# CARBOHYDRATE AND END GROUP ANALYSES OF OVINE FSH

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SUMMARY The carbohydrate content and terminal amino acids of a highly purified preparation of ovine follicle stimulating hormone (FSH) were determined. The purified FSH contained high amounts of hexosamine and sialic acid. The C-terminal amino acids were serine and glutamic acid while serine and phenylalanine were found at the N-termini.

## INTRODUCTION

Several laboratories have previously reported the carbohydrate content (1-3) and terminal amino acids (4) of ovine follicle stimulating hormone (FSH). The relative potencies of these preparations ranged from 30 to 45.7 in terms of NIH-FSH-S1. Sherwood et al. (5) recently described a procedure for obtaining highly purified ovine FSH with biological activity equivalent to 133 units of NIH-FSH-S1 per mg. This report presents the results of carbohydrate and end group analyses of ovine FSH prepared by this method.

## EXPERIMENTAL

Preparation of the FSH. Ovine FSH was isolated from several kilograms of pituitary glands according to the method of Sherwood et al. (5) and dried to constant weight in a vacuum desiccator over anhydrous calcium sulfate (Drierite) at room temperature. The amount of moisture remaining in the FSH after drying by this procedure was determined by additional drying of small samples in vacuo over Drierite at 110°. Protein determinations were done by the

method of Lowry et al. (6).

The purified FSH used in these studies was assayed by the augmentation method of Steelman and Pohley (7) and found to contain an average of 157 units of NIH-FSH-S1 per mg.

Chemicals. Orcinol was purified before use. All other chemicals were reagent grade.

Carbohydrate analyses. Total hexose was determined by the orcinol-$H_2SO_4$ procedure of Winzler (8) as modified by Francois et al. (9). Glucosamine and galactosamine were quantitated on a Beckman Model 120C Amino Acid Analyzer using a 50 minute buffer change. Under these conditions the hexosamines are eluted well after phenylalanine. The FSH was hydrolyzed under nitrogen in 4 N HCl (1 mg/ml) at 100° for 4 hours to release the hexosamines. The acid was removed in vacuo over KOH and $H_2SO_4$ at 0°. Fucose was determined by the method of Dische and Shettles (10) as utilized by Dische (11). Sialic acid was quantitated by the thiobarbituric acid method of Warren (12) after hydrolysis of the hormone samples in 0.1 N $H_2SO_4$ at 80° for one hour.

C-terminal analysis. A modification of the hydrazinolysis procedure of Fraenkel-Conrat and Tsung (13) was used in this study. The sample (100 nmoles) was dried over $P_2O_5$ in a vacuum oven at 80° for a minimum of 20 hours. Anhydrous hydrazine (0.5 ml) was added, the test tube evacuated and sealed and the sample hydrolyzed for 24 hours at 80°. The hydrazinolyzate was dried in a desiccator over concentrated $H_2SO_4$ and KOH, dissolved in 2.0 ml water and extracted successively with 3.0 ml of heptaldehyde for 0.5, 1.0 and 2.0 hours with constant shaking; once with 2.0 ml of ethyl acetate followed by 3.0 ml ether. The aqueous phase was heated to 80° for 10 minutes to remove the remaining ether, freeze-dried and stored until it was quantitated on the amino acid analyzer. Loss of in-

dividual amino acids during hydrazinolysis was estemated by treatment of an aliquot (100 nmoles) of the Beckman amino acid standard calibration mixture as described above. Hydrazinolysis data are corrected for procedural losses.

N-terminal analysis. N-terminal analyses was carried out on native and desialyzed ovine FSH. Desialylation was accomplished by heating in 0.1 N $H_2SO_4$ at 80° for one hour. The dansyl chloride reaction and the identification of the dansyl amino acids were performed with 45 nmoles of FSH according to the procedure of Gros and Labouesse (14). Dansyl phenylalanine was used for the determination of a standard curve for each quantitative determination. Crystalline bovine insulin (compliments of Eli Lilly, Indianapolis, Ind.) was used as a standard protein to determine the efficiency of the dansylation procedure so a correction factor could be applied to recovery values for ovine FSH.

## RESULTS AND DISCUSSION

Carbohydrate and protein content of purified FSH. The carbo hydrate and protein content of the FSH used in this study is compared in Table I with results reported by other laboratories. The most noteworthy differences in the carbohydrate content of purified FSH are the relatively high values for hexosamine and sialic acid.

Decreased biologic activity has been reported following removal of sialic acid from FSH and human chorionic gonadotropin (HCG) (15-18). Loss of 0.3% sialic acid resulted in 37% reduction in the biological potency of HCG (15). Progressive desialylation of HCG up to 25% reduced the biologic activity about 75% (16). When more than 10% sialic acid was removed from human urinary FSH a progressive loss of biological activity occurred with increasing desialylation (17). Morell et al. (18) demonstrated that the liver rapidly removed desialyzed proteins from the circulation suggesting

Table I. Carbohydrate and Protein Content of Purified Ovine FSH.*

| Chemical Component | de la Llosa et al. (1) | Papkoff et al. (2) | Cahill et al. (3) | Present study |
|---|---|---|---|---|
| Total Hexose | 12.5 | 5.7 | 9.7 | 8.9 |
| Mannose | | | 3.1 | |
| Galactose | | | 3.6 | |
| Total Hexosamine | | 4.5 | 4.5 | |
| Glucosamine | 9.0 | | 3.1 | 7.1 |
| Galactosamine | 0.0 | | 1.4 | 1.1 |
| Fucose | 0.5 | 0.6 | 0.6 | 1.2 |
| Sialic Acid | 5.0 | 2.8 | 5.4 | 6.0 |
| Protein | 56.0 | | 81.2 | 70.6 |
| Water | | | | 2.1 |
| Total | 83.0 | ** | 98.4 - 101.4*** | 97.0 |

* Results are expressed as percentages of the total dry weight of the sample.

** Not given since a protein value was not reported.

*** Total dependent on whether or not the total hexose content or the content of mannose and galactose is used.

that this may be a normal catabolic pathway for the gonadotropic hormones.

It is probable that the high biological activity of the purified FSH used in the present study is due to the relatively high sialic acid content. Although the sialic acid content of the FSH preparation studied by Cahill et al. (3) is only 10% less than the amount reported here, its relative potency is considerably less (43 x NIH-FSH-S1). It is conceivable that the 6% sialic acid found in the present study does not represent a maximum value since the sialic acid residues of proteins are labile and may be cleaved during the purification procedure. Preparations of purified ovine FSH containing more than 200 units of NIH-FSH-S1 per mg of dry weight have been obtained in this laboratory (unpublished data); unfortunately sialic acid determinations were not done on these individual preparations.

C-terminal amino acids. The results of C-terminal amino acid analyses are summarized in Table II. Hydrazinolysis resulted in high, equimolar recoveries of serine and glutamic acid.

Table II. Free Amino Acids of Ovine FSH after Hydrazinolysis.*

| Amino Acid | Recovery** |
|---|---|
| Serine | 1.28 |
| Glutamic Acid | 1.20 |
| Glycine | 0.15 |

* Hydrazinolysis for 20 hours at 110; values in mole per mole based on a MW of 33,000.

** Results are the average of duplicate runs on a single preparation. Variation between duplicates was not greater than 2%.

N-terminal amino acids. The results of N-terminal amino acid analyses are presented in Table III. Phenylalanine was the only amino acid recovered in measurable quantities from the native FSH molecule. Serine, glycine, proline and isoleucine were visible on the thin layer plate under ultraviolet light but could not be quantitated. However, when the FSH was desialyzed, approximately one mole each of serine and phenylalanine were recovered per mole of glycoprotein. Moderate quantities of proline and glycine were also recovered.

Table III. N-terminal Amino Acids of Ovine FSH Before and After Desialylation.*

| Amino Acid | Recovery** | |
|---|---|---|
| | Native FSH | Desialylated FSH |
| Serine | --‡ | 0.91 |
| Glycine | --‡ | 0.32 |
| Phenylalanine | 0.17 | 1.13 |
| Proline | --‡ | 0.48 |
| Isoleucine | --‡ | 0.16 |

* Values expressed as mole per mole based on a MW of 33,000.

** Results represent a single determination on a single preparation.

‡ These amino acids were visable on the thin layer plate under UV light but could not be quantitated.

The results of our N- and C-terminal analyses are not in agree ment with Papkoff and Ekblad (4) who reported that serine and leucine are the C-terminal amino acids and proline and glycine N-terminals for their preparation of ovine FSH.

The authors cannot explain the multiplicity of spots observed

with the dansyl chloride procedure although the possibility exists that certain labile bonds at the terminal positions of the molecule may be cleaved during the purification procedure. However, it is interesting that two of the secondary spots we observed (proline and glycine) correspond to the N-terminal amino acids identified by Papkoff and Ekblad (4). We would like to speculate that the diversity in end group analyses reported for various hormone preparations may also be related to the observed differences in biological potency.

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