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PERIODATE OXIDATION, ACID HYDROLYSIS, AND STRUCTURE–ACTIVITY RELATIONSHIPS OF HUMAN-PITUITARY, FOLLICLE-STIMULATING HORMONE AND HUMAN CHORIONIC GONADOTROPHIN

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ABSTRACT

The component analyses and structures of highly purified, human-pituitary, follicle-stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) have been studied. By means of Smith degradation, a partial linkage-analysis of the carbohydrate units in FSH and hCG has been achieved. These data, coupled with those for enzymic hydrolysis and oxidation of the carbohydrate moiety of FSH, allowed the tentative proposal of the sequence *N*-acetylneuraminyl-(2→2)-D-galacto-pyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranosyl-(1→6)-D-mannopyranose for some of the chain termini of FSH. Mild, acid hydrolysis of FSH gave fragments of low molecular weight which retained some *in vivo* and *in vitro* biological activity. The structure–activity relationship of the carbohydrate-chain termini of FSH was investigated by oxidation, or by derivatisation of the 6-aldehyde end-groups produced by the action of D-galactose oxidase on *N*-acetylneuraminic acid-free FSH.

INTRODUCTION

Although the carbohydrate compositions of several preparations of human-pituitary, follicle-stimulating hormone (FSH)[1–5] and human chorionic gonadotrophin (hCG)[6–9] have been reported, there is relatively little information on the arrangement of the carbohydrate residues[10–12]. Further studies of the carbohydrate moieties of high-activity FSH and hCG have now been made. The FSH used in these studies[13] is of much higher *in vivo* biological activity and greater stability than that used previously, but is only available in relatively small amounts. Thus, only analytical techniques that could be used on this microscale were permitted. The results of various analytical and degradative methods applied to FSH are compared with those previously obtained for the less-active preparations. In particular, comparison is made with the periodate-oxidation study[11] of FSH (CP1, 760 i.u./mg) and the methylation[12], enzyme[14], and mild acid hydrolysis[15] studies of FSH (CP150, 1500 i.u./mg). Further work on the structure–activity relationship[16–18] in FSH is also reported.

Evidence indicating a generalized role for the terminal sialic acid residues of circulating glycoproteins, including FSH, has been published[19]. It has long been accepted that the terminal *N*-acetylneuraminic acid groups of FSH are necessary for *in vivo*[20], but not for *in vitro*, biological activity[21]. From these results, it seems probable that bound neuraminic acid is essential for successful transport from the subcutaneous injection site to the ovary. The mechanism by which this is achieved is still unknown, but can be investigated by modifying the carbohydrate termini. The effect on FSH activity of modifying the terminal *N*-acetylneuraminyl-D-galactosyl groups has now been examined.

EXPERIMENTAL AND RESULTS

Measurement of activities. — Biological *in vivo* activity of FSH samples was determined by the ovarian augmentation method in mice[22], and the *in vitro* activity by the method of Boggis and Ryle[23]. Immunological activity was determined by a double antibody radioimmunoassay[24]. The standard employed for bioassay was calibrated in terms of the Second International Reference Preparation of human Menopausal Gonadotrophin, and that for radioimmunoassay was the preparation MRC 69/104.

The activity of hCG samples was detected by an *in vitro* assay[25] using Pregnyl (Organon) as standard.

Component analysis. — *N*-Acetylneuraminic acid was determined spectrophotometrically as described by Warren[26], after liberation by acid hydrolysis; values were corrected for acid-catalysed degradation. Amino acid and 2-amino-2-deoxyhexose contents were determined with a Locarte amino acid analyser, after hydrolysis in 6M hydrochloric acid at 108° for 24 h. Free fucose and other neutral and basic monosaccharides were determined, after hydrolysis in methanolic hydrogen chloride (1.5M) (and *N*-acetylation where appropriate), by g.l.c. of their trimethylsilyl ethers on 10% silicone ester 30 on Celite[27].

Preparation of gonadotrophins. — Human-pituitary FSH, type FSH-CPDS (potency 7,200 i.u., by bioassay *in vivo*) was prepared as described previously[13,24], except that only the fraction eluted between 75 and 125mM ammonium acetate was used in the Sephadex G-100 gel-filtration stage. Human chorionic gonadotrophin (batch H45B, 9,300 i.u./mg) was kindly provided by Dr. H. van Hell at N.V. Organon (Oss, Holland). Component analyses of the hormones are reported in Table I.

