Purification and Characterization of Follicle-Stimulating Hormone from Sheep Pituitary Glands*

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ABSTRACT: Purified follicle-stimulating hormone (FSH) was prepared from extracts of fresh sheep pituitary glands by successive chromatographies on Sephadex G-100 and DEAE-Sephadex. The FSH obtained was essentially homogeneous as indicated by chromatography on Sephadex G-100, disc electrophoresis using polyacrylamide gel, and ultracentrifugal analysis. Its sedimentation constant $s_{20,w}$ was 2.63 S and the molecular weight was estimated to be 32,500 ± 1500 by ultracentrifugal analysis and chromatographic analysis on Sephadex G-100.

The FSH activity of this preparation was approximately 44 times that of the NIH-FSH-S1 standard as determined by the Steelman–Pohley assay [Steelman, S. L., and Pohley, F. M. (1953), *Endocrinology 53*, 604], and there was a low level of luteinizing hormone (LH) activity as indicated by the ovarian ascorbic acid depletion assay.

Several methods have been reported during recent years for obtaining purified FSH[1] preparations from sheep pituitary glands (Steelman and Segaloff, 1959; Ellis, 1958; Duraiswami *et al.*, 1964; Papkoff *et al.*, 1964). These preparations were not studied extensively from the physical and chemical standpoints. In a previous paper (Hashimoto *et al.*, 1965), preliminary results were reported on the preparation of FSH which was shown to be homogeneous by polyacrylamide gel electrophoresis and ultracentrifugal analysis. The present report gives additional information on the purification of this FSH, and its biological and physicochemical properties.

Materials and Methods

Materials. Fresh frozen whole sheep pituitary glands were obtained from Pitco Biologicals, N. Y., and stored at −18° prior to extraction.

Gel Filtration and Ion-Exchange Chromatography. Sephadex G-100 (bead form) and DEAE-Sephadex A-50 (medium) were used for the purification of the FSH. The size of the column was chosen according to the amounts of sample to be applied.

Disc Electrophoresis. Disc electrophoresis in polyacrylamide gel was carried out at 4° and pH 8.6 according to the procedure of Davis (1964) and Ornstein (1964), and in acetate buffer at pH 4.5 according to the method of Reisfeld *et al.* (1962).

Ultracentrifugal Analysis. The FSH preparation (5–10 mg) was dissolved in 0.5–1.0 ml of 0.02 M, pH 5, acetate buffer containing 0.1 M sodium chloride, and dialyzed against the same buffer for 4–12 hr. If a precipitate formed, it was removed by centrifugation at 10,000 rpm for 15 min. This precipitate was presumably denatured hormone since it was inactive but had the same amino acid composition as the active soluble hormone. The solution outside the dialysis tubing was used as the control. The procedure for ultracentrifugal analysis is described in the Appendix.

Chromatographic Analysis on Sephadex G-100. Determination of the molecular weight of the FSH preparation was performed at 4° by the method of Whitaker (1963) using gel filtration on Sephadex G-100. Column dimensions were 1.1 × 60 cm and the flow rate was regulated to 4.5 ml/hr. The eluents were 0.01 and 0.1 M acetate buffers of pH 6.0 containing 0.1 M sodium chloride. Waldenström's macroglobulin (human γ^M-globulin) used for the determination of the void volume V_0 and human γ^G-globulin as reference were provided by Drs. H. F. Deutsch and T. Suzuki, Department of Physiological Chemistry, University of Wisconsin. The molecular weights of the γ^M-globulin and γ^G-globulin were estimated by them to be near 750,000 (Kovacs and Daune, 1961) and 160,000 (Williams *et al.*, 1952), respectively. The bovine serum albumin, pepsin (each two times crystallized), and trypsin (three times crystallized) used as references were obtained, respectively, from the Nutritional Biochemicals Corp., the Sigma Chemical Co. and the Worthington Biochemical Corp.

Ultraviolet Absorption Spectrum. The absorption spectrum of a 0.0396% solution of FSH in 0.02 M, pH 5.0, acetate buffer containing 0.1 M sodium chloride was measured at 25° on a Beckman DU spectrophotometer. The buffer solution was used as the blank.

