *c*. The column was then used to determine the Stokes radii of native human LH and subunits, applied as ^{125}I-labeled proteins, assuming the validity of the theory developed by Laurent and Killander (1964) which relates the elution volume to the effective molecular radius of standards and unknowns.

The column was jacketed and run at 8° in the upward direction with a constant flow rate maintained by a peristaltic pump. The column was equilibrated with and developed in 0.20 M NaCl–0.01 M phosphate (pH 7.5), as previously reported by Ryan (1969) and Ryan *et al.* (1971).

With sedimentation coefficients and Stokes radii determined in this manner, the molecular weights (M) were estimated from the relation

$$M = \frac{6\pi\eta_0(N)a(s_{20,w})}{(1 - \bar{V}\rho_0)} \quad (1)$$

where $s_{20,w}$, a, and $\bar{V}$ are the sedimentation coefficient, Stokes radius, and partial specific volume, respectively, for the protein in water at 20°, η_0 and ρ_0 are the viscosity and density, respectively, of water at 20°, and N is Avogadro's number.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were done using a Beckman Spinco Model E ultracentrifuge equipped with an RTIC temperature control unit. Interference optics, using sapphire windows for the centerpiece, were employed in the meniscus depletion technique of Yphantis (1964); 270–280 μg of each protein was dissolved in 1.0 ml of 0.10 M NaCl and dialyzed at 4° for 14 hr *vs.* 50 ml of the same solvent whose measured pH was 5.9. The final dialysate was used as the reference solvent in the centrifugation cell. Protein solution or reference solvent (100 μl) and 20 μl of an FC-43 solution gave a 3-mm liquid column height in the double sector centerpiece. The runs were 9–10 hr in duration but two sequential photographs taken 1 hr apart at the end of each run were used to verify that equilibrium had been reached in each case. A Gaertner comparator was used to measure fringe displacements on the photographic plates and the average displacement ($Y - Y_0$) of five fringes was determined.

At each speed photographs were also taken in which the reference solvent was placed in both sides of the double sector centerpiece. Measurements on these photographs were used to correct the experimental fringe displacements in the actual run at that speed. The corrected displacements were plotted as ln $(Y - Y_0)$ *vs.* r^2 where r is the distance from the center of rotation. The speeds shown in Table III are instrument control settings. The angular velocities used in the molecular weight calculations were determined by timing the revolution counter for 15–20 min, and the temperature was taken from the calibration data for the RTIC control unit.

Amino Acid Analysis. Amino acid compositions of the various preparations were determined by hydrolyzing the proteins in sealed evacuated tubes with constant boiling 6 N hydrochloric acid (Pierce) at 110° for 24 or 48 hr. The acid was removed under vacuum with a Buchler-Evapomix before redissolving the hydrolysates in buffer prior to their passage through a Beckman Model 121 automatic amino acid analyzer using two-column methodology. The cystine and methionine contents were determined as cysteic acid and methionine

sulfone in 18-hr hydrolysates of performic acid oxidized samples. Norleucine was used as an internal standard and the compositions reported have been corrected for norleucine recovery.

Reaction with p-Mercuribenzoate. The spectrophotometric method of Boyer (1954), using neutral pH and monitoring the reaction at 250 nm, was used to test for the presence of free sulfhydryl groups.

Spectrophotometric Determination of Tyrosine and Tryptophan. The method given by Goodwin and Morton (1946) in which the absorbance of the protein in 0.10 M NaOH is measured at 294.4 and 280 nm was used to determine the tyrosine and tryptophan contents and their ratio.

Absorption Spectra of Human LH and Subunits. The absorption spectra of the hormone and its subunits were recorded with an American Instrument Co. DW-2 spectrophotometer over the wavelength range 230–330 nm using 1-cm path length quartz cells. The instrument was operated in the split beam scanning mode with the base line for this wavelength range adjusted by a bank of trimpots with solvent in both sample and reference beams. The hormone and subunit preparations used for these spectra were dissolved in 0.01 M ammonium bicarbonate solutions, lyophilized, and then relyophilized from glass distilled water three times before weighing and dissolving in the solvent, 0.20 M NaCl–0.01 M phosphate (total), pH 7.5. The protein concentrations were in the range 500–550 μg/ml. The base line was checked prior to and immediately after each scan.