Periodate-oxidation studies of FSH and hCG. — An aqueous solution of the gonadotrophin preparation (2 mg, 1 ml) at 4° was treated with sodium metaperiodate to give a final concentration of 16mM and kept at 4° in total darkness for 24 h. Excess of periodate was reduced with ethylene glycol (2 μl), and after 20 min, sodium borohydride (1.5 mg) was added during 2 h. Excess of borohydride was then decomposed by the dropwise addition of 0.2M hydrochloric acid to pH 4. The solutions were fractionated by gel filtration on a column of Bio-Gel P-6 (bed volume, 24 ml). Fractions containing the excluded material of high molecular weight were combined and

TABLE I

COMPONENT ANALYSIS OF HUMAN PITUITARY, FOLLICLE-STIMULATING HORMONE AND HUMAN CHORIONIC GONADOTROPHIN

Component	*Number of residues/mole*	
	FSH[a]	*HCG*[b]
Aspartic acid	15.2 (19)[c]	17.5
Threonine	17.3 (18)	14.2
Serine	13.8 (12)	15.3
Glutamic acid	25.0 (25)	17.2
Proline	12.2 (14)	29.2
Glycine	11.4 (17)	15.6
Alanine	11.4 (12)	14.3
Cystine (0.5)	15.2 (14)	11.1
Valine	12.5 (15)	13.8
Methionine	3.2 (3)	3.1
Isoleucine	7.3 (7)	5.4
Leucine	10.5 (13)	14.3
Tyrosine	12.4 (8)	5.3
Phenylalanine	7.6 (8)	6.5
Histidine	7.0 (6)	4.6
Lysine	12.4 (13)	10.1
Arginine	8.2 (8)	13.3
L-Fucose	2.0 (2)	1.5
D-Galactose	13.4 (9)	16.6
Mannose	19.4 (20)	19.4
2-Amino-2-deoxyglucose	14.5 (19)	15.5
2-Amino-2-deoxygalactose	1.6 (1)	5.0
N-Acetylneuraminic acid (by acid hydrolysis)	10.6 (6)	14.8
N-Acetylneuraminic acid (by enzymic hydrolysis)	11.1	16.2

[a]Mol. wt. 35,000 (Ref. 1). [b]Mol. wt. 40,000 (Ref. 7, as amended[33]). [c]CP150[14], assuming a mol. wt. of 35,000.

lyophilised, and samples were hydrolysed and analysed by g.l.c. Further samples of the products were chromatographed on Dowex resin AG-1 (x8, sulphate form, 200–400 mesh) at 50° with 86% ethanol[28]. The column eluate was continuously monitored automatically for formaldehyde after oxidation with periodate[29]. Because glyceraldehyde and glycerol were eluted in similar positions, a further sample of the oxidised gonadotrophin was reduced with sodium borohydride (glyceraldehyde→ glycerol) before fractionation. The original glyceraldehyde content was then calculated from the difference of the two glycerol contents. The results of the periodate-oxidation study are shown in Table II.

Mild, acid hydrolysis of FSH. — Two samples of the FSH (1 mg) were incubated at pH 0.0 and 20° for 1 and 2 h (2.0 ml)[15], and then immediately fractionated on a Sephadex G-25 column (bed volume, 34 ml) at 0–4°, using water as eluant (Fig. 1). The elution profiles for the two products were identical. Fractions containing the included (low molecular weight) and excluded (high molecular weight) material

TABLE II

PERIODATE-OXIDATION ANALYSIS OF HUMAN-PITUITARY FSH AND hCG

Component	*Moles/mole of FSH*		*Moles/mole of hCG*	
	Before oxidation	*After oxidation*	*Before oxidation*	*After oxidation*
Mannose	19.4	6.5	19.4	4.8
D-Galactose	13.4	4.7	16.6	5.6
L-Fucose	2.0	0.0	1.5	0.0
2-Amino-2-deoxyglucose	14.5	0.3	15.5	0.6
2-Amino-2-deoxygalactose	1.6	n.d.[a]	5.0	n.d.[a]
N-Acetylneuraminic acid	10.6	1.2	14.8	0.7
Propylene glycol	—	1.5	—	1.0
Ethylene glycol	—	12.0	—	14.2
Glycerol (including glyceraldehyde)	—	10.6	—	10.4
Erythritol	—	0.0	—	0.0
Threitol	—	0.0	—	0.0
Glyceraldehyde	—	3.9	—	3.4

[a]n.d., not determined.

