Ultracentrifugal Studies of Human Luteinizing Hormone and Its Subunits: Dependence on Protein Concentration and Ionic Strength†

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ABSTRACT: Purified human pituitary luteinizing hormone (hLH) and its α and β subunits have been examined by physical methods. Freshly prepared hLH showed three closely adjacent bands on electrophoresis in polyacrylamide gels under alkaline conditions, but on standing even in the freeze-dried condition additional bands appeared. α and β subunits gave bands which were quite different from the main hLH bands but comparable with the additional bands, as well as traces of hLH bands. hLH was investigated at three ionic strengths (0.1, 0.2, 0.5) at pH 5.9 ± 0.1. Sedimentation velocity experiments demonstrated a complex system of association-dissociation which was further investigated by sedimentation equilibrium. Association occurred at the higher protein concentrations at each ionic strength, but to a significantly higher level at I = 0.5. Only at I = 0.1, pH 5.8, was there a clear indication over a range of protein concentration of the occurrence of a species of molecular weight 32 000 ± 2000, in fair agreement only with the sum of the molecular weights of the α and β subunits. At higher ionic strengths, there were indications of dissociation at low protein concentration (<0.10 g/100 mL) as well as association at higher values (>0.20 g/100 mL). In view of the occurrence of molecular weights less than 28 000 at I = 0.2, hLH was treated in terms of a monomer of molecular weight 14 000, and some evidence was obtained for tetramer formation ($4M \rightleftharpoons M_4$). At higher ionic strength this model does not apply and it is thought that indefinite association may be occurring to some extent also. The α subunit gave indications of association from its sedimentation coefficient vs. concentration plot, and sedimentation equilibrium (at pH 5.9, I = 0.1) demonstrated molecular weights increasing with increasing concentration. Evidence for tetramer formation also was obtained. The β subunit, in spite of an apparently simple sedimentation coefficient-concentration curve, showed molecular weights varying from well below 14 000 to beyond 20 000. There is evidence to suggest that the isolated α and β subunits, even on standing as dry solid, are not stable but give rise to lower molecular weight products. Aged freeze-dried hLH did not show such impurity.

There is considerable variation in the molecular weight of hLH as reported from a number of laboratories and as determined by various methods. Detailed studies of sedimentation equilibrium at pH 3.6 (Squire et al., 1962) suggested that the hormone was polydisperse with a minimum molecular weight of 26 000. It was subsequently shown that oLH (Li and Starman, 1964) and hLH (Ryan, 1968) dissociated into subunits at acid pH. This dissociation may have contributed to the inhomogeneity observed at pH 3.6, resulting in a minimum molecular weight somewhat lower than that of the intact hormone. Later studies by Squire and Benson (1966) confirmed this interpretation. Reichert and Jiang (1965) obtained molecular weight values of 45 000 from gel filtration studies and 25 000 to 30 000 by sucrose density gradient centrifugation. More recent studies have utilized the method of Siegel and Monty (1966) by which molecular weight is calculated from a "Stokes radius" determined by gel filtration and the sedimentation coefficient determined in the ultracentrifuge. In these studies, sedimentation coefficients determined at a single protein concentration varied from 2.3 (Rathnam and Saxena, 1970) to 2.8 (Ryan et al., 1970) and the calculated molecular weight from 27 000 to 33 000, respectively. Roos et al. (1975) report values of 3.2 and 3.5 S for component II and III, respectively, of their hLH preparation. The corresponding molecular weight as determined by the Yphantis meniscus depletion technique was 37 000 for each component. Bishop and Ryan (1973) have also examined preparations of hLH and its subunits particularly by sedimentation equilibrium using the Yphantis meniscus depletion technique. They suggest approximate molecular weights of 30 000 and 15 000 for hLH and its two subunits but on examination of their data, which covers only a short range of concentration, it seems clear that aggregation reactions are also occurring which have been ignored.

In the present work, an attempt has been made to derive reliable molecular weights for hLH and its subunits. The great scarcity of the materials has made it necessary to use not only freshly prepared but freeze-dried materials, some of which had been stored at room temperature for at least several months. Such preparations are used as standards for diagnostic and other purposes. It is conceivable that some of these preparations could have aged irreversibly and a comparison with results for fresh material was considered important.

Materials and Methods

Reagents. Urea (99.5%) and various buffer salts used in this work were of Analar grade.

Purification of hLH. Fraction DEAE-I[1] of hLH prepared from acetone-dried human pituitaries by the method of

† From the Department of Biochemistry, University of Cambridge, Cambridge, England. Supported by funds from the Population Council, the Ernest Oppenheimer Fund, and partially by funds from the National Institute of Health (Grant HD-9140-H). Part of this work was presented at the Vth International Congress of Endocrinology, July 1976, held in Hamburg, West Germany.

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[1] Abbreviations used: hLH, Human luteinizing hormone; oLH, ovine luteinizing hormone; OAAD, ovarian ascorbic acid depletion; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; CM, carboxymethyl; TSH, thyrotropic hormone; FSH, follicle stimulating hormone.

Stockell Hartree (1966) was further purified by ion-exchange chromatography on CM-cellulose (Whatman microgranular CM-32)[2] with all operations performed at 4 °C. The sample was dissolved in the starting buffer (0.08 M sodium acetate adjusted to pH 5.0 with acetic acid) and was applied to a CM-cellulose column (46 × 1 cm) previously equilibrated with the same buffer. After elution of unretarded, biologically inactive material absorbing at 280 nm, a linear gradient was applied from 0.14 to 0.16 M sodium acetate at pH 5.0. The flow rate of the column was 50 mL h^{-1} (100 mL of bed volume)$^{-1}$. Fractions of 4.5 mL each were collected and the protein concentration was monitored by absorption at 280 nm. Fractions containing hLH were eluted with the gradient and were pooled, dialyzed against several changes of distilled water for 3 days, and freeze-dried. The product referred to as CM-hLH was used throughout this work. The yield was 48% by weight from DEAE-I and the biological activity was 5 to 7 mg of NIH-LH-S1/mg (Nureddin et al., 1972).