End Group Analysis. N-Terminal amino acids were identified by the 5-dimethylaminonaphthalene-1-sulfonyl procedure as described by Gray (1972) using the thin layer chromatographic methods described by Woods and Wang (1967). C-Terminal amino acids were determined by carboxypeptidase A digestion as described by Ambler (1972) followed by amino acid analysis using the Beckman Model 121 analyzer.

Results

Purity of the Native Hormone and the Isolated Subunits. The native LH had a radioimmunologic potency 38.1 (±0.2) times greater than LER 907, a commonly used impure reference preparation, and is comparable in potency to other purified preparations of human LH. Contamination with TSH was found to be 0.105 mU/mg by radioimmunoassay. This would suggest a 2.6% contamination on a mass basis assuming that purified TSH has a potency of 4 U/mg. Radioimmunologic assay for FSH indicated a potency of 1.61 mg of LER 907/mg or contamination with FSH of less than 1% on a mass basis. Immunoelectrophoresis against potent antisera to human serum proteins showed no precipitin arcs, while reaction with an antiserum to crude human pituitary extract showed a single precipitin arc.

When assayed in the radioreceptor assay, which more closely reflects biological activity, the native hormone had a potency of 3.82 NIH LH S1 U/mg. The isolated α and β subunits contained less than 0.076 NIH S1 U/mg or less than 2% of the activity of the native hormone.

Radioimmunoassays were used to identify the α and β subunits and to make rough appraisals of the degree of cross-contamination. These data are presented in Table I. These assays are based on the structural and antigenic similarities between human LH and HCG. The data indicate that what we call LHα (CMC-U-I) is similar to HCGα and what we call LHβ (CMC-U-II and DEAE-S-U-I) is similar to HCGβ. Furthermore, the data suggest that LHα contains relatively little LHβ or native LH, and LHβ contains relatively little LHα or native LH.

TABLE I: Radioimmunologic Assays of the Isolated Subunits of LH.

Preparation	α Assay (mg of HCGα/mg ± SE)	β Assay (mg of HCGβ/mg ± SE)
LHα (CMC-U-I)	0.635 ± 0.069	0.057 ± 0.024
$LH\beta_1$ (CMC-U-II)	0.056 ± 0.007	1.260 ± 0.31
$LH\beta_2$ (DEAE-SU-I)	0.021 ± 0.009	1.620 ± 0.72

Intrinsic Viscosity of Native Hormone. The data for viscosity determined at 25, 35, and 45° are presented in Figure 2. When the relative viscosity (η/η_0) is plotted as a function of concentration, the initial slope is the intrinsic viscosity [η].[2] The intrinsic viscosities obtained in this manner are 4.95 cm³/g at 25° and 5.75 cm³/g as the average of the two higher temperatures, possibly indicating a moderate expansion of the molecule, an increase in asymmetry, or both. The intrinsic viscosity at 25° will be used with the sedimentation coefficient discussed below to compute a molecular weight for the native hormone.

$\bar{V}$ of Native Hormone. The protein concentration estimated in the manner described under Methods was 10.409 mg/ml and we assumed a measurement error of ±2% in this value. This introduces an error of about ±1% in the $\bar{V}$ estimate. Considering this possible error alone, we obtained a $\bar{V}$ estimate of 0.714 ± 0.006 cm³/mg. This compares tolerably well, when one considers the error term, with the value of 0.702 cm³/g calculated by the method of Cohn and Edsall (1943) from the known amino and carbohydrate compositions and using the sugar residue $\bar{V}$ values given by Gibbons (1966).[3] Calculated $\bar{V}$ values for α and β subunits are given in Table II for comparison. For the sake of consistency in the estimations of molecular weights reported herein, the calculated $\bar{V}$ was used for both the native hormone and the subunits. We report the measured $\bar{V}$ as reasonable experimental justification for using the Cohn and Edsall procedure. We also use this experimental $\bar{V}$ below, combined with the intrinsic viscosity and sedimentation coefficient, to compute the molecular weight of the native hormone.

