Periodate Oxidation Studies of Human Pituitary Follicle-Stimulating Hormone

By J. F. KENNEDY

Department of Chemistry, University of Birmingham, Birmingham 15

AND W. R. BUTT

Department of Clinical Endocrinology, Birmingham and Midland Hospital for Women, Birmingham 11

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A preparation of human pituitary follicle-stimulating hormone was subjected to periodate oxidation, borohydride reduction and acid hydrolysis. Comparison of the analysis of the remaining intact carbohydrate and amino acid units with the analyses of the original material and identification of the carbohydrate fragments permit some structural assignments to the molecule of follicle-stimulating hormone. The results of radioimmunological assay of fragments of the molecule of follicle-stimulating hormone suggest that, although the carbohydrate component is essential for biological activity, it is not a requirement for immunological activity, which appears to be a function of the protein moiety.

FSH* has been prepared from the pituitary glands of a number of animal species including man. The complete component analyses on a number of these preparations have been reported (Butt, 1967; Barker, Gray, Kennedy & Butt, 1969), but no information is available on the primary structure. The present paper is a study of the periodate oxidation of a preparation of human pituitary FSH, and the results are discussed with regard to the structure of the carbohydrate moiety of the glycoprotein.

MATERIALS AND METHODS

Preparation of FSH. The preparation CP1 was obtained from acetone-dried powder of pituitary glands as described by Butt, Crooke & Cunningham (1961). The biological activity of this preparation, assayed by the ovarian-augmentation method in mice (Brown, 1955) was 760 i.u. of FSH (95% fiducial limits 460–1220)/mg.; the standard was a laboratory sub-standard calibrated in terms of the second international reference preparation for human menopausal gonadotrophin. Radioimmunoassays were performed with bentonite as a solid phase for the antibody, as described by Butt & Lynch (1968).

Determination of D-galactose. The samples and appropriate controls and standards were hydrolysed in 2M-HCl at 100° for 4 hr. in sealed tubes. The HCl was removed by repeated rotary evaporation *in vacuo* at 20° and the residues were dissolved in water (250 μ l.). D-Galactose was then determined by a modification (P. J. Somers, unpublished

work) of the enzymic method of Avigad, Amaral, Asensio & Horecker (1962). Galactose oxidase (Seravac Laboratories Ltd., Maidenhead, Berks.) (5 mg.) and peroxidase (ex horseradish; C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) (2.5 mg.) were dissolved in 0.1M-sodium phosphate buffer, pH 7.0 (15 ml.), o-dianisidine solution (10 mg./ml. in methanol; 250 μ l.) was added and the solution was diluted to 25 ml. with water. The samples (250 μ l.) were mixed with this enzyme solution (250 μ l.) and incubated at 25° for 2 hr. Extinctions were then read at 460 nm.

Determination of hexose and 6-deoxyhexose. A modification (A. R. Law & P. J. Somers, unpublished work) of the cysteine-H₂SO₄ reaction (Dische, Shettles & Osnos, 1949) was employed. Samples (100 μ l.) containing 0–10 μ g. of total hexose were cooled to 0° and chilled 86% (v/v) H₂SO₄ containing L-cysteine hydrochloride (700 μ g./ml.) (500 μ l.) was added to the solution with immediate thorough mixing. After 3 min. at 100°, the solutions were maintained at 20° for 40 min. Extinctions at 400 and 412 nm. were determined and the hexose and 6-deoxyhexose contents calculated from simultaneous equations by using the extinctions of the standards at the two wavelengths and the value obtained for D-galactose by the enzymic method.

Determination of 2-amino-2-deoxyhexose. Samples and standards of 2-amino-2-deoxy-D-glucose and -galactose were hydrolysed in 3.9M-HCl at 100° for 9 hr. in sealed tubes. The HCl was then removed as before and the residues were dissolved in 0.3M-HCl (100 μ l.). These solutions were separately fractionated on a column (80 cm. long $\times$ 0.6 cm. diam.) of the sulphonic acid exchange group resin AG50W (X8; H⁺ form; 100–200 mesh) pre-equilibrated with 0.3M-HCl. The elution rate in this reagent was 0.16 ml./min. and the eluate was continuously monitored fluorimetrically, for formaldehyde released after periodate oxidation by an automated version (Cho Tun, Kennedy,

* Abbreviation: FSH, follicle-stimulating hormone.

Stacey & Woodbury, 1969) of the pentane-2,4-dione method described by Belman (1963). The 2-amino-2-deoxyhexose contents of the sample were calculated by integration of the areas under the peaks and comparison with the standards. In one instance, a scaled-down version of the Svennerholm (1956) modification of the Elson-Morgan assay (Elson & Morgan, 1933) was used.

Determination of N-acetylneuraminic acid. The analyses were performed by a scaled-down version of the procedure of Warren (1959) after hydrolysis in 0.1 M-HCl at 80° for 1 hr.

Periodate oxidation. An aqