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Some Chemical and Physical Properties of Human Pituitary Follicle-Stimulating Hormone*

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ABSTRACT: A procedure has been developed for the purification of human follicle-stimulating hormone (FSH) which employs ammonium sulfate and pH fractionation, chromatography on sulfoethyl Sephadex C-50 and carboxymethyl Sephadex C-50, and gel filtration on Sephadex G-100. The purified human FSH has been characterized by disk and free-boundary electrophoresis, ultracentrifugation, terminal group analysis, and amino acid and carbohydrate content. The isoelectric point is located at pH 5.6. Terminal groups were found to be absent by the fluorodinitrobenzene and carboxypeptidase procedures. The hormone was found to contain 3.9% hexose, 2.4% hexosamine, 0.4% fucose, and 1.4% sialic acid. These results have been compared with those of ovine FSH.

Studies on the purification of human pituitary follicle-stimulating hormone (FSH)[1] have recently been reported (Roos and Gemzell, 1964, 1965; Amir *et al.*, 1966; Parlow *et al.*, 1965). These studies have all remarked on the instability of the hormone. The factors responsible for the observed losses of biological potency are not, however, clear at this time. Virtually nothing has been reported on the chemistry of human FSH, information which could possibly shed light on the instability of the hormone. In addition, the most successful of the above cited studies (Roos and Gemzell, 1964, 1965) leaves much to be desired because of the low yield of hormone obtained as well as the reliance on technically difficult methods of purification (*i.e.*, preparative polyacrylamide gel electrophoresis). We have endeavored to develop a simple purification procedure which would result in a good yield of highly purified FSH. The purification procedure and some of the properties of the product are described in this paper.

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[1] Abbreviations used: FSH, follicle-stimulating hormone; ICSH, interstitial cell stimulating hormone; GH, human growth hormone; FDNB, fluorodinitrobenzene.

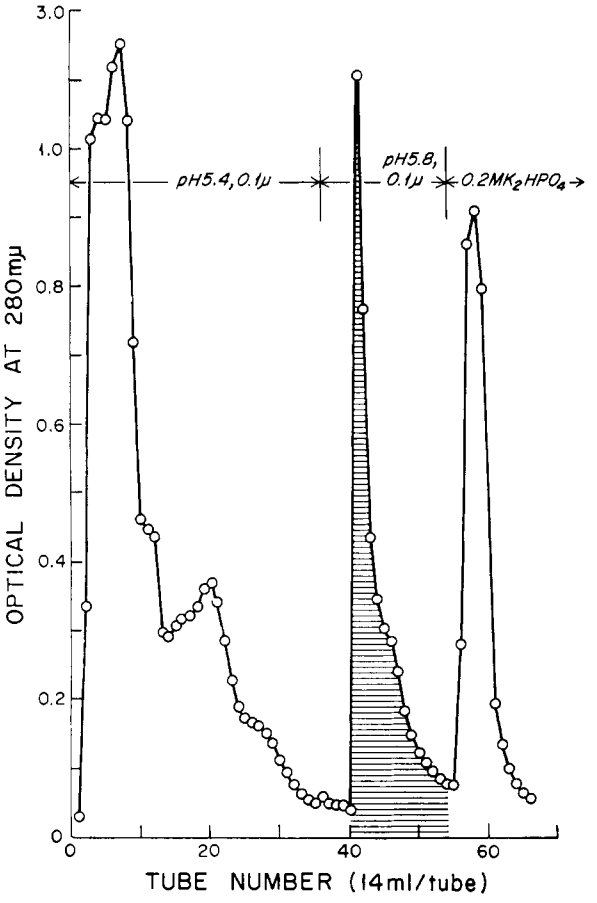

FIGURE 1: Chromatography of crude human FSH on a 2.5 × 21 cm column of sulfoethyl Sephadex C-50. Approximately 800 mg was applied to the column. FSH activity is found in the shaded fraction.