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[1] The abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; OAAD, ovarian ascorbic acid depletion.

TABLE I: Purification of Follicle-Stimulating Hormone from Sheep Pituitary Glands.

Fractionation Procedure		Protein g/kg	Activity	
Step	Fraction	Fresh Tissue	Units[a]	Recov[b] (%)
I	Extraction extract E	4.69 ± 0.57 (10)[c]	0.45	100
II	Chromatography on Sephadex G-100 in NaCl (0.05 M)			
	II-F2	1.80 ± 0.14 (10)		96
	Rechromatography in NaCl (0.004 M)			
	II-F2-1[d]	0.76 ± 0.16 (5)	2.6	76
	II-F2-2	0.76 ± 0.10 (5)		17
III	Chromatography on DEAE-Sephadex in phosphate (M)			
	III-F1 (0.01)	(3)		
	III-F2 (0.02)	0.004 (3)		
	III-F3 (0.05)	0.031 (3)		1
	III-F4 (0.075)	0.087 (3)		19
	III-F5[e] (0.10)	0.013 (3)		20
	III-5a	0.018 (3)		
	NaCl (0.10) III-F6 (0.025)	0.352 (3)		12
IV	Chromatography on Sephadex G-100 in NaCl (0.004 M)			
	IV-F2	0.011 (3)		22
	Rechromatography on G-100 in acetate (0.025 M)			
	IV-F2-1	0.011 (2)	17	11
	IV-F2-2 (FSH)	0.0044 (2)	44	11

[a] One unit is equivalent to the FSH activity in 1 mg of NIH-FSH-S1 standard as determined by the method of Steelman and Pohley (1953). [b] Based on the increase in the weight of the ovaries of intact immature rats. [c] Figures in parentheses indicate the number of experiments on which the results are based. [d] Used in step III. [e] Used in step IV.

Protein. The protein content of the fractions was determined by absorption at 280 mμ and by the biuret method (Gornall *et al.*, 1949) using two-times-crystallized bovine serum albumin as a standard. The nitrogen content was determined by the Kjeldahl method as modified by Hiller *et al.* (1948).

Biological Activity. The gonadotropic activities of the fractions obtained in each step during purification were estimated on the basis of the increase in the weight of the ovaries of intact immature female rats. The percentage values given in Table I are based on these estimations. Extract E, fraction II-F2-1, and the FSH preparations, IV-F2-1 and IV-F2-2, were assayed by the method of Steelman and Pohley (1953). The latter two FSH preparations were also assayed by the increase in the weight of the ovaries and ventral prostates of hypophysectomized 29-day-old rats (Greep *et al.*, 1942), the ovarian ascorbic acid depletion (OAAD) method of Parlow (1961), and for thyroid-stimulating hormone (TSH) activity by the method of Greenspan *et al.* (1956) which is dependent on the uptake of ^{32}P by the chick thyroid. The intact rats were obtained from the Holtzman Co., Madison, Wis., and the hypophysectomized rats from the Hormone Aassy Laboratories, Chicago, Ill. The preparations of FSH and LH used as standards were supplied by the Endocrinology Study Section, National Institutes of Health. One unit of FSH and 1 unit of LH are equivalent in activities, respectively, to 1 mg of NIH-FSH-S1 and NIH-LH-S1 standards.

Experimental Procedure and Results

Purification. The purification procedures and results of the fractionation of gonadotropic extract E to obtain the FSH are summarized in Table I. The operations were carried out at 4° unless indicated otherwise.

STEP I. EXTRACTION. The fresh glands (1 kg) were ground finely and extracted by a modification (Duraiswami *et al.*, 1964) of the method of Koenig and King (1950). The hormone and other proteins were precipitated at −18° by the gradual addition of acetone to extract E, recovered, and dried by lyophilization. The FSH activity of this extract is expressed in terms of the NIH-FSH-S1 standard (Table I).