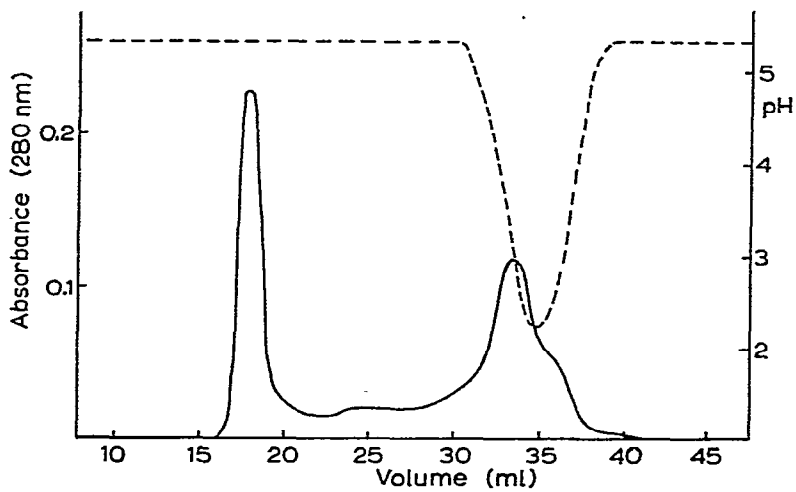

Fig. 1. Fractionation of FSH on Sephadex G-25 eluted with water, after hydrolysis (pH 0.0, 20°, 2 h). (———), Absorbance at 280 nm; (– – –), pH.

were combined and analysed as above, and their biological and immunological activities determined (Table III). A 1:1 mixture of the materials of high and low molecular weight was incubated in phosphate buffer (pH 7.0, 0.1M) at 37° for 24 h. The biological and immunological activities of the incubate, together with those of the similarly and separately treated original materials were also determined (Table IV).

TABLE III

ANALYSIS OF FRAGMENTS FROM ACID TREATMENT OF HUMAN-PITUITARY FSH

	High Mol. Wt.		*Low Mol. Wt.*	
Yield (%) from column (absorbance, 280 nm)	50		50	
Activity (i.u./mg) *in vivo* assay	780	450	550	320
in vitro assay	~1170	380	450–1100	~350
radioimmunoassay	1170	730	340	110
Protein (%)	59.5		70.7	
Amino acid composition[a]				
Aspartic acid	14.2		14.0	
Threonine	15.8		14.9	
Serine	12.1		17.1	
Glutamic acid	18.5		20.0	
Proline	10.9		10.3	
Glycine	10.7		16.0	
Alanine	10.2		11.8	
Cystine (0.5)	7.3		5.3	
Valine	11.4		11.7	
Methionine	2.2		0	
Isoleucine	6.4		5.9	
Leucine	9.1		8.9	
Tyrosine	11.4		9.7	
Phenylalanine	6.9		6.3	
Histidine	4.8		6.4	
Lysine	10.0		10.0	
Arginine	7.0		7.8	
Carbohydrate composition (%)				
L-Fucose	2.1		0.9	
D-Galactose	8.3		5.3	
Mannose	9.1		6.2	
2-Acetamido-2-deoxyglucose	7.4		8.5	
2-Acetamido-2-deoxygalactose	[b]		[b]	
N-Acetylneuraminic acid	13.7		8.3	

[a]Ratio to lysine = 10. [b]Not determined.

TABLE IV

ACTIVITY OF FRAGMENTS OF FSH[a] AFTER INCUBATION AT pH 7 AND 37° FOR 24 H

	Activity (i.u./mg)		
	High mol. wt.	*Low mol. wt.*	*Mixed high and low mol. wt.*
In vivo assay	520	⩾100	270
In vitro assay	870	0–60	310
Radioimmunological assay	1,330	500	700

[a]Produced by mild, acid hydrolysis and separated on Sephadex G-25.