Separation of the Subunits of hLH. Dissociation of CM-hLH and separation of the subunits were performed by a modification of the chromatographic procedure of Stockell Hartree et al. (1971). After incubation of the hormone overnight at 4 °C in 8 M urea, chromatography was performed on CM-32. The column size, conditions, and buffers were identical with those used for preparation of CM-hLH, except that freshly prepared 4 M urea was incorporated in all buffers in order to prevent reassociation of subunits during chromatography. The α subunit was recovered in the unadsorbed fraction eluted with the starting buffer and the β subunit was eluted with 0.14–0.16 M sodium acetate buffer, pH 5.0 (Figure 1). The isolated subunit fractions were each pooled, dialyzed, and freeze-dried as described above. Subunits prepared by this procedure were similar to previously described preparations in their biological activities, amino acid compositions, and polyacrylamide gel electrophoretic patterns, and they could be recombined to give rise to the original $\alpha\beta$ hormone with approximately 50% recovery of activity (Nureddin et al., 1972; Stockell Hartree et al., 1971). The weight yield of each subunit was 32% of the CM-hLH starting material.

Disc Gel Electrophoresis. The method of Davis (1964) was followed. No stacking gel was used. The 50-μg sample, dissolved in 0.1 mL of 40% sucrose, was applied directly on the resolving gel by layering it under the buffer. In order to introduce the protein into the gel, a current of 1.0 mA/tube (5-mm diameter) was used for 15 min and then 2.5 mA/tube for 2.5 h. The gels were stained with Amido black.

Bioassay of LH Activity. This was performed by the ovarian ascorbic acid depletion (OAAD) method of Parlow (1961) as modified by Bell et al. (1965) with NIH-LH-S1 used as a standard. The hLH preparations and standards were each tested at two dose levels.

Ultracentrifuge Studies

Sedimentation Velocity and Diffusion. Sedimentation velocity experiments on CM-hLH and on the α and β subunits were carried out in the Beckman Spinco Model E ultracentrifuge at 59 780 rpm using a valve-type synthetic boundary cell. The temperature was accurately measured throughout the experiments and maintained in the range 19–21 to ± 0.1 °C. Correction of sedimentation coefficients to the viscosity and density of water at 20 °C was carried out assuming a partial specific volume of 0.71 and correction factors based upon viscosity and density measurements made in this laboratory. The sedimentation velocity examination was performed at different protein concentrations (0.04–0.7 g/100 mL) in phosphate–NaCl buffers within the pH range 5.4–6.0 at ionic strengths 0.1, 0.2, and 0.5. Photographs were normally taken at 8-min intervals and at 4-min intervals when the lower protein concentrations were used. Photographic plates were measured on a projection microcomparator (Precision Grinding Ltd.), using the peak positions for the location of the boundaries. Sedimentation velocity coefficients may normally be used to calculate the molecular weight of a protein as defined by the Svedberg equation:

$$M = \frac{RTs^\circ}{D^\circ(1 - \bar{v}\rho_0)} \quad (1)$$

where R and T are the gas constant and absolute temperature respectively, $\bar{v}$ is the partial specific volume of the protein [calculated for hLH as 0.71 from its composition (Ryan et al., 1970)], and ρ_0 the density of the solvent; s° and D° are the sedimentation and diffusion coefficients, respectively, at infinite dilution, obtained by extrapolation.

The diffusion coefficient, D, was determined at as low a speed as could be achieved without introducing rotational instability (usually 4326 rpm). For such speeds and times up to 12 h, the term $\omega^2 st$ (where ω is angular velocity and t the time in seconds), for the values involved in this work, was small compared with unity and was therefore ignored. The diffusion coefficient was calculated from the slope of a plot of $(A/H)^2$ as a function of time using the following equation:

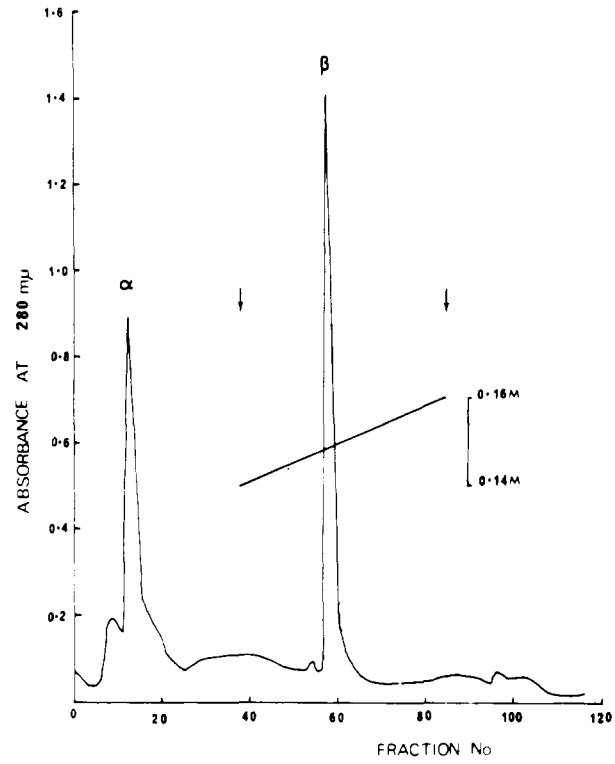

FIGURE 1: Separation of the subunits of CM-hLH by ion-exchange chromatography on CM-32. The α subunit was eluted with the equilibrium buffer: 0.08 M sodium acetate–acetic acid, pH 5.0. The arrows indicate the beginning and end of a linear gradient between 0.14 and 0.16 M sodium acetate–acetic acid buffer, pH 5.0. A 1 M solution of the same buffer was applied at the end of gradient. All buffers included freshly prepared 4 M urea. Fractions (4.5 mL) were collected and all operations performed at 4 °C. The size of the column was 46 × 1 cm.

[2]A preliminary report has been published by Nureddin et al. (1972).

$$D = \frac{1}{4\pi t}\left[\frac{A}{H}\right]^2 \quad (2)$$

where A and H are the area and height of the schlieren peak, corrected for magnification in the radial direction.

Sedimentation Equilibrium. Earlier sedimentation equilibrium experiments were performed in a synthetic boundary, capillary type double-sector cell equipped with an aluminium-filled Epon centerpiece. Sapphire cell windows were used for all experiments. Photographs on both the Rayleigh interference and schlieren optical systems were taken almost simultaneously when required using the push-pull slit assembly. In order to obtain the best schlieren picture, the phase-plate angle was varied from 45 to 70 °C. The positions of the lower and upper menisci were located precisely from a schlieren picture taken at 90° bar angle. A solution blank, to correct for baseline curvature, was also performed using the same synthetic boundary cell. The cell was filled to a level below the lower capillary, using a microsyringe. Buffer dialysate (0.13 mL) was introduced into the left-hand compartment, and, in the right-hand side, 0.01 mL of fluorocarbon FC 43 (Minnesota Mining Manufacturing Co., London, England) followed by 0.11 mL of protein solution (0.2–0.3 g/100 mL) well-dialyzed against the chosen phosphate–NaCl buffer. The effective initial concentration (in terms of schlieren area or interference fringes) was determined after the main equilibration experiment. Thus, after homogenizing the contents of each channel by gently shaking, 0.25 mL of buffer dialysate was added to the left-hand solvent compartment, and, on accelerating, this was sufficient for solvent to flow over into the solution side. Photographs of the solvent solution interface under the equilibration conditions then gave the effective initial concentration. The method of Hexner et al. (1961) was followed to determine a suitable speed and time of overspeeding. The approach to equilibrium was monitored by measuring the displacement of a single interference fringe across the cell image. When such displacements were identical with $\pm 10\ \mu$ for two consecutive pictures, then the experiment was terminated.

An apparent weight-average molecular weight, at a given radial position, r, in the cell, may be calculated from

$$M_{w,r}^{\text{app}} = \frac{1\,\mathrm{d}\ln c}{F\,\mathrm{d}(r^2)} \quad (3)$$

where

$$\frac{1}{F} = \frac{2RT}{(1-\bar{\nu}\rho_0)\omega^2} \quad (4)$$

For this purpose, the concentration (c) at the meniscus, c_m, was calculated from the equation for the conservation of mass:

$$c_0 - c_m = \frac{r_b^2(c_b - c_m) - \int_{c_m}^{c_b} r^2\,\mathrm{d}c}{r_b^2 - r_m^2} \quad (5)$$

In this equation the subscripts m and b refer respectively to the meniscus and bottom of the solution column. Equation 5 was evaluated mainly according to the method described by Richards et al. (1968). The fringe number corresponding to the initial concentration, c_0, was obtained by measuring the number of fringes across the boundary in the interference pattern of a synthetic boundary experiment. Alternately it could be obtained by measuring the corresponding schlieren area and applying a correction factor, CF, defined by Chervenka (1966):

$$\mathrm{CF} = \frac{A\tan\theta}{J} \quad (6)$$

where θ is the bar angle, A the schlieren area in cm^2, and J the number of fringes. A mean CF of 0.01065 was obtained for experiments carried out at bar angles of 45, 50, and 60°. To relate fringe numbers to concentration in g/100 mL, it can be assumed that one fringe corresponds to 0.0236 g/100 mL. This was obtained by counting fringes in a synthetic boundary experiment for which the concentration in g of dry CM-hLH protein/100 mL was known (a 10% moisture content of the freeze-dried material was assumed). The same factor has been assumed for the α subunit.

Where the plot of ln c vs. R^2 (magnified radial distance) possessed definite curvature, the slope at a given point was obtained and checked using least-squares criteria as recommended by Yphantis (1964). In some cases, the Chervenka (1966) procedure, in which $(1/r)(\mathrm{d}c/\mathrm{d}r)$ is plotted against Δc, is defined by

$$c = C_{ref} + \Delta c \quad (7)$$

C_{ref} being usually the concentration at the meniscus. In such cases the apparent z-average molecular weight at any point in the cell $M_{z,r}^{\text{app}}$ is related to the slope by

$$M_{z,r}^{\text{app}} = \text{slope}\,\frac{RT}{(1-\bar{\nu}\rho_0)\omega^2} \quad (8)$$

In later work an improved sedimentation equilibrium procedure has been introduced, in which interference optics only were used and the fluorocarbon layer was omitted. This technique has made it possible to avoid the calculation based on the assumption of conservation of mass. Well-dialyzed protein solution (0.07 mL) was introduced into the right-hand limb of the charcoal filled epon double sector cell provided with a capillary 3 mm from the base of the channels. Dialysate (0.16 mL) was introduced into the left-hand limb and upon acceleration of the rotor, the slow layering of dialysate on the protein solution which occurred, was complete before a speed of 5000 rpm was reached. At first the fringe system was discontinuous at the junction of solution and solvent but, when this discontinuity had just disappeared, a full length interference picture was taken. The solution column was approximately 3-mm high. Then, using a modified short shift mechanism (incorporating an Electronic Sequence Timer, Engel and Gibbs T5/110AS)[3] with a suitable "gate" in front of the photographic plate, a succession of interference pictures were taken which contained only a short length of both air fringes and of the solution column near the meniscus (Charlwood, 1965). Pictures were taken at 2-min intervals for approximately 60 min, at 4-min intervals for 120 min, and at successively long intervals over the next 24 h. Using the air fringes as markers, these pictures made it possible to follow and measure accurately the increasing concentration at the meniscus (to better than $^1/_{10}$ of a fringe number) arising from backward diffusion of the solute. Finally, when equilibrium had been established, the "gate" was removed. A series of complete interference pictures were taken. After aligning such plates on the stage of a two-dimensional microcomparator by means of the outside air-fringes, the interference pattern was analyzed as described for the earlier experiments.