STEP II. CHROMATOGRAPHY ON SEPHADEX G-100. The FSH in the above extract was partially purified by two consecutive chromatographies on Sephadex G-100. The extract was dissolved in 30 ml of 0.05 M sodium chloride and centrifuged, and the supernatant

was applied to a 6 × 96 cm column of Sephadex G-100 equilibrated with 0.05 M sodium chloride. Fractions of 12–14 ml were collected/15 min. The activity was determined by administration of aliquots pooled from each of four consecutive tubes to intact immature female rats. The tubes showing gonadotropic activity were combined to give fraction II-F2 (Figure 1 and Table I) which was assayed and lyophilized. A 2.5-fold purification was accomplished by this operation. Fraction II-F2 was rechromatographed on a column of Sephadex G-100 (4.6 or 6.0 × 115 cm) which was equilibrated with 0.004 M sodium chloride. The chromatogram consisted of two main peaks of activity which were recovered separately to give fractions II-F2-1 and II-F2-2 (Table I).

STEP III. CHROMATOGRAPHY ON DEAE-SEPHADEX. The FSH of fraction II-F2-1 was further purified by chromatography on DEAE-Sephadex. The quantities of protein applied to the columns ranged from 50 mg to 1 g. The size of the columns was proportional to the amount of protein chromatographed. Buffer systems consisting of bisodium phosphate or potassium sodium phosphate of pH 7.0 were used. A sample of 658 mg of protein was dissolved in 0.01 M phosphate buffer, dialyzed against this buffer, and applied to a 4.2 × 50 cm column previously equilibrated with the same buffer. The column was developed first with this buffer, then stepwise with 0.02, 0.05, 0.075, and 0.1 M, pH 7.0, phosphate buffers, and finally with the 0.1 M phosphate containing 0.25 M sodium chloride. The chromatogram is illustrated in Figure 2 and the results are summarized in Table I. The fractions eluted with 0.01, 0.02, and 0.05 M phosphate buffers (fractions III-F1, F2, and F3) showed little FSH activity, but the major part of the LH activity was recovered in these fractions. The main FSH fraction of low protein content was eluted with 0.1 M phosphate buffer and recovered in fraction III-F5. FSH activity and a low level of LH activity were also recovered in fractions III-F4 and F6.

STEP IV. FINAL PURIFICATION. Fraction III-F5 which showed high FSH activity was desalted and fractionated on a 3.4 × 70 cm column of Sephadex G-100 equilibrated with 0.004 M sodium chloride. The major part of the FSH activity was recovered in the second peak designated as fraction IV-F2. The final purification was effected by passing fraction IV-F2 through a column of Sephadex G-100 equilibrated with 0.025 M acetate buffer at pH 5.0 or 0.01 M acetate buffer at pH 6.0 containing 0.1 M sodium chloride. Two protein absorption peaks (Figure 3) were obtained. The most highly purified FSH from the biological and chemical standpoints was recovered in fraction IV-F2-2 (Table I).

Biological Studies. The recoveries of the gonadotropic activities of the different fractions are given in Table I as percentages of the activity of extract E. These percentage values are based on the increase in the weight of the ovaries of intact female rats and are rough estimations of the gonadotropic activity of the fractions. The rather great loss in over-all gonadotropic activity from the purified fractions is due to the removal of LH from the fractions during the purification and to loss of activity during fractionation.

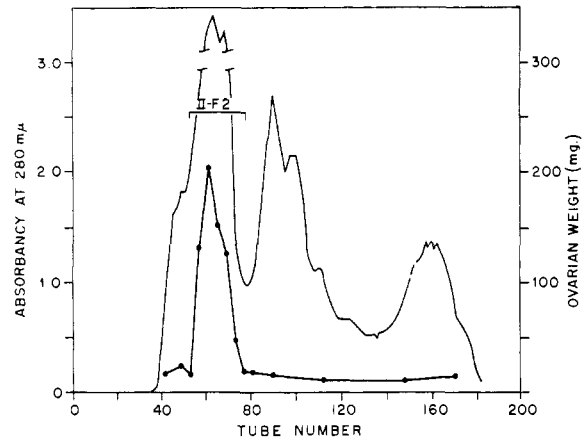

FIGURE 1: Chromatography of extract E from sheep pituitary glands (step I) on Sephadex G-100 in 0.05 M sodium chloride solution. Column dimensions were 6 × 96 cm and 14 ml was collected/tube per 15 min. ———, absorbancy at 280 mμ; –·–·–·–, weight of ovaries in milligrams. The brackets denote the fractions that were pooled to give II-F2 for subsequent rechromatography.