Desialylisation treatment of FSH and hCG. — Solutions of the gonadotrophins (1 mg/ml, 1 ml) were incubated with neuraminidase (*ex. Vibrio cholerae*, Koch–Light Labs. Ltd., 500 units, 1 ml) at 37° for 24 h. The hydrolysates were fractionated by gel filtration on Bio-Gel P-6 (bed volume, 24 ml) and then by ion-exchange chromatography on CM-cellulose, to remove material of low molecular weight and neuraminidase, respectively. The *N*-acetylneuraminic acid released and the biological and immunological (for FSH), or biological *in vitro* (for hCG), activities of the remaining materials of high molecular weight (gonadotrophin core) were determined (Tables I and V).

TABLE V

ACTIVITY OF GONADOTROPHINS AFTER NEURAMINIDASE TREATMENT

	FSH assays (i.u./mg)[a]		
	in vivo	*in vitro*	*Immunological*
Original FSH	7,200	10,000	7,670
Desialylised FSH	⩾300	1,140	2,580
		hCG assay (i.u./mg)	
Original hCG		10,900	
Desialylised hCG		20–200[a]	

[a]Assuming absorbance at 280 nm = 1.0 at a concentration of 1 mg/ml.

Reactions on desialylised FSH. — *N*-Acetylneuraminic acid-free FSH, prepared by acid hydrolysis (pH 2, 1 h, 80°), was assayed for terminal D-galactose residues by the D-galactose oxidase method of Barker *et al.*[1], but using ammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate)[30] (1.6 mg/ml in phosphate buffer (0.1M, pH 7.0)) in place of *o*-dianisidine in methanol and determining the absorbances at 415 nm; 65% of the available D-galactose residues were exposed by removal of the *N*-acetylneuraminic acid.

Samples of *N*-acetylneuraminic acid-free and oxidised (by D-galactose oxidase) FSH were further oxidised with iodine (10mM, 0.1 ml) and sodium carbonate (5%, 6 μl) for 30 min at 20° or converted into the bisulphite complex[31] (300 mg of sodium bisulphite/ml, 0.2 μl). The derivatised samples and unreacted *N*-acetylneuraminic acid-free FSH were separately dialysed against sodium acetate buffer (0.02M, pH 5.0, 2 × 1 litre) at 0–4° before testing for the biological and immunological activities. No change relative to the unreacted *N*-acetylneuraminic acid-free FSH was noted.

DISCUSSION

The component analysis of the highly purified FSH (CPDS, 7,200 i.u./mg) shows great similarity to that of the less-active preparation[1,14] (CP150, 1500 i.u./mg; Table I). This less-active preparation of FSH (CP150) had been used to prepare the

highly purified FSH (CPDS), giving a 4.8-fold increase in activity. The component and structural similarity between the two preparations, however, indicates that only closely related molecules have been removed in the final purification process. The presence of 2-acetamido-2-deoxygalactose answers previous queries about the significance of its appearance, in small amounts, in less-active preparations[1], as it can no longer be thought to be present solely in impurities. The major component differences are the increased D-galactose (4.4 moles/mole of FSH), 2-amino-2-deoxyglucose (4.5 moles/mole of FSH), and N-acetylneuraminic acid contents (4.6 moles/mole of FSH) (Table I). These increases may be explained by addition of terminal groups containing these three residues to the previously found, terminal D-mannose residues (4.3 moles/mole of FSH)[12]. The component analysis of hCG is similar to that found previously for preparations of similar activity[8,9].

The products obtained from the periodate oxidation of FSH are similar in type and amount to those obtained in an earlier periodate-oxidation study[11] (on CP1, 760 i.u./mg), and substantially in agreement with the methylation analysis[12] (on CP150, 1500 i.u./mg). The conclusions which may be reached from the present oxidation studies (Table VI) for the two hormones are analogous, indicating structural similarities between their carbohydrate moieties.