Sedimentation Constants, Stokes Radii, and Molecular Weights Determined from Sucrose Density Gradient Centrifugation and Gel Filtration. Data are presented in Table II. The native hormone had a sedimentation coefficient, $s_{20,w}$ of 2.63 S and a Stokes radius of 30.4 Å. This measured sedimentation coefficient assumes, incorrectly, no difference in $\bar{V}$ between the unknown (native LH) and the standard marker

[2] The plot of $[\eta/(\eta_0 - 1)]/c$ *vs.* c is a more sensitive method of analysis. When working at low concentrations, and therefore a limited range of c, the scatter in the data for that plotting method is more apparent than in the plot we have used. Nonetheless, we have made these plots and estimated the intrinsic viscosity (the Y intercept at $c \approx 0$) using least-squares regression analysis. The values were 4.88 cm³/g at 25°, 5.98 cm³/g at 35°, and 5.79 cm³/g at 45°.

[3] The $\bar{V}$ of the native hormone and the α and β subunits were calculated using the amino acid composition data presented in Table IV, sequence data obtained from various sources as cited in the text, and the carbohydrate data of Braikevitch and Hartree (1970), Kathan *et al.* (1967), and Closset *et al.* (1972). It should be noted in this connection that the percentages of carbohydrate in the native molecule and isolated α and isolated β subunits are approximately 20.7, 28.4, and 13.4%, respectively.

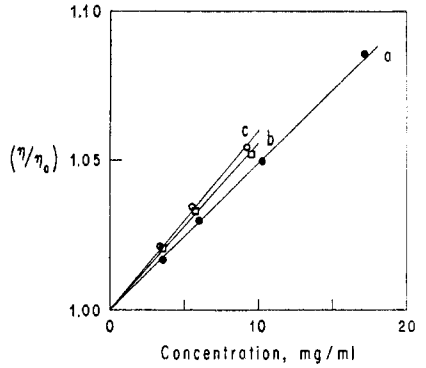

FIGURE 2: Viscosity data for human luteinizing hormone. The solvent employed in the Cannon-Ubbelohde viscometer was 0.20 M NaCl–0.01 M phosphate (pH 7.5). Line a is through data for 25°, line b for 45°, and line c for 35°.

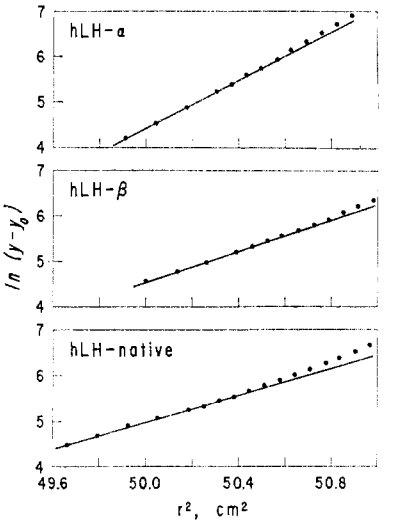

FIGURE 3: Sedimentation equilibrium of human LH and its subunits by meniscus depletion method. The nominal rotor speeds for the runs illustrated were 28,000 rpm for human LH native, 48,000 rpm for human LHα, and 44,000 rpm for human LHβ. The temperature was near 25° for all runs and the protein concentrations (before dialysis) were 0.27–0.28 mg/ml in 0.10 M NaCl (pH 5.9) solutions.

proteins. Correction for this difference, using the empirically derived factor presented by Ryan *et al.* (1971), yields a sedimentation coefficient, $s_{20,w}$, of 2.56 S. The mol wt of the native hormone calculated from the Stokes radius, corrected $s_{20,w}$, and a $\bar{V}$ of 0.702 is 28,800.

LHα was found to have Stokes radius of 21.7 Å, a calculated $\bar{V}$ of 0.68, an uncorrected $s_{20,w}$ of 1.68 S, a $\bar{V}$ corrected $s_{20,w}$ of 1.52 S, and a calculated mol wt of 11,800.

LHβ (preparation CMC-U-II) was found to have a radius of 21.5 Å, a $\bar{V}$ of 0.71, a $\bar{V}$ corrected $s_{20,w}$ of 1.70 S, and a mol wt of 14,380.