Results of assays of the FSH preparation (fraction IV-F2-2) using intact and hypophysectomized rats are summarized in Table II. When total doses of 7, 15, and 20 μg of FSH were given to intact female and male rats, high FSH activity and a low LH response

TABLE II: Biological Action of Purified Follicle-Stimulating Hormone.[a]

Rats		Total dose of Protein (μg)	Activity	
			FSH Ovaries (mg)	LH Ventral Prostates (mg)
Kind	Age[b]			
Intact	21	0 (saline)	16	35
		7	52	33
		15	109	41
		20	114	43
Hypophysectomized	29	0 (saline)	10	11
		9	44	
		18	56	20
		100		25

[a] FSH fraction IV-F2-2 (Table I). Results from assays using three to five rats per level of hormone. [b] Age at time injections were started.

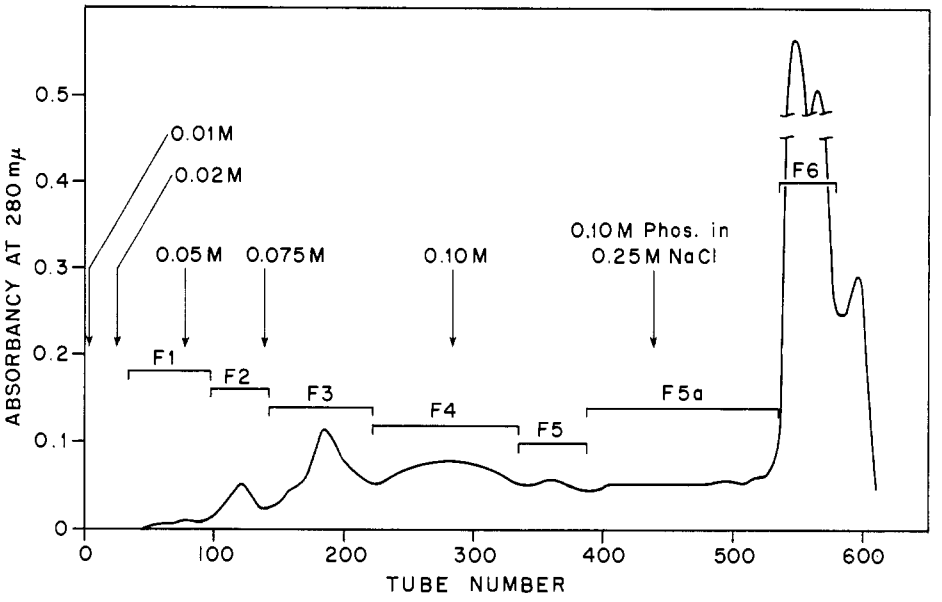

FIGURE 2: Fraction II-F2-1 (658 mg) obtained from rechromatography on Sephadex G-100 (step II) was chromatographed on a 4.2 × 50 cm column of DEAE-Sephadex. The initial buffer was 0.01 M phosphate at pH 7.0 followed by stepwise elution with pH 7 phosphate buffers of increasing concentrations as indicated. The eluate was collected at the rate of 6 ml/tube per 20 min. Tubes within the brackets were combined to give the fractions indicated.