Theoretical. In further sections of this paper, it will be shown in several cases that the measured weight-average molecular weight increases with increasing concentration, and since the protein preparations concerned are well defined though not necessarily completely homogeneous, it seems clear that as-

[3]Obtainable from Engel and Gibbs Ltd., Borchanwood, Flstree Way Herts.

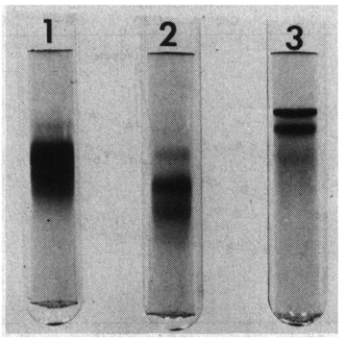

FIGURE 2: Polyacrylamide gel electrophoretic patterns for CM-hLH and its subunits in Tris–glycine buffer, pH 8.3. Gels 1, 2, and 3 refer to CM-hLH, α subunit, and β subunit, respectively.

sociation–dissociation systems are involved. Milthorpe et al. (1975) have suggested the evaluation of a function Ω and its extrapolation to zero concentration where

$$\Omega(r) = \frac{\bar{c}(r) \exp \phi_1 M_1(r_F^2 - r^2)}{\bar{c}(r_F)} \tag{9}$$

and

$$\lim_{c \to 0} \Omega = \frac{a_1(r_F)}{\bar{c}(r_F)} \approx \frac{c_1(r_F)}{\bar{c}(r_F)} \tag{10}$$

In these equations, the subscript 1 indicates the monomer of molecular weight M_1, $\phi_1 = (1 - \bar{\nu}_1\rho)\omega^2/2RT$, r_F refers to a convenient reference position in the cell, and a_1 to the activity of monomer. At the low protein concentrations and near isoelectric conditions of these components, it is assumed that $a_1 \approx c_1$. Evaluation of Ω requires an assumption of the molecular weight value of the monomeric species involved.

If the system is free from thermodynamic nonideality, then the limiting value of Ω allows the evaluation of the monomeric concentration at the reference position and, therefore, throughout the solution column. Thus, the weight fraction of monomer f_1 $(= (c_1/\bar{c}))$, as a function of total concentration $\bar{c}$ and monomer concentration c_1, may be obtained. To check for the occurrence of the reaction,

$$n\mathrm{M}_1 \rightleftharpoons \mathrm{M}_n \tag{11}$$

the plot $(1 - f_1)/f_1$ vs. $(c_1)^{n-1}$ may be used. A linear plot through the origin, with $(n - 1)$ assuming a particular integral value, then yields the value of n (see, e.g., Chun et al., 1972). Alternatively, indefinite association is tested by the occurrence of a linear plot of $(1 - (f_1)^{1/2})$ vs. c_1.

Two particular difficulties have arisen. Firstly, in attempting the extrapolation of Ω to zero concentration, irregularity in the curve with upturning of the curve at low concentrations has occurred in some cases and particularly where other evidence suggests the occurrence of low-molecular-weight impurity. Secondly, very significant linear plots of $(1 - f_1)/f_1$ against c_1^3 have been noted but such plots do not pass through the origin.

Results and Discussion

The preparations of CM-hLH, α subunit, and β subunit used for these studies were not homogeneous (Figure 2) by polyacrylamide gel electrophoresis. Fresh preparations of CM-hLH showed three major components, presumably the three biologically active components isolated by Peckham and Parlow (1969) and by Rathnam and Saxena (1971) and three of the four components present in Roos et al. (1975) preparation.

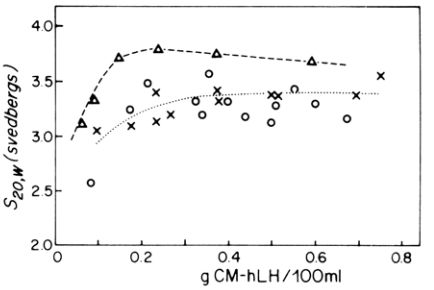

FIGURE 3: Plot of $s_{20,w}$ vs. protein concentration of CM-hLH preparation in the following phosphate–NaCl buffers: (X) $I = 0.1$, pH 5.85; (O) $I = 0.2$, pH 5.90; (Δ) $I = 0.5$, pH 5.45.

Since these components have been shown to be closely similar in structure with only minor differences in amino acid or carbohydrate composition (Peckham and Parlow, 1969; Rathnam and Saxena, 1971; Roos et al., 1975), CM-hLH was used for these studies without further purification. The α subunit used in this work contained one main electrophoretic component with a significant proportion of faster moving material and a trace of slower moving CM-hLH. The β subunit possessed two main components moving more slowly than the hLH components with a trace of material which was probably residual hLH. It is worth mentioning that a sample of CM-hLH which had stood for several months in the dry state gave, in addition to three main bands, additional bands corresponding in position with those for α and β subunits.

Human Luteinizing Hormone. A study of the sedimentation velocity behavior of CM-hLH as a function of protein concentration and ionic strength shows unusual features. Figure 3 indicates that, indeed, some type of association–dissociation occurs which is a function of both the concentration of the hormone and the ionic strength of the medium. In all cases the schlieren peaks were well-defined and symmetrical and remained so throughout the experiment. The absence of significant quantities of more rapidly sedimenting or trailing material was noteworthy.

At all ionic strengths, the sedimentation coefficients were found to increase with concentration at low concentrations, but above a certain concentration level were found to remain constant or decrease slowly. The low concentration increase was most gradual for $I = 0.1$, pH 5.85, little change being observed above 0.40 g/100 mL where a value of 3.4 S was obtained. At a concentration of 0.04 g/100 mL, Roos et al. (1975) obtained a value of 3.2 S and 3.4 S for components II and III, respectively. However, a direct comparison cannot be made since these measurements were performed at pH 7.0. At $I = 0.2$, and pH 5.90, the scatter of the experimental points was unusually large. Diffusion coefficients were evaluated at $I = 0.1$ for protein concentrations of 0.4 and 0.2 g/100 mL as 6.45 and 8.4×10^{-7} cm^2 s^{-1}, respectively. In both instances a plot of $(A/H)^2$ as a function of time was linear, indicating that the samples studied were homogeneous (Squire and Li, 1961). Using for the corresponding sedimentation coefficients the values 3.4 and 3.2 S, the calculated molecular weights are 44 300 and 32 000. The lower $s_{20,w}$ value below 0.2 g/100 mL indicates some dissociation of the LH molecule. At $I = 0.5$, the sedimentation vs. concentration curve is displaced to higher s values by approximately 0.4 S unit. It would seem that, at the higher protein concentrations, the higher ionic strength (0.5) tends to promote association to a higher level than the lower ionic strengths. The sedimentation velocity results of Figure 3 as a whole indicate complex behavior by the CM-hLH molecule.

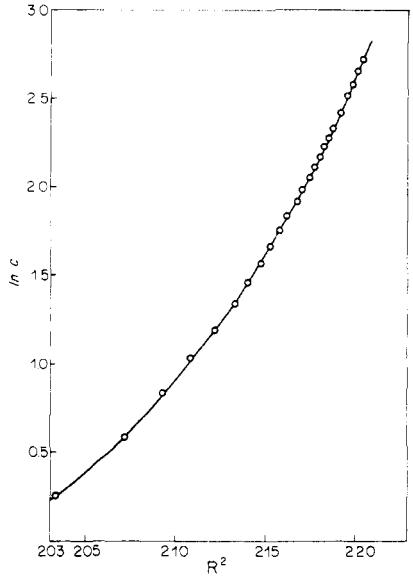

FIGURE 4: Sedimentation equilibrium plot of ln c (c in fringes) vs. R^2 (R = magnified radial distance) for CM-hLH preparation in phosphate–NaCl at I = 0.2, pH 5.90 at 20 °C. Speed = 18 000 rpm.

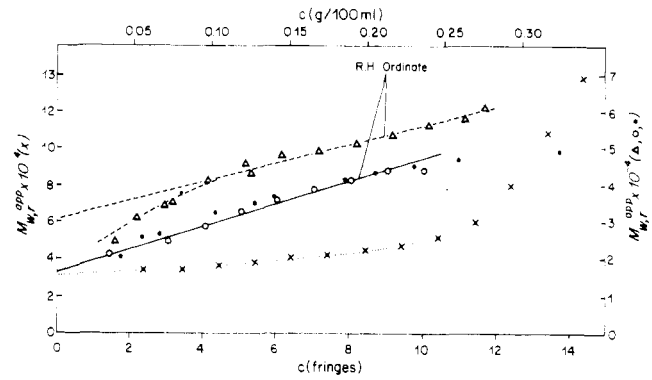

FIGURE 5: Plot of $M_{w,r}^{app}$ vs. c (c in fringes and g/100 mL) from sedimentation equilibrium for CM-hLH preparation in the following buffers. (X) I = 0.1, pH 5.85; (○) I = 0.2, pH 5.70, fresh freeze-dried material, initial loading concentration = 0.92 mg/mL; (●) I = 0.2, pH 5.90, 3 year old freeze-dried material, initial loading concentration = 4.0 mg/mL; (△) I = 0.5, pH 5.45.

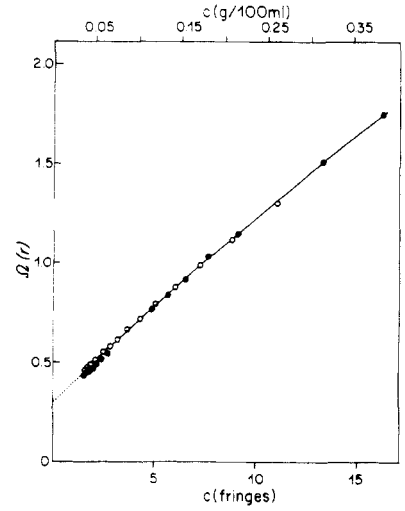

FIGURE 6: Plot of Ω function (eq 9) vs. concentration for CM-hLH in phosphate–NaCl buffer at I = 0.2, pH 5.85 at 20 °C at 16 000 (●—●) and 18 000 (○—○) rpm.

Sedimentation equilibrium measurements are relevant in clarifying the nature of the molecular processes involved. Since these experiments were performed at protein concentrations below 0.5 g/100 mL, at appreciable ionic strength and pH values near isoelectric (pH ca. 6.4; Rathnam and Saxena, 1970), it is thought that deviations from ideality will be small. In all cases, plots of ln c vs. R^2 (Figure 4) were distinctly curved and concave to the ordinate axis. Figure 5 summarizes the molecular weight data for I = 0.1, 0.2, and 0.5. At I = 0.1, the limiting value of $M_{w,r}^{app}$ indicates that the molecular weight 32 000–34 000 is valid at low concentrations (<0.10 g/100 mL), in general agreement with the value obtained from sedimentation and diffusion measurements at low concentrations and with that reported by Roos et al. (1975) from composition data. The presence of significant quantities of degradation products thus seems excluded. However, significant aggregation occurs at higher protein concentrations which, as shown by sedimentation velocity analysis, cannot be ascribed to irreversible aggregation or heterogeneity of the sample. At I = 0.2, $M_{w,r}^{app}$ values also vary with protein concentration and at very low concentration (<0.10 g/100 mL) fall considerably below 28 000, the approximate sum of the molecular weights of the α and β subunits, as given in previous work (Bishop and Ryan, 1973). Thus, dissociation of the CM-hLH molecule at low protein concentration seems to occur. At higher protein concentration, values considerably higher than 28 000 are observed. A similar situation appears to occur at I = 0.5, where even higher $M_{w,r}^{app}$ values are obtained at protein concentrations greater than 0.2 g/100 mL. Chervenka (1966) type plots give general confirmatory evidence.

The most detailed experimental study was performed at I = 0.2, pH 5.85, from which the relevant curve of Figure 5 is taken. The presence of two sets of symbols should be noted. The good agreement between them, together with the sedimentation velocity analysis and with the straight line behavior of plots of $(A/H)^2$ vs. time, suggests strongly the absence of significant quantities of irreversibly aggregated or degraded material. $M_{w,r}^{app}$ values range continuously from about 25 000 at 0.05 g/100 mL to more than 50 000 at 0.3 g/100 mL with signs of reaching a limiting value. It is clear that a polymerizing unit smaller than 28 000 has to be envisaged. Accordingly, and in view of complications later described for α and β subunits, a simple model assuming the polymerization of a single 14 000 unit has been investigated. Accordingly, the Ω function (eq 9) was evaluated and plotted against concentration. Figure 6 contains such plots for two runs at different speed; though at low concentrations, there is some divergence of calculated points for the two experiments, this is considered to be within experimental error. The extrapolation limit was used to obtain $a_1(r_F) \approx c_1(r_F)$ (eq 10) from which c_1 and therefore f_1 were deduced throughout the cell. Plots of $(1 - f_1)/f_1$ against c_1, c_1^2, c_1^3, etc. were therefore made and of $(1 - (f_1)^{1/2})$ against c_1. Figure 7 shows a plot of $(1 - f_1)/f_1$ against c_1^3 which is linear over most of the concentration range (<0.25 g/100 mL), indicating that a tetramer of M_1 = 14 000 is formed. However, some reservation must be expressed since the plot does not pass through the origin. It should be mentioned that the assumption of M_1 values other than 14 000 (e.g., M_1 = 14 000 ± 1000) does not affect the linearity of $(1 - f_1)/f_1$ plots, though clearly the slopes observed are modified.

At higher ionic strength, I = 0.5, it is clear from Figure 5 that molecular weights in excess of a tetramer occur so that a plot of $(1 - f_1)/f_1$ vs. c_1^3 was found to deviate from linearity. Nor did a plot of $(1 - (f_1)^{1/2})$ vs. c_1 prove linear, which would have indicated indefinite association. It seems possible that

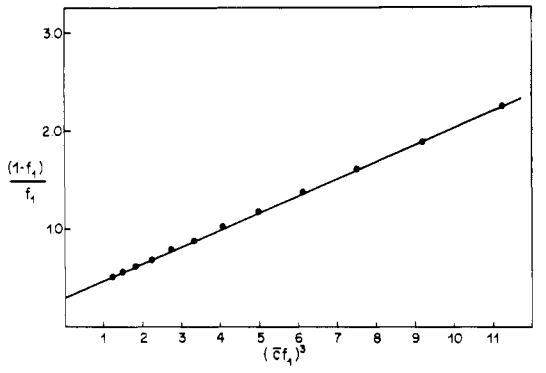

FIGURE 7: Plot of $(1 - f_1)/f_1$ vs. $(\bar{c}f_1)^3$ to test the model $4M \rightleftharpoons M_4$ for CM-hLH in phosphate–NaCl buffer at $I = 0.2$, pH 5.85 and 20 °C.

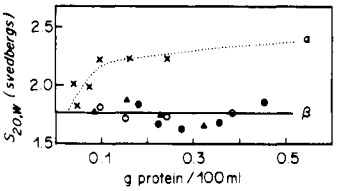

FIGURE 8: Plot of $s_{20,w}$ vs. protein concentration for α (X) and β (O, ▲, ●) subunits in phosphate–NaCl buffer at $I = 0.1$, pH 5.85. (Different symbols indicate different preparations.)

both mechanisms may occur simultaneously at the higher ionic strengths. However, since it is later suggested that both α and β subunits alone tend to undergo association reactions, it is clear that the complete description of the hLH system must also include consideration of the behavior of the α and β subunits alone. This will require detailed knowledge of the individual association reactions which is not yet available.

Protein association–dissociation reactions are known to be very widespread and it would seem that they occur with some frequency amongst the pituitary glycoprotein hormones. In addition to the studies reported here on hLH, ox TSH (Shome et al., 1968), human FSH (Papkoff et al., 1967; Rathnam and Saxena, 1972), and ovine FSH (Papkoff et al., 1967) have also been reported to show concentration-dependent aggregation. It is relevant to note here that the concentration of the various hormones in the blood is apparently of the order of 10^{-6} g/100 mL. Whether the dissociation into subunits observed under these experimental conditions occurs also in the blood cannot be ascertained at this point. The concentration at the target tissue may, however, be much greater, in which case the association phenomenon obtained in these studies could well occur there.

α Subunit. The sedimentation velocity behavior of the α subunit of CM-hLH as a function of protein concentration at pH 5.85 and $I = 0.1$ is shown in Figure 8. The $s_{20,w}$ value of the subunit increases markedly with increasing concentration of the protein above a concentration of 0.05 g/100 mL, indicating probably that aggregation of some type is taking place. At higher concentrations, a much more gradual increase is observed. Thus, extrapolation to zero concentration is not ordinarily meaningful. A single symmetrical schlieren peak was observed at all protein concentrations studied.

$D_{20,w}$ values of 7.13×10^{-7} and 7.22×10^{-7} cm^2 s^{-1} were obtained for the α subunit at concentrations of 0.35 and 0.22 g/100 mL, respectively. The corresponding sedimentation coefficients at these concentrations were 2.32 and 2.23 S. Molecular weight values of 25 500 and 25 400 were calculated from these values using eq 1 with $\bar{\nu} = 0.71$, but these are not

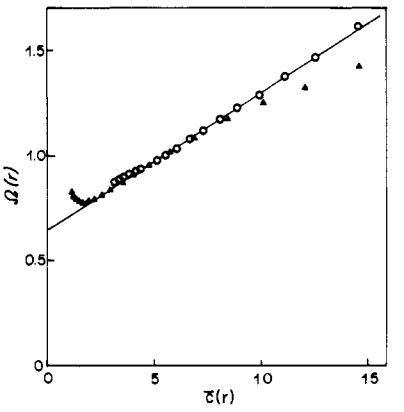

FIGURE 9: Plot of Ω (eq 9) function vs. $\bar{c}$ for α subunit in phosphate–NaCl buffer at $I = 0.1$, pH 5.85, at 20 °C. Preparation and speed (rpm) are respectively: (O) fresh, 16 200; (▲) aged, 24 000.

to be regarded as accurate since concentration effects have been neglected. A plot of $(A/H)^2$ vs. time was linear in both instances. It was not possible to obtain reliable diffusion coefficients at concentrations lower than 0.1 g/100 mL where a change in the slope of the $s_{20,w}$ vs. concentration plot is observed so that the corresponding molecular weights are not available.

Sedimentation equilibrium work is reported for a fresh preparation of the α subunit of CM-hLH at $I = 0.1$ and pH 5.85. The curve of ln c vs. R^2 is concave upward, thus indicating polydispersity or the presence of association reactions. The plot of $M_{w,r}{}^{app}$ vs. concentration was linear and extrapolation to zero concentration leads to an apparent molecular weight of 11 000. Neglecting the small possible deviations from ideal solution behavior, a value of nearly 40 000 at the bottom of the cell is obtained. This together with the anomalous $s_{20,w}$ curve of Figure 8 would seem to indicate that association of the α subunit to dimers and higher aggregates occurs. Chervenka (1966) type plots also showed pronounced concavity toward the ordinate axis, the calculated $M_{z,r}{}^{app}$ values varying from approximately 15 000 near the meniscus to values in excess of 40 000 near the cell base. It is of interest that Papkoff and Ekblad (1970) obtained a polymeric form of the α subunit of ovine FSH, although detailed studies of the association have not been performed. Ultracentrifugal studies by Pierce and co-workers (1971) on the α subunit of ox TSH also indicate that mobile equilibria exist between monomers and aggregates of this subunit. The studies of Bewley et al. (1974) on ovine α subunit indicated that the system was nonaggregating as judged by sedimentation velocity experiments. However, as shown later for the β subunit of hLH this could be misleading.

In extrapolating the function Ω (assuming $M_1 = 14\,000$) to zero concentration (Figure 9) for a fresh α preparation, a slight upturn from the calculated points at low concentration was observed which made extrapolation uncertain. However, for a similar examination of an old α sample (stored in the dry state at room temperature for 3 years), the upturn of calculated points at low concentration was more pronounced though coincidence of points is obtained throughout a significant portion of their common concentration range. It would thus seem that on long standing, in the dry state, a low-molecular-weight contaminant is formed in the α material. Using the extrapolation for the fresh α preparation, c_1 was calculated by means of equation 10 and $(1 - f_1)/f_1$ was plotted against different functions of c_1. A good approach to linearity was obtained from

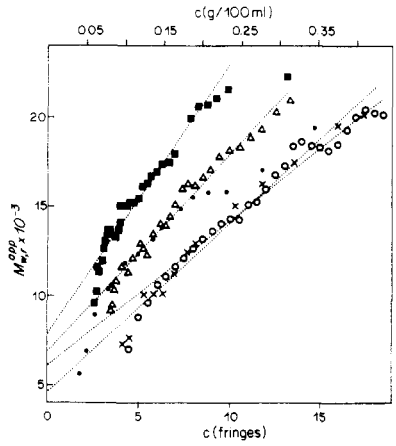

FIGURE 10: Plot of $M_{w,r}^{app}$ vs. c for β subunit from sedimentation equilibrium data in the following buffers: (△, ●) I = 0.1, pH 5.85; (x, ○) I = 0.2, pH 5.90; (■) I = 0.5, pH 5.45.

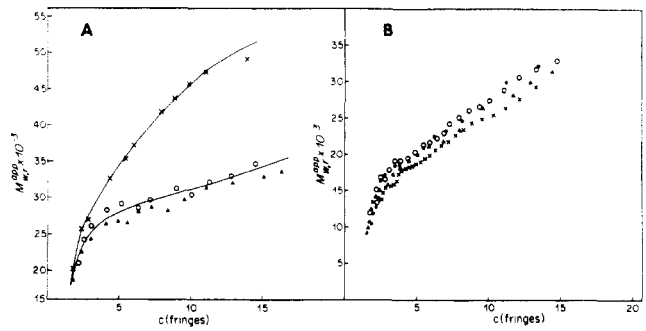

FIGURE 11: (A) Comparison of plots of $M_{w,r}^{app}$ vs. protein concentration, c (in fringes), from sedimentation equilibrium data for bLH and hLH. All runs were performed at I = 0.2, T = 20 °C, pH 6.0, and at an initial loading concentration of 4.0 mg/mL. Preparation and speed (rpm) are respectively: (X) hLH, 18 000; (○) bLH, 16 000; (▲) bLH, 18 000. (B) Plot of $M_{w,r}^{app}$ vs. protein concentration, c (in fringes), from sedimentation equilibrium for different runs of β-bLH. All runs were performed at I = 0.2, pH 6.0, and at an initial loading concentration of 4.0 mg/mL. Speed (rpm) and temperature (°C) are respectively: (X and ○) 20 000, 5; (▲) 21 996, 20; (●) 20 000, 20.