Sedimentation Equilibrium. Plots of the data are shown in Figure 3. All plots show upward curvature to some degree. We have used only the lower and middle parts of the curves to estimate molecular weights. The data from these regions of each run were analyzed by a computerized least-squares program to yield the lines shown in Figure 3 and the slopes used in the molecular weight calculation using the equation

$$M = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{\mathrm{d} \ln (Y - Y_0)}{\mathrm{d}r^2} \qquad (2)$$

In this equation, ω is the angular velocity of rotation in radians/second, $\bar{V}$ is the partial specific volume of the protein, and ρ is the density of the solvent. Calculated molecular weights are given in Table III. Only minimal variations were noted when each preparation was examined at two different speeds. The native hormone had a mol wt of 28,850 while the α and β subunits were 15,750 and 15,305, respectively. The calculated $\bar{V}$ values presented in Table II were used for these estimations.

Amino Acid Composition, Tyrosine–Tryptophan Ratios, and Free Sulfhydryl and End Group Analyses. The results of amino acid analysis are presented in Table IV and are compared to the values reported by other investigators. The relatively high contents of proline and half-cystine should be noted. The major differences between the α and β subunits occur with lysine, arginine, serine, proline, and isoleucine.

As determined spectrophotometrically, at a protein concentration of 1.770×10^{-5} M, assuming a mol wt of 28,300 for native human LH, the tyrosine content was found to be 7.13 residues/molecule, the tryptophan content was 1.37 residues/molecule, and the tyrosine/tryptophan ratio was 5.21. These numbers compare favorably with those reported by Braikevitch and Hartree (1970) and Papkoff and Li (1970).

Employing cysteine as a standard in the *p*-mercuribenzoate method of Boyer (1954) and correcting for the absorbance due to the protein itself, no free sulfhydryl groups were detected.

N-Terminal valine and serine were found in the native hormone using the 5-dimethylaminonaphthalene-1-sulfonyl procedure and also by Edman degradation in the Beckman

TABLE II: Physical Properties of LH and Its Subunits as Determined by Density Gradient Centrifugation and Gel Filtration.

	$\bar{V}$ (cm³/g)	$s_{20,w}$ Measured	$s_{20,w}$ Corrected[a]	Stokes Radius (Å)	Mol Wt[b]
Native	0.702[c]	2.63	2.56	30.4	28,800
	0.714 ± 0.006[d]				
α (CMC-U-I)	0.68[c]	1.68	1.52	21.7	11,800
β (CMC-U-II)	0.71[c]	1.78	1.70	21.5	14,380

[a] Corrected for the difference in $\bar{V}$ between the unknown and the standard proteins as discussed by Ryan *et al.* (1971). [b] Calculated by eq 1; see text. [c] Calculated from composition data; see text. [d] Experimentally determined.

TABLE III: Molecular Weights Determined by Sedimentation Equilibrium.

	Speed (rpm)	$\bar{V}$ (cm³/g)	Mol Wt	Av Mol Wt
Native	32,000	0.702	28,100 ± 700	28,850
	28,000	0.702	29,600 ± 700	
α Chain	52,000	0.680	15,400 ± 350	15,750
	48,000	0.680	16,100 ± 400	
β Chain	44,000	0.710	14,315 ± 350	15,305
	40,000	0.710	16,295 ± 400	

TABLE IV: Amino Acid Compositions of Human Luteinizing Hormone and Its Subunits.[a]