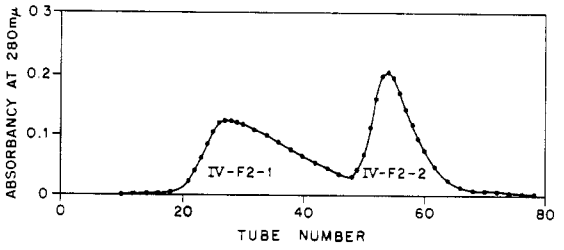

FIGURE 3: Gel filtration of partially purified FSH fraction IV-F2. Three milliliters of fraction IV-F2 corresponding to 12 mg of protein was chromatographed on a 2.8 × 44 cm column of Sephadex G-100 equilibrated with 0.025 M, pH 5.0, acetate buffer. Fractions of 3.0 ml were collected.

were obtained as indicated by the great increase in the weight of the ovaries and the slight increase in the weight of the ventral prostates. Similar results were obtained when hypophysectomized rats were used.

Quantitative assays for FSH were done by the method of Steelman and Pohley (1953) and the results are given in Table I. When the FSH (IV-F2-2) was assayed at 2- and 4-μg levels the activity was 44 times the NIH-FSH-S1 standard whereas fraction IV-F2-1 was 17 times as active as the standard. Fractions IV-F2-2 and IV-F2-1 contained 0.06 and 0.42 unit of LH activity, respectively, in terms of the NIH-LH-S1 standard as determined by the ovarian ascorbic acid depletion method of Parlow (1961). TSH activity was not detected by the chick thyroid assay method (Greenspan *et al.*, 1956).

Physicochemical Characterization

The FSH preparation (fraction IV-F2-2) was used for the following physical studies.

Disc Electrophoresis. The homogeneity of the FSH preparation was indicated by disc electrophoresis using polyacrylamide gel at pH 8.6 in Tris–glycine buffer (Davis, 1964; Ornstein, 1964), and in acetate buffer at pH 4.5 according to the procedure of Reisfeld *et al.* (1962). The electropherograms showed one zone (R_F 0.60–0.63) at pH 8.6 (Figure 4) as reported by Hashimoto *et al.* (1965). The mobilities were close to the value for bovine serum albumin (R_F 0.6) which was used as a reference. Of the initial FSH activity, 40–60% was recovered by extracting the corresponding zones from unstained polyacrylamide gels. The FSH migrated little from the origin at pH 4.5 as determined by the method of Reisfeld *et al.* (1962).

Ultracentrifugal Analysis. The FSH obtained from two experiments was investigated for homogeneity using a Spinco Model E ultracentrifuge (see Appendix). The runs were made at 59,780 rpm using 0.24 and 0.14% solutions of FSH in pH 5.0, 0.02 M, acetate buffer containing 0.1 M NaCl. The sedimentation patterns are represented in Figure 5. The FSH appeared to be monodisperse and the $s_{20,w}$ values were calculated to be 2.63 S.

Gel Filtration on Sephadex G-100 Using the Method of Whitaker (1963). The FSH preparation (IV-F2-2) when chromatographed on Sephadex G-100 according

FIGURE 4: Disc electrophoresis of FSH fraction IV-F2-2 in polyacrylamide gel with pH 8.6 Tris–glycine buffer for 1.5 hr at 3 ma. The origin is indicated by O and the FSH zone by Z.

to the method of Whitaker (1963) showed a single sharp peak (Figure 6). The ratio of elution volume to void volume (V_e/V_0) of the reference proteins determined in this experiment is shown in Table III. As the molecular weight of the γ^M-globulin was confirmed by Deutsch and Suzuki (Materials and Methods) to be approximately 750,000 (Kovacs and Daune, 1961), the elution volume V_e of the γ^M-globulin was taken as the void volume V_0. This V_0 volume and the elution volumes V_e for the reference proteins were used in determining the V_e/V_0 values. When these values were plotted *vs.* the logarithms of the molecular weights for the reference proteins, a linear correlation was obtained with the exception of γ^G-globulin in the 0.01 M acetate–0.1 M sodium chloride buffer. On the basis of this relationship and V_e/V_0 values of 1.74 and 1.62 obtained for the hormone, molecular weights of 32,000 and 31,000 were found for the FSH (Table III).