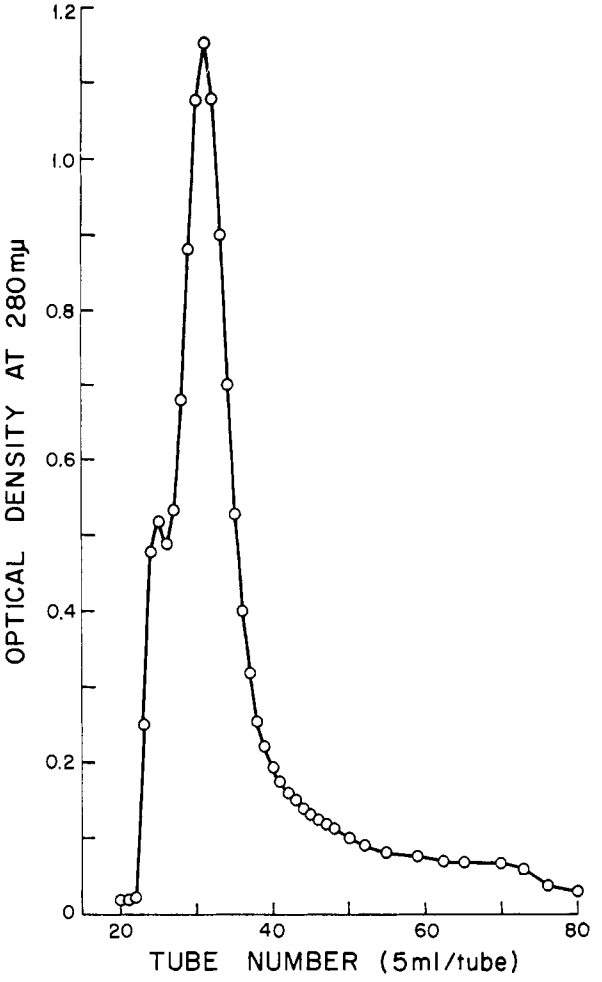

FIGURE 2: Gel filtration on a 2.5 × 90 cm column of Sephadex G-100 of partially purified human FSH. The column was equilibrated with a pH 5.4, 0.033 ionic strength acetate buffer. Approximately 75 mg was applied to the column.

Methods and Materials

The human pituitaries employed in these studies were obtained at autopsies and stored in the frozen state until used. Sulfoethyl Sephadex (SE-C-50), carboxymethyl Sephadex (CM-C-50), cation-exchange gels, and Sephadex G-100 were all products of Pharmacia, Inc., and prepared for use according to the manufacturer's directions. All column experiments were run at room temperature (22–25°). Effluents from columns were monitored for protein content by measurement of the absorbance at 280 mμ in a Beckman DU spectrophotometer. Dialyses were performed in a running tap water bath maintained at 1°. Final preparations were dialyzed in addition against changes of distilled water for 24 hr. FSH activity was determined by the augmentation test (Steelman and Pohley, 1953) and ICSH activity was measured by the ventral prostate test (Greep *et al.*, 1941). Free-boundary electrophoresis experiments were conducted at 1° in a Spinco Model H electrophoresis diffusion apparatus. Other methods employed in the characterization studies will be cited therein.

Purification

The initial steps in the purification of human FSH are similar to those previously employed for the extraction of human growth hormone (GH) (Li and Papkoff, 1956; Li *et al.*, 1962). Frozen human pituitaries are mixed with two volumes of 0.9% saline and homogenized in a Waring Blendor for 30 sec. The suspension is adjusted to a pH of 7.5 and stirred for 2 hr at 0°. The suspension is centrifuged to remove the insoluble residue and 311 g of $(NH_4)_2SO_4$ is added to the supernatant fluid. The precipitate which forms (ICSH and GH concentrates) is removed by centrifugation and 210 g of $(NH_4)_2SO_4$ is added to the supernatant fluid. Following centrifugation, the precipitate is dissolved in water, dialyzed, and lyophilized. This fraction has averaged 2.0 g/100 g of frozen glands and has been stored at room temperature in the lyophilized state.