Amino Acid	Present Work	Closset *et al.*[b]	Hartree *et al.*[c]	Saxena and Rathnam[c]
	(a) Native Hormone			
Lys	4.4	4.1	4.0	3.9
His	2.4	2.5	2.6	2.9
Arg	4.7	6.3	6.3	4.7
Asp	7.4	7.0	6.5	7.2
Thr	7.4	6.5	7.5	7.0
Ser	6.9	6.8	7.2	6.9
Glu	8.4	8.8	9.5	8.0
Pro	9.4	13.4	10.2	13.0
Gly	6.1	6.5	6.5	5.5
Ala	4.8	6.1	4.8	6.0
Half-Cys	11.1	7.9	8.9	7.6
Val	8.2	8.7	8.7	8.7
Met	3.1	1.7	1.9	1.8
Ile	3.3	3.5	3.0	5.9
Leu	6.2	5.8	6.3	5.6
Tyr	2.9	2.9	2.8	2.4
Phe	3.0	2.1	3.8	3.6
	(b) α Subunit			
Lys	5.5	6.1	6.0	4.6
His	3.1	3.3	2.9	2.7
Arg	3.7	3.6	4.5	3.8
Asp	6.1	5.2	6.1	7.9
Thr	10.2	8.2	8.2	8.0
Ser	8.5	8.2	7.8	7.0
Glu	10.6	10.2	10.2	8.9
Pro	8.0	7.4	9.2	9.4
Gly	5.2	6.9	5.3	5.5
Ala	4.8	5.0	4.6	5.7
Half-Cys	8.2	8.3	9.9	8.9
Val	8.0	7.7	7.9	8.7
Met	3.5	3.8	2.6	1.9
Ile	1.2	1.4	1.7	5.0
Leu	5.3	5.2	5.1	5.0
Tyr	3.5	4.4	3.6	2.7
Phe	4.3	4.6	4.4	4.1
	(c) β Subunit			
	CMCU-II	DEAE-S-U-I		
Lys	4.1	1.8	2.5	2.3	3.3
His	2.0	2.3	2.7		3.1
Arg	5.3	8.1	8.7	8.2	5.6
Asp	7.3	6.9	6.2	6.6	6.5
Thr	8.9	8.9	6.1	6.0	6.1
Ser	5.4	5.8	6.0	5.7	6.7
Glu	8.6	5.4	7.2	7.3	7.1
Pro	13.5	13.2	14.8	13.5	16.6
Gly	7.9	7.1	8.3	7.0	5.5
Ala	5.6	4.5	5.0	4.7	6.3
Half-Cys	7.2	10.0	7.2	9.9	6.7
Val	6.6	8.6	9.1	9.7	8.7
Met		1.8	2.0	1.5	1.7
Ile	4.2	4.0	3.9	3.8	5.0
Leu	5.9	7.5	7.1	7.4	6.2
Tyr	1.3	1.1	2.0	2.0	2.1
Phe	2.3	1.4	2.1	1.9	3.2

[a] Amino acid compositions reported as residues/100 residues analyzed. [b] 1962. [c] 1971.

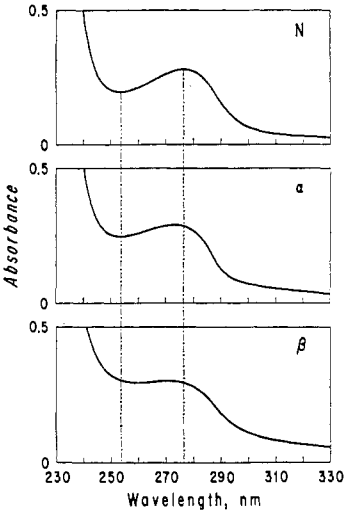

FIGURE 4: Absorption spectra of human LH and its subunits. Protein concentrations in the 0.20 M NaCl–0.01 M phosphate (pH 7.5) solvent were: native, 0.515 mg/ml; α subunit, 0.537 mg/ml; β subunit 0.514 mg/ml. Quartz cells of 3-ml capacity and 1-cm path length were used. The ambient temperature was 25 ± 1° but the cells were not otherwise thermostated.

automatic sequenator. Valine was found at the first step in the Edman degradation of LHα and serine at the first step of LHβ (Keutmann, H. T., Bishop, W. H., Ryan, R. J., and Potts, J., unpublished data). Carboxypeptidase digestion of the α subunit yielded serine first and in largest yield, with subsequent and smaller yields of lysine, histidine, and tyrosine. Carboxypeptidase treatment of the β-subunit preparation was not performed.

Ultraviolet Absorption Spectra of Human LH and Subunits. In Figure 4 are shown the absorption spectra with the peak and trough of the native spectrum indicated for comparison with the subunit spectra. It is seen that the subunit spectra are different from each other and from the native. The absorption maximum of native LH was 276.1 nm and the molar extinction coefficient was 1.54×10^4. The absorption maxima and molar extinction coefficients of LHα and LHβ were, respectively, 274.3 nm and 7.56×10^3 and 272.2 nm and 8.69×10^3.

Figure 5 is a *calculated* difference absorption spectrum constructed from the data in Figure 4.