Ultraviolet Absorption Spectrum. The absorbances of a 0.0396% solution of FSH were 0.400 at 255 mμ, 0.402 at 260 mμ, 0.451 at 277 mμ, 0.431 at 280 mμ, and 0.262 at 290 mμ. The curve is a typical absorption pattern of a protein solution with absorption maximum at 277 mμ and minimum at 255 mμ. There was essentially no increase in absorption at 290 mμ which is an indication of a lack of tryptophan, and this was confirmed by amino acid analysis. The absorbancy of a 1% FSH solution at 280 mμ was calculated to be 10.9. When the carbohydrate content (14%, unpublished data) of the FSH is considered, the absorbancy is calculated to be 9.4.

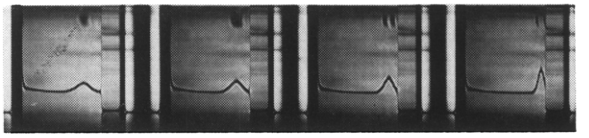

FIGURE 5: Ultracentrifugal pattern of the FSH preparation (fraction IV-F2-2) at a concentration of 0.24% in 0.02 M, pH 5.2, acetate buffer containing 0.1 M NaCl at 20° and 59,780 rpm. The picture shown was taken at a bar angle of 60° and at time intervals of 16 min. The direction of the sedimentation is toward the left.

TABLE III: V_e/V_0 Values and Molecular Weights of Proteins Used in Determining the Molecular Weight of Follicle-Stimulating Hormone.

Proteins	V_e/V_0 Values (pH 6.0 buffers) 0.01 M Acetate–0.1 M NaCl	0.1 M Acetate–0.1 M NaCl	Mol Wt[a]
Human γ^M-globulin	1.00	1.00	750,000
Human γ^G-globulin	1.24	1.22	160,000
Bovine serum albumin	1.38	1.41	70,000
Pepsin	1.69	1.58	35,500
Trypsin	1.85	1.68	23,800
FSH	1.74		32,000
FSH		1.62	31,000

[a] These molecular weights are reported in the following papers: γ^M-globulin (Kovacs and Daune, 1961), human γ^G-globulin (Williams *et al.*, 1952), bovine serum albumin (Phelps and Putnam, 1960), pepsin (Blumenfeld and Perlmann, 1959), and trypsin (Cunningham, 1954).

Discussion

The Koenig and King method (1950) was used for extracting both FSH and LH from fresh ground sheep pituitary glands. This extract provided a suitable starting material for the preparation of FSH by a method consisting of four steps (Table I). The purification was effected by gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephadex. The chromatography on DEAE-Sephadex requires approximately 5 days but it yields FSH of high purity and high activity as indicated by physical studies and bioassays, respectively. The activity of

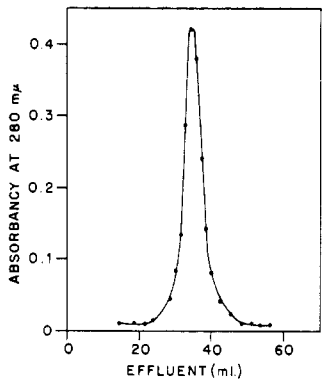

FIGURE 6: Chromatography of purified FSH on Sephadex G-100. One milliliter containing 8 mg of protein was applied to a 1.1 × 60 cm column equilibrated with 0.1 M, pH 6.0, acetate buffer containing 0.1 M sodium chloride. Fractions of 1.5 ml were collected/20 min.

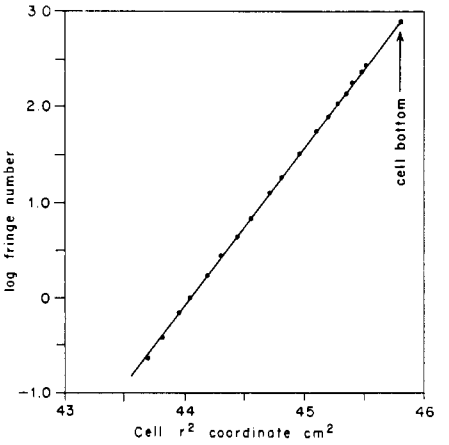

FIGURE 7: Sedimentation equilibrium of the FSH preparation in 0.02 M, pH 5.0, acetate buffer containing 0.1 M sodium chloride.

this FSH was 44 times that of the NIH-FSH-S1 standard as determined by the Steelman and Pohley bioassay (1953) (Table I) and 0.06 unit of LH based on the NIH-LH-S1 standard. The sum of the activities of the two FSH fractions (IV-F2-1 and IV-F2-2) obtained in the final step of the procedure was 20% of the total activity of the original extract E. Papkoff *et al.* (1964) reported FSH prepared by a different procedure which had essentially the same activity as the above preparation IV-F2-2.