c_1[3], though again the line did not pass through the origin. The use of slightly different Ω intercepts did not impair linearity, but did affect the position of the line. A plot of $(1 - (f_1)^{1/2})$ vs. c_1 was not linear, thus, excluding indefinite association. The apparent conclusion of tetrameric formation is tentative and only by the use of fresh and highly purified preparations will a more definite conclusion be possible. As to the molecular weight of the α subunit, it is not possible from this work on its own to give a sound value, but the value 14 000 indicated by amino acid analysis (Stockell Hartree et al., 1971) would seem to be in general conformity with this work. Values in the literature vary between 11 800 and 15 750 (Bishop and Ryan, 1973) to 21 000 (Reichert and Lawson, 1973) and it would seem probable that the association reactions indicated above are responsible.

β Subunit. This hormone-specific subunit appeared from sedimentation velocity work to be a simpler system. As shown in Figure 8, the sedimentation velocity behavior of this subunit is largely independent of concentration, apparently indicating a monodisperse, nonaggregating, and nondissociating system. The schlieren peaks were symmetrical for all concentrations studied and an $s_{20,w}^0$ of 1.76 S is obtained.

Diffusion coefficients of 7.98×10^{-7} and 7.82×10^{-7} cm^2 s^{-1} were obtained at protein concentrations of 0.35 and 0.22 g/100 mL, respectively. The plot of $(A/H)^2$ vs. time was linear in both instances and an average molecular weight of 18 800 was calculated using eq 1 with $\bar{\nu}$ = 0.71.

Sedimentation equilibrium measurements rapidly removed the illusion of the simplicity of the β subunit. Measurements were performed at three ionic strengths with a pH of 5.9 ± 0.1 and an initial loading concentration of 0.3–0.4 g/100 mL. The layering technique was used exclusively for these experiments so that no assumption of conservation of mass was involved. In all cases, the plot of ln c vs. R^2 possessed definite curvature and molecular weights were calculated by the Yphantis least-squares procedure (1964). Figure 10 summarizes these calculations at the different ionic strengths. It is clear that, in each case, measured molecular weight values fall considerably below 14 000 at low concentrations as well as much higher at the higher concentrations. Evidence that the low values are due to the instability of the isolated β subunit was obtained by consideration of the Ω (eq 9) function. While this function for the hLH molecule varied monotonically with concentration allowing reasonable extrapolation, the curve for the β subunit showed very marked deviation upward, comparable though more marked than that for the α subunit. It should be mentioned that these β samples had been made 3 years previously though they had been stored in the dry state. A different preparation of β-hLH showed a much smaller deviation from monotonic behavior.[4] The apparent dissociation of β-hLH to give rise to an average molecular weight much less than 14 000 is difficult to reconcile with the suggestion of a definite amino acid composition (Stockell Hartree et al., 1971; Rathnam and Saxena, 1971) and a calculated molecular weight from its chemical composition of 14 407 (Bishop and Ryan, 1973). However, Reichert and Lawson (1973) have shown that, upon reduction of β-hLH with mercaptoethanol, a fast moving component (FMC) with a molecular weight less than 10 000 is visible in gel electrophoresis performed in the presence of sodium dodecyl sulfate. Their Figure 6 also suggests the complete loss of the original main β band and its replacement by faster moving material presumably with a molecular weight lower than 14 000 but higher than FMC. These findings together with the present work therefore throw considerable doubt upon the stability of the β subunit, which can only be resolved by further work. Nor can the presence of a tightly bound component be excluded at this point.

The above findings concerning association reactions of both α and β subunits have considerable bearing on the slowness of recovery of biological activity on mixing α and β subunits (Stockell Hartree et al., 1971). Each subunit may be largely in an associated and unreactive form which undergoes reaction with its opposite partner only after dissociation.

Comparison of Human and Bovine LH Systems. Through the kindness of Dr. J. G. Pierce who gave us samples of bovine LH and its β subunit, a comparison has been made of the human and bovine LH systems. Bovine LH in phosphate-NaCl at I = 0.2, pH 6.0, gave molecular weights from sedimentation equilibrium which varied continuously from much less than 28 000 at low concentration (<0.3 g/100 mL) with signs of approach to limiting values (Figure 11A). Treating this system as for hLH with a monomer of M_1 = 14 000, it was found that a monomer–tetramer model was not applicable but there was support for monomer ⇌ trimer. Similarly (Figure 11B) the β subunit under similar conditions gave molecular

[4]This sample refers to preparation LER-1793-B. It had been prepared at least 1 year prior to this work. Dr. L. E. Reichert, personal communication.

weights ranging from considerably less than 14 000 at low concentration (<0.05 g/100 mL) to greater than 30 000 at higher concentrations (>0.30 g/100 mL). After evaluating the Ω function, extrapolation to zero concentration was rendered doubtful by the upturn of the calculated values, as in the case of human β subunit. Analysis of the system has not therefore been carried further. However, as shown in Figure 11A, it is clear that in its main features the bovine LH system is similar to the human, but the dissociation and association reactions differ quantitatively for the two systems.

Acknowledgments

The authors thank Dr. A. Stockell Hartree for supplying fraction DEAE-I of hLH which was used for further purification and the subsequent work reported in this paper. We also thank Dr. J. G. Pierce for samples of bLH and its β subunit and for fruitful discussions with him. A sample of β-hLH LER-1793-B was a gift of the National Institute of Arthritis, Metabolism and Digestive Diseases and the National Pituitary Agency of the University of Maryland. Thanks are due also to Mrs. R. Shownkeen and to Mr. D. Reed who has provided excellent technical assistance in the ultracentrifugal experiments.

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