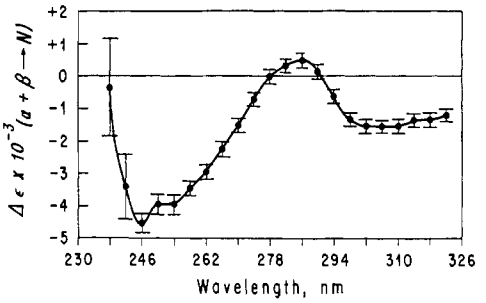

FIGURE 5: Calculated difference molar extinction coefficient, $\Delta\epsilon \times 10^{-3}$, for the process $\alpha + \beta \rightarrow$ native. The curve was constructed from the spectra in Figure 4 using assumptions discussed in the text. The bars through the points are approximate errors in the difference extinction coefficient incurred by estimating optical densities from the chart paper. These error limits do not include the possible larger errors resulting from inexact protein concentration estimates.

TABLE V: Sedimentation Coefficients and Stokes Radii of Various Preparations of Native Human LH.

Preparation	$s_{20,w}$ Uncorrected[a]	$s_{20,w}$ $\bar{V}$ Corrected[b]	Stokes Radius (Å)[a]
RR 9765 B	2.71 (7)	2.64	29.5 (4)
RR 41267 B	2.69 (2)	2.62	29.7 (2)
AP DEAE-2[c]	2.78 (1)	2.71	31.9 (2)
AH DEAE[d]	2.67 (1)	2.60	
AH IRC2[d]	2.69 (2)	2.62	30.4 (1)
LER 960[c]			30.8 (3)
Av	2.71 (13)	2.63	30.3 (12)

[a] Parenthetical numbers refer to the number of observations. The average was weighted by the number of observations for each preparation. [b] $\bar{V}$ correction according to the empirical data of Ryan *et al.* (1971). [c] Gift of the National Pituitary Agency. [d] Gift of Dr. Anne Stockell Hartree, Cambridge, England.

Discussion

The preparation of native LH used in these physical and chemical studies and used for the preparation of subunits is comparable in purity and potency to those used by Hartree (1966) and her coworkers, Closset *et al.* (1972) and Reichert *et al.* (1970). Indeed, direct comparison by radioimmunoassay with Reichert's preparation LER 960 and Hartree's preparation DEAE-I indicated no difference in potency.

Methods used for the preparation of subunits deserve some comment. The countercurrent system of Papkoff and Samy (1967) which has been used very successfully for the preparation of subunits from ovine and bovine LH has not been satisfactory for human LH. Two problems exist. First, the human hormone does not readily dissociate in this solvent system and secondly the β subunit is not as soluble in the organic phase as the ovine and bovine hormones. These problems can be overcome by preincubation in guanidine and by increasing the concentrations of ammonium sulfate and dichloroacetic acid (Lee and Ryan, 1971b). However, our own efforts to scale this procedure to a preparative method were frustrated by poor yields, particularly of the β subunit.

In the urea CMC method of Nureddin *et al.* (1971), it appears unlikely that the less than theoretical yield of the α subunit can be accounted for by incomplete dissociation in 9 M urea or recombination of α and β to form native hormone while being chromatographed in 4.5 M urea. Instead, we would suggest that two populations of the α subunit exist. Those with the highest content of sialic acid, and therefore more acidic, do not bind to CMC while those with a lower sialic acid content stick to the exchanger. The preparation of a β subunit by elution from CMC and gel filtration relies upon recombination of a nonstoichiometric mixture of α and β to form native hormone and an excess of the β subunit. The possibility exists that the denaturing solvents could produce irreversible structural changes that render either α or β incompetent to recombine. If this were the case, then the G-100 fraction should contain a mixture of free α and β since they have similar Stokes radii. The immunologic data presented in Table I suggest that this is not so, at least in any major degree. The DEAE-Sephadex method of Closset *et al.* (1972) produced a satisfactorily purified β subunit but the yield we obtained was appreciably less than they reported. The reason for this difference is not apparent.