Ellis (1958) and Steelman and Segaloff (1959) reported the purification of sheep FSH using columns of DEAE-cellulose which resulted in preparations 30–40 times as active as the Armour Standard which is equivalent to 11–14 units of NIH-FSH-S1 standard calculated on the basis of 2.8 Armour units/unit of NIH-FSH-S1. DEAE-cellulose was used by Duraiswami *et al.* (1964) to prepare sheep FSH 10 times as active as the NIH-FSH-S1 standard. The chromatography was carried out in the presence of 6 M urea and the final purification was done by starch gel electrophoresis. The degree of homogeneity of these preparations was not reported.

Physicochemical properties of FSH preparations from sheep pituitary glands have been studied by Li and Pedersen (1952) and Papkoff *et al.* (1964). The former reported a molecular weight of 67,000 for their FSH preparation (Li *et al.*, 1949) and its sedimentation coefficient $s_{20,w}$ was 4.7 S, but in a recent paper Papkoff *et al.* (1964) reported the sedimentation coefficient of sheep FSH as 2.5 S. The molecular weight of this FSH preparation was not reported.

The molecular weight of the FSH (fraction IV-F2-2) reported in this study was estimated to be 32,500 ± 1500 by the method of Whitaker (1963) and by ultracentrifugal analysis (see Appendix). Although the results from the physicochemical analyses indicate that this FSH preparation was essentially homogeneous, a low level of LH activity 0.06 times that of the NIH-LH-S1 standard was indicated by the ovarian ascorbic acid depletion assay.

Appendix

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The sedimentation velocity of FSH preparations obtained from sheep pituitary glands by the method described in this paper was studied by use of a Model E ultracentrifuge. The FSH preparation sedimented as a homogeneous species as shown in Figure 5. The sedimentation coefficients obtained at 20° after correction for solvent density and viscosity (0.02 M, pH 5.0, acetate buffer containing 0.1 M sodium chloride) at FSH concentrations of 2.4 and 1.4 mg/ml had the same value of 2.63 S, indicating little or no concentration dependence. On the basis of the estimation of the decrease in concentration by radial dilution, it is concluded that the wide spreading of the boundary at low concentration was not likely due to the heterogeneity of the protein preparation.

The method introduced by Yphantis (1964) for the determination of molecular weights with moderate precision and requiring only a small sample was used for the molecular weight determination. Samples at FSH concentrations lower than 2.4 mg/ml were added to the multichannel centerpiece. A solution column of approximately 3 mm was used. The rotor temperature was 20° and the speed was 29,500 rpm. The equilibrium was reached after 50 hr of centrifugation. Five fringes (three constructive bright fringes and two destructive dark interference fringes) were read along the radical distance from the center of rotation by use of a Gaertner microcomparator. For a homogeneous ideal system a plot of the logarithm

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of fringe displacement *vs.* the square of the radial distance should give a straight line. This was found to be true for the FSH preparation as shown in Figure 7. The weight-average molecular weights calculated from the slopes of the plots for two different FSH preparations each at 2.4 and 1.4 mg/ml were 31,100 and 33,900 g/mole, respectively, using 0.72 ml/g for the partial specific volume.

The partial specific volume was determined by a 50-cm linear density-gradient column made of mixtures of water saturated with monochlorobenzene and 1-chlorobutane (Linderström-Lang and Lanz, 1938). The column was calibrated with standard KCl solutions of known density. There is a degree of uncertainty in the partial specific volume because of the difficulties in determining the solute concentration accurately.

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