Further purification is achieved with batches of approximately 10 g of the crude FSH fraction. In a

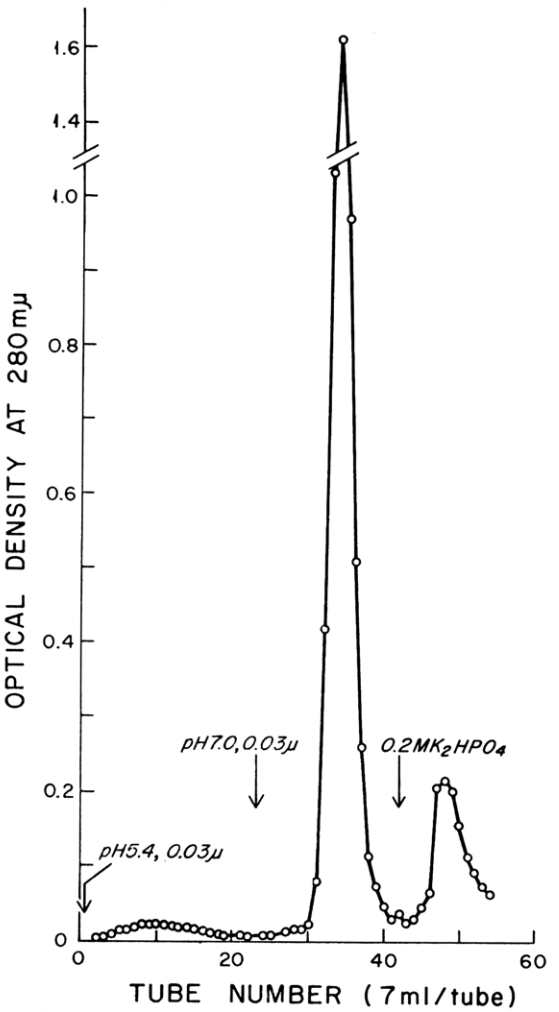

FIGURE 3: Chromatography of human FSH on a 2.5 × 16 cm column of carboxymethyl Sephadex C-50. Approximately 60 mg was applied to the column.

typical experiment, 9.5 g of crude FSH is dissolved in 800 ml of cold water and the pH is adjusted to 3.5 by the addition of freshly prepared 0.2 M HPO_3. The copious, pigmented precipitate which forms is removed by centrifugation and the supernatant fluid is adjusted to pH 6.5–7.0 with 1 N NaOH. Solid $(NH_4)_2SO_4$ (311 g/l of supernatant fluid) is added and the small resultant precipitate removed by centrifugation. Additional $(NH_4)_2SO_4$ (210 g/l) is added and the precipitate obtained after centrifugation is dissolved in about 50 ml of water and dialyzed overnight. The volume of the dialyzed solution is determined and one-fourth its volume of 1 M K_2HPO_4 is added. The solution is heated to 55–60° for 2–3 min and immediately chilled. The solution is dialyzed against running tap water 24 hr and lyophilized, yielding 370 mg.

The fraction obtained as described above is further purified by chromatography on sulfoethyl Sephadex C-50 (SE-C-50). In this system the partially purified FSH is dissolved in a pH 5.4, 0.1 ionic strength acetate buffer and applied to a 100-ml column of SE-C-50 equilibrated to the same buffer. Approximately 60% of the applied material passes through the column unabsorbed and is inert with respect to FSH activity. The FSH fraction is eluted with a pH 5.8, 0.1 ionic strength (potassium phosphate) buffer and amounts to about 25% of the material are applied. The remaining protein is eluted with 0.2 M K_2HPO_4 and is inert. A typical SE-C-50 experiment is seen in Figure 1.