The Stokes radius and sedimentation constant of the preparation of native LH, reported herein, should be compared with the values we obtained, using identical techniques, for other preparations of native LH (Table V). The molecular weight of native LH calculated from the Stokes radius and sedimentation constant (28,800) is in excellent agreement with the value of 28,850 obtained by sedimentation equilibrium. Both are also in good agreement with the value of 28,050 calculated with the Scheraga–Mandelkern equation (1953)[4] using the intrinsic viscosity $[\eta]$ (4.95 cm^3/g), $\bar{V}$ (0.714 cm^3/g), and sedimentation constant $s_{20,w}$ (2.56 S)

$$M = \frac{4.69 \times 10^3 (s_{20,w})^{3/2} [\eta]^{1/2}}{(1 - \bar{V}\rho)^{3/2}} \quad (3)$$

When the calculated $\bar{V}$, 0.702 cm^3/g is used in this equation, a mol wt of 26,400 is obtained. All three hydrodynamic estimates agree excellently with a chemical mol wt of 28,260 estimated from amino acid composition, sequence data (Closset *et al.*, 1973; Shome and Parlow, 1973; Sairam *et al.*, 1972; and Keutmann, H. T., Bishop, W. H., Potts, J., and Ryan, R. J., unpublished data), and the carbohydrate composition data (summarized by Closset *et al.* (1972), Kathan *et al.* (1967), and Braikevitch and Hartree (1970)).

We presume, though we have no direct proof, that the heterogeneity evident in the sedimentation equilibrium plots shown in Figure 3 is a result of aggregation. Whether this presumed aggregation is kinetically reversible or irreversible and, if reversible, on what time scale and how complicated the scheme of equilibria, are points on which we have no direct information at present. However, we do point out that Nureddin *et al.* (1971), using sedimentation velocity, have found a concentration-dependent sedimentation coefficient for native human LH in the approximate concentration range which we estimate obtains through the region of upward curvature. In earlier work on native human LH, Squire *et al.* (1962) found a similar upward curvature in the sedimentation equilibrium data and concluded that a reversible concentration dependence was not sufficient to account for the whole effect and that irreversible aggregates were present. We can claim no clearer interpretation for our sedimentation equilibrium runs. In any case, we can, by extrapolation of the lines and reconverting from logarithms, estimate on a mass basis the fractions of total protein in the centrifugation cell which are not in the form of the monomer of the assumed molecular weight. These figures are 10.2, 4.6, and 1.9% for the native, α, and β proteins, respectively. The error estimates in molecular weight for the individual sedimentation runs shown in Table III were calculated using essentially the method discussed by Yphantis (1964).

The intrinsic viscosity may be used to estimate the axial ratio of the equivalent ellipsoid of revolution for the protein molecule. Following Tanford's (1961) discussion of the Einstein–Simha equation and using the measured $\bar{V}$ of human LH while assuming a solvation of 0.2 g of solvent/g of protein, we compute the Simha asymmetry factor to be 5.3. This corresponds to an axial ratio of about 4:1 for the equivalent prolate ellipsoid. A similar value of approximately 5:1 was

[4] The value of the constant β incorporated into this form of the equation was 2.16×10^6, which is reasonable for molecules with axial ratios up to about 5:1 (Schachman, 1957).

obtained using the sedimentation velocity data. The axial ratio obtained in this manner is insensitive to the small differences between the measured and calculated $\bar{V}$. The calculation is, however, significantly affected by the degree of solvation of the protein, which is a difficult parameter to estimate. A value of 0.2 g/g is generally assumed. This may be, and probably is, unrealistically low for carbohydrate-containing proteins, particularly one like native LH which is approximately one-fifth carbohydrate. Thus, the axial ratio of 4 or 5 to 1 most probably represents an upper limit of the degree of asymmetry of native LH. An assumption of a solvation of 0.4 g/g yields an axial ratio estimate of about 3.5:1.

We know of no published data on the isolated subunits of human LH to compare with the Stokes radii, sedimentation constants, and molecular weights reported herein. The molecular weights of LHα calculated from the sedimentation constant and Stokes radius, sedimentation equilibrium, and chemical composition were 11,800, 15,750, and 13,853, respectively. The molecular weight calculated from the Stokes radius and sedimentation constant may be too low because of the empirical "$\bar{V}$ correction" applied to the sedimentation constant. Since the calculated partial specific volume, 0.68 cm^3/g, of the α subunit is lowest and deviates most from that of the standard proteins used in the density gradient sedimentation, its "$\bar{V}$ correction" to $s_{20,w}$ is largest. In order to maintain consistency, we have reported the calculated molecular weight using the corrected $s_{20,w}$. If this correction is neglected for the α subunit, a mol wt of 13,100 is obtained, in better agreement with the chemical estimate. It seems possible that the correction to $s_{20,w}$ for the α subunit is simply too large. The sedimentation equilibrium value may be too high because of the presence of high molecular weight material as mentioned above.

The calculated molecular weights of LHβ by hydrodynamic techniques, 14,380 ($s_{20,w}$, a) and 15,305 (sedimentation equilibrium), are both in good agreement with the value of 14,407 calculated from chemical composition.

As demonstrated in Table IV, the amino acid composition data show generally good agreement with results from other laboratories. The data for N-terminal residue analysis of the native hormone and isolated subunits are consistent with the N-terminal valine of the human LHα subunit given by Sairam *et al.* (1972) and the partial sequence of human LHα by Inagami *et al.* (1972) as well as the N-terminal serine found by Closset *et al.* (1973) and Shome and Parlow (1973) in human LHβ. The results of the carboxypeptidase digestion of CMC-U-I are consistent with the C-terminal sequence of both human LHα and HCCα (Sairam *et al.*, 1972; Bahl *et al.*, 1972; Morgan and Canfield, 1972). Our results indicating the absence of free sulfhydryl groups are in agreement with Bahl's (1969) work on HCG who checked for their presence with *p*-chloromercuribenzoate and by the carboxyamidomethylation reaction on the unreduced protein.

The calculated difference spectrum shown in Figure 5 is based on several assumptions: (a) the weighed proteins are free of significant contaminants and are perfectly dry; (b) molecular weights of 28,260 for native human LH and 13,850 and 14,410 for the α and β subunits, respectively. We must emphasize that the calculated difference extinction coefficients were between native hormone and subunits prepared by the 9 M urea incubation and chromatographic procedures described above. As such, this difference spectrum should be interpreted cautiously because it is subject to both operational and conceptual difficulties. Relatively small errors in estimates of concentration could result in artifactual displacements of the ordinate. In addition, part of the population of subunit molecules may be irreversibly altered due to the denaturing conditions employed in subunit isolation. In this case a problem could arise regarding what one means by "native subunit" since virtually all preparative procedures for subunit involve strongly denaturing conditions. (Experimental work is in progress which attempts to circumvent these problems.) In this connection, we should point out that Ekblad *et al.* (1970) have observed circular dichroic spectra of ovine FSH in 8 M urea which are radically altered compared to native ovine FSH but which revert to the native spectra essentially completely when the urea is dialyzed away.

Although some degree of irreversible denaturation may have occurred in the isolated subunits, we do not feel that they have been totally or largely unfolded to highly disordered systems resembling random coils. Several lines of evidence suggest the retention of some organized structure in the isolated subunits. First, it is estimated that human LHα has five intrachain disulfide bonds and human LHβ has six such bonds. Secondly, on incubation of the isolated α and β subunits we were able to regenerate 15% of the biological activity of the native molecule. This is less than we would like to have seen; however, our data do not allow us to conclude whether the low yield was due to "some denaturation" or less than ideal conditions for recombination. Thirdly, the isolated subunits were recognized by antibodies against α and β subunits. Lastly, estimates of frictional coefficients derived from the experimental data are not compatible with very approximate frictional coefficients calculated for random coils of the same chain length.

With these reservations, we speculate that the curve in Figure 5 has very generally the shape of a "red shift" aromatic difference spectrum with a positive peak near 285 nm and a crossover from negative to positive difference extinction near 278 nm (Wetlaufer, 1962; Donovan, 1969). The tendency for the calculated spectrum to become flat near 300 nm suggests an error in the concentration estimate which may have shifted the whole curve somewhat toward negative difference extinction without major alteration in the peak of trough wavelength positions. Such an error would cause a crossover which would be shifted from its true position to a slightly longer wavelength. Without pressing the interpretation, we can at least suggest that the structural combination of α and β subunits to form the native hormone results in the transfer of aromatic side chains, within one or both subunits, from a more aqueous exposed to a less aqueous exposed, possibly buried, environment within the native molecule. The change in environment could arise simply from segregation of these side chains away from solvent at the sites of interaction between subunit surfaces or from more profound structural alterations within the subunits involving actual relative motions of structural elements. In other optical studies on ovine LH, Jirgensons and Ward (1970) measured the circular dichroism of the native hormone and both subunits. Their data also suggested conformational changes in one or both subunits when the two combine to form the native hormone.

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