TABLE VI

THE CARBOHYDRATE LINKAGES[a] OF HUMAN-PITUITARY FSH AND OF hCG

Component	*In FSH (moles/mole)*	*In hCG (moles/mole)*
Fucose, 1- and 2-substituted, or terminal non-reducing	2.0	1.5
Mannose, 1- and 3-substituted, or branchpoint	6.5	4.8
Mannose, 1- and 2-, or 1- and 6-substituted, or terminal non-reducing	12.9	14.6
Galactose, 1- and 3-substituted, or branchpoint	4.7	5.6
Galactose, 1- and 2-, or 1- and 6-substituted, or terminal non-reducing	8.7	11.0
2-Amino-2-deoxy-D-glucose, 1- and 6-substituted, or terminal non-reducing	14.2	14.9
2-Amino-2-deoxy-D-galactose, 1- and 6-substituted, or terminal non-reducing	1.6[b]	5.0[b]

[a]As determined by periodate-oxidation analysis. [b]Approximate values.

Of the D-galactose residues of FSH, 65% (8.7 moles/mole of FSH) were susceptible to D-galactose oxidase after removal of the terminal *N*-acetylneuraminic acid residues (10.6 moles/mole of FSH). Although the exact response of terminal D-galactose residues in FSH in the assay cannot be assumed to be identical with that of the D-galactose standard[32], the result indicates that all, or most, of the *N*-acetylneuraminic acid residues are linked directly to D-galactose residues. Acid hydrolysis, as opposed to enzymic hydrolysis, was used for the production of *N*-acetylneuraminic

acid-free FSH, since only the terminal residues were under investigation, and interference by the enzyme and its removal by chromatography were avoided. Combination of this finding with the composition analysis and the periodate-oxidation results leads to the conclusion that some of the carbohydrate chains of FSH terminate with the group *N*-acetylneuraminyl-(2→2)-D-galactopyranosyl-(1→6)-(2-acetamido-2-deoxy-D-glucopyranosyl)-(1→6)-D-mannopyranose, which is similar to that postulated[7] for hCG.

The results of the analysis of the fractions of FSH after mild, acid hydrolysis (Table III) agree substantially with those obtained[15] for a less-pure preparation of FSH (CP150, 2,000 i.u./mg). In particular, the distribution of serine, glycine, histidine, and *N*-acetylneuraminic acid show marked similarity. This again demonstrates the lability of FSH under acid conditions somewhat milder than those neccessary to hydrolyse the *N*-acetylneuraminic acid residues. This may be due to a partial separation into sub-units, where the unit containing the higher proportion of carbohydrate residues has a higher, apparent molecular weight on gel filtration, due more to an increased content of bound water than to an actual, increased molecular weight. The mild, acid hydrolysis decreased the specific activity of the FSH, but gave material of low molecular weight (included material on Sephadex G-25 has mol. wt. 5,000) with *in vitro* and *in vivo* biological, and immunological activities. This demonstrated that a fragment(s) of the FSH molecule containing the biologically active site(s) can be produced. The low stability of these fragments was indicated by the loss of *in vivo* and *in vitro* biological activities after incubation (pH 7, 37°, 24 h; Table IV). This might be due to denaturation of a portion of an active glycoprotein when not stabilised by the rest of the molecule. The separated fragments of the FSH molecule could not be reunited by conditions expected to reunite sub-units of FSH, thus indicating that they were not integral sub-units.

The loss of biological *in vivo* activity by the enzymic removal of terminal *N*-acetylneuraminic acid from FSH is now accepted[20]. The biological *in vitro* activity was, however, retained to the extent of 44% of the remaining immunological activity (Table V), which indicates that this *in vitro* activity is partially independent of the *N*-acetylneuraminic acid content. It is therefore clear that the terminal *N*-acetylneuraminic acid residues are involved in the transport of the FSH molecule to the ovary, for without them the FSH does not arrive at the ovary in an active state (biological *in vivo* assay) but shows activity if forcibly transported to the ovary (biological *in vitro* assay). This result is in agreement with the work of Morell *et al*[19].

N-Acetylneuraminic acid-free hCG retained ~1% of the original biological activity. This might be due to incomplete desialylisation, but is more probably due to use of an assay[25] intended for luteinising hormone rather than hCG, and which measures different biological properties than the usual hCH assays.

The lack of any change in the activities on removal of the *N*-acetylneuraminic acid groups of FSH and conversion of the exposed D-galactose residues into D-galacturonic acid and other acidic groups indicates that the chemical function of the terminal *N*-acetylneuraminic acid residues is not simply to provide an acidic environ-

ment at the carbohydrate-chain termini. These residues, therefore, must have other, more-specific functions, involving the structure of the whole FSH molecule.

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