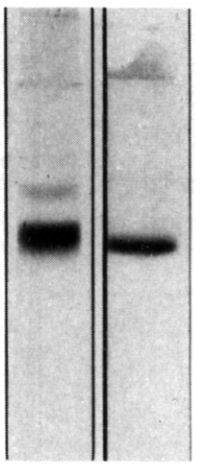

FIGURE 4: Disc electrophoresis of purified human FSH. Left: pH 8.3, 7.5% gel, 56 min, 4 ma, 100-μg sample, migration toward anode. Right: pH 4.5, 12% gel, 80 min, 6 ma, 65-μg sample, migration toward cathode.

The tubes comprising the active fraction are combined and $(NH_4)_2SO_4$ added (560 g/l) to precipitate the protein. After centrifugation, the precipitate is dissolved in 5 ml of water and applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated with a pH 5.4, 0.033 ionic strength acetate buffer. Typical results are seen in Figure 2. The FSH activity is found in the major peak. The tubes from this area (27–38 in Figure 2) are pooled and applied to a 40-ml column of carboxymethyl Sephadex C-50 (CM-C-50), equilibrated to the pH 5.4, 0.033 ionic strength acetate buffer employed for the gel filtration. As seen in Figure 3, virtually all the protein is absorbed to the gel and is eluted with a pH 7.0, 0.033 ionic strength (potassium phosphate) buffer. This fraction which contains the human FSH is dialyzed against distilled water and lyophilized, yielding 55 mg of purified hormone from 9.5 g of the crude FSH fraction.

Characterization Studies

Electrophoresis. The purified human FSH was studied by disc electrophoresis in columns of polyacrylamide (Reisfeld *et al.*, 1962; Clarke, 1965) and by the classical free-boundary technique. Typical patterns obtained by the disk electrophoresis technique at pH 4.5 and 8.3 are seen in Figure 4. It is evident that the preparation consists in the main of a single major component. In the experiment run in the pH 8.3 buffer (left pattern,

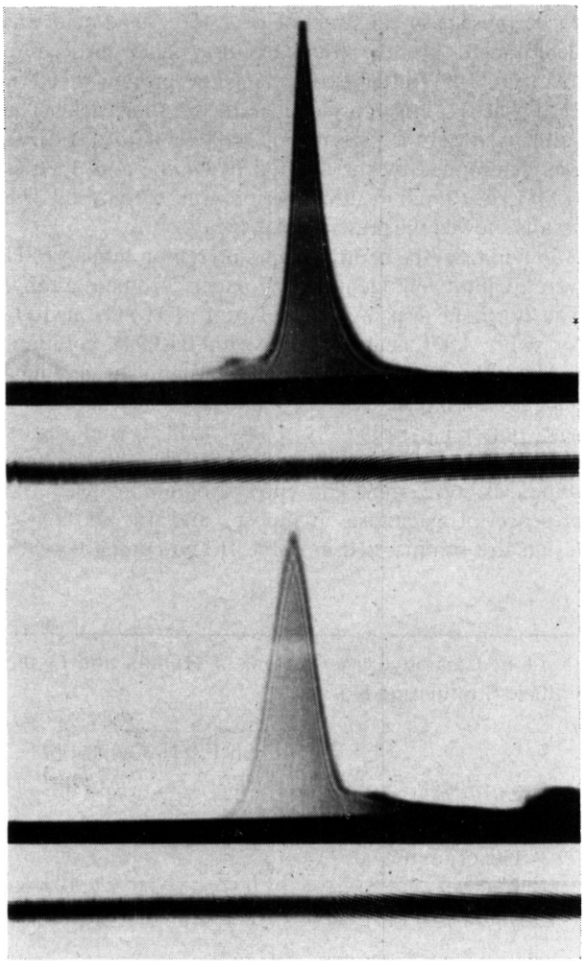

FIGURE 5: Free-boundary electrophoresis patterns of human FSH. Conditions: 0.5% protein solution; pH 4.2, 0.05 ionic strength sodium acetate buffer; potential gradient of 5.8 v/cm; picture taken after 10,800 sec of electrophoresis.

Figure 4) two very faint bands can be seen preceding the intensely stained major band. In a separate experiment, five columns of polyacrylamide gel, each containing 100 μg of hormone, were run and matched with a stained column. Areas corresponding to the three bands were cut out, eluted, and analyzed for protein (Lowry *et al.*, 1951). The concentration of protein in the eluates corresponding to the two faint bands were below the level of detection; the main band, therefore, accounts for at least 90% of the total. Bioassay showed that the main component possessed FSH activity.

Free-boundary experiments at pH values 4.2, 5.2, 6.0, and 7.2 confirmed the results obtained by the disk electrophoresis experiments (see Figure 5). In addition, when the electrophoretic mobilities, calculated from the descending limb patterns, are plotted as a function of pH, the isoelectric point is determined to be 5.6. This is shown in Figure 6.

Ultracentrifugation. Sedimentation velocity experi-

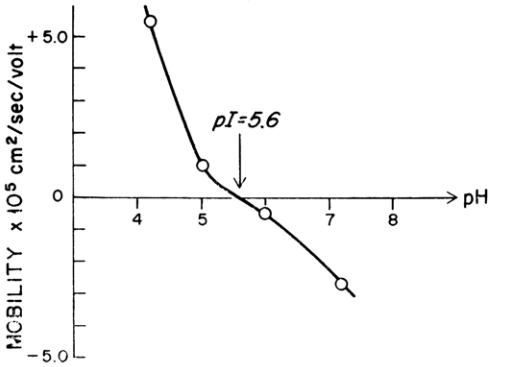

FIGURE 6: The electrophoretic mobility of purified human FSH plotted as a function of pH.

ments were performed in the Spinco Model E ultracentrifuge. Solutions of the hormone (10 mg/ml) were dialyzed against the buffer employed for 24 hr prior to analysis. In 0.1 M $NaHCO_3$ a sedimentation coefficient of 4.63 S was obtained, whereas at pH 1.3 (0.07 M HCl–0.03 M KCl) a value of 3.65 S was obtained. The significance of these values which are appreciably higher than the value of 2.95 S previously reported (Roos and Gemzell, 1964) will be further discussed.

Spectrophotometric Analysis. The ultraviolet absorption spectrum of the purified FSH was determined in acidic and alkaline solutions in the range 230–370 mμ. Spectra were obtained which were typical of proteins containing only tyrosine, tryptophan, and phenylalanine as the major ultraviolet-adsorbing moieties. In addition, the tyrosine and tryptophan content were determined from the measurements obtained in 0.1 M NaOH (Goodwin and Morton, 1946). These results showed the presence of 4.47% tyrosine and 1.65% tryptophan. In neutral and acidic solutions the absorption maximum is between 275 and 277 mμ. A solution containing 1 mg of FSH/ml gives an optical density of 0.920.

Amino Acid Analysis. Samples of human FSH were dissolved in constant boiling HCl (1 mg/ml of acid) and hydrolyzed in sealed, evacuated tubes at 110° for 20 and 70 hr. Following hydrolysis, they were brought to dryness in a vacuum desiccator over NaOH. Analyses were made in a Beckman amino acid analyzer (Spackman *et al.*, 1958). The data, corrected for hydrolytic destruction, are tabulated in Table I. The amino acid analysis, together with the carbohydrate composition reported below, accounted for an 87% recovery of material, uncorrected for moisture and ash.

Terminal Groups. The dinitrophenylation technique (Sanger, 1945) was employed for the detection of amino-terminal residues in the human FSH preparation. The protein (11.3 mg) was dissolved in 1 ml of 1% $NaHCO_3$; 2 ml of 2% FDNB in ethanol was added and allowed to react for 2 hr at room temperature. Excess reagent was extracted with ether. The dinitrophenyl (DNP) protein solution was dialyzed against distilled water and lyophylized, yielding 11.8 mg of

TABLE I: Amino Acid Composition of Human and Ovine Follicle-Stimulating Hormones.

Amino Acid	Residues/25,000 g	
	Human[a]	Ovine[b]
Lysine	15.0	9.4
Histidine	4.7	3.5
Arginine	6.5	5.1
Aspartic	20.3	10.5
Threonine	8.4	10.8
Serine	10.7	8.4
Glutamic acid	15.3	11.7
Proline	9.9	10.7
Glycine	12.8	6.9
Alanine	14.8	8.6
Half-cystine	10.7	9.3
Valine	11.4	7.3
Methionine	1.7	1.5
Isoleucine	3.9	4.9
Leucine	15.1	6.2
Tyrosine	6.7	5.4
Phenylalanine	8.2	5.0
Tryptophan	2.2[c]	0.9[c]

[a] Corrected for hydrolytic destruction. [b] Uncorrected for hydrolytic destruction; taken from Papkoff *et al.* (1967). [c] Spectrophotometric determination.

DNP protein. Following hydrolysis of the DNP protein in 1 ml of 5.7 N HCl at 110° for 20 hr in a sealed, evacuated tube, the hydrolysate was diluted and extracted three times with ether. The ether-soluble fraction was chromatographed in the systems described by Levy (1954). The only spot found attributable to a DNP-amino acid was a trace of valine. Elution and spectrophotometric estimation showed this amounted to only 0.005 μmole/11.8 mg of DNP protein. Analysis of the aqueous fraction of the hydrolysate by paper electrophoresis did not reveal the presence of any α-DNP-arginine. It was concluded that in all probability, human FSH did not possess a free amino-terminal group.

Similar results were obtained when the carboxyl terminus was examined by reaction with carboxypeptidase A. Human FSH was treated with carboxypeptidase A (enzyme:substrate = 1:25, 1% $NaHCO_3$, room temperature, 20 hr) and the liberated amino acids were determined by the dinitrophenylation technique. Traces of DNP-threonine and DNP-serine were detected and estimated to be present in the amount of 0.01 and 0.015 μmole, respectively, per 25 mg of human FSH.

Carbohydrate Components. The hexose content of the human FSH preparation was determined by the orcinol–sulfuric acid procedure (Winzler, 1955) using mannose as a standard. The results showed the presence of 3.9% hexose. Hexosamine was estimated (Rondle and Morgan, 1955; Marshall and Neuberger, 1960) to be present in the amount of 2.4%. Sialic acid was determined by the thiobarbituric acid procedure (Warren, 1959) after hydrolyzing the protein in 0.1 N H_2SO_4 at 80° for 1 hr to liberate the sialic acid. The results showed the presence of 1.4% sialic acid. Fucose was determined by the method of Dische and Shettles (1948); a 10-min heating period was employed. The results showed the presence of 0.4% fucose.

In addition, the neutral sugars present in human FSH were qualitatively identified by paper chromatography. The hormone was hydrolyzed in 1 N H_2SO_4 at 100° for 6 hr. After neutralization with $Ba(OH)_2$ solution, the hydrolysate was concentrated and applied to Whatman No. 1 paper. The chromatogram was developed with butanol–pyridine–0.1 N HCl (5:3:2) and sugars were detected with alkaline silver nitrate. Appropriate standards were run. The chromatogram showed the presence of galactose, mannose, and fucose. These results are summarized in Table II and compared with

TABLE II: Carbohydrate Content of Human and Ovine Follicle-Stimulating Hormone.

Sugar	Human FSH (%)	Ovine FSH[a] (%)
Hexose	3.9	5.7
Hexosamine	2.4	4.5
Fucose	0.4	1.1
Sialic acid	1.4	2.8

[a] Taken from Papkoff *et al.* (1967).

results previously obtained with ovine FSH (Papkoff *et al.*, 1967).

Biological Activity. Preparations of purified human FSH assayed after lyophilization have consistently shown an activity equivalent to 40–60 times the NIH-FSH-S1 standard.[2] After several weeks, however, during which time the lyophilized preparation was stored at room temperature, the specific activity had decreased to approximately 50% of the initial potency. Contamination with ICSH activity appeared to be present to the extent of about 1% when assayed by the ventral prostate test and compared with a human ICSH preparation (Papkoff, 1966).

Discussion

We have previously developed a method for the purification of ovine FSH and described some of the properties (Papkoff, 1966; Papkoff *et al.*, 1967). It is of interest to compare the two species of FSH with

[2] A more detailed study of the biological characteristics of human FSH as well as information regarding its stability under various conditions will be presented elsewhere.

respect to their behavior in fractionation procedures as well as chemical and physical properties. In comparing the purification procedures, we find a major difference in behavior in the conditions necessary for successful chromatography on sulfoethyl Sephadex C-50. The human hormone is adsorbed at pH 5.4 and eluted with a pH 5.8 buffer. Ovine FSH, however, is completely unadsorbed on SE-C-50 under these conditions and requires a pH of 4.0 in order to be adsorbed to the gel. This is undoubtedly a reflection, in part, of the difference in the isoelectric points of the species of FSH. We have determined the isoelectric point of human FSH to be 5.6; ovine FSH, however, is isoelectric near pH 4.5 (Raacke *et al.*, 1958). It is of interest to note that the isoelectric point determined for human ICSH is 5.4 (Squire *et al.*, 1962), a value very close to human FSH.

The behavior of the human FSH in the ultracentrifuge is similar to that observed with ovine FSH in that the results are suggestive of an aggregation phenomenon. However, the sedimentation coefficients determined in 0.1 M $NaHCO_3$ and pH 1.3 (4.65 and 3.45 S) are both significantly higher than the value of 2.95 reported by Roos and Gemzell (1964). Their conditions, however, varied from ours in that the sample analyzed had not been lyophilized and a different buffer system was employed. Nevertheless, a more extensive study is in order not only to characterize the sedimentation behavior of human FSH but to determine the molecular weight as well.

Human FSH, like ovine FSH, does not yield any terminal amino acids on analysis. The amino acid content of human FSH shown in Table I is unremarkable in that all the commonly expected amino acids are present. The amino acid analyses, together with the carbohydrate composition, account for 87% of the dry weight of the material, uncorrected for moisture and ash. There are differences noted when the human FSH amino acid composition is compared with that of ovine FSH. Of special interest is the tyrosine and tryptophan content; both are present to a greater extent in the human FSH. As a result, solutions of human FSH give a higher optical density at 280 mμ than do solutions of ovine FSH. It should be recalled that Roos and Gemzell (1964) in assaying their human FSH preparations assumed an optical density of 1.0 for a 1-mg/ml solution. This, it turns out, is fairly close to the value of 0.920 which we have determined.

Although there are demonstrated differences in the amino acid content of human and ovine FSH, greater variations are seen when the carbohydrate composition is compared. With respect to each carbohydrate component measured, the amount present in human FSH is lower than in ovine FSH. Indeed, it appears the human FSH possesses approximately one-half the carbohydrate content of ovine FSH. In the case of ICSH, another gonadotropin found in pituitaries, we have previously shown that the carbohydrate content of ovine, bovine, and human ICSH, while differing slightly, are about the same (Papkoff, 1966).

The lack of biological stability remains a perplexing problem. We have conjectured in the past this is due to the action of enzymes. In the case of ovine FSH (Papkoff *et al.*, 1967), this was primarily caused by proteolytic enzymes similar to those described by Adams and Smith (1951). The crude human FSH ammonium sulfate fraction has demonstrable proteolytic enzyme activity at pH 4.0. To minimize this we have employed the heating procedure described above. In experiments where this step has been omitted the final product has an activity only 10–15 times the NIH-FSH-S1 standard. It also possesses considerably less sialic acid than material which has been heat treated. It is known FSH preparations treated with neuraminidase rapidly lose biological activity, presumably due to release of sialic acid (Ellis, 1961; Papkoff, 1965; Amir *et al.*, 1966). It is possible that the heat treatment not only inactivates proteolytic enzymes but certain carbohydrases as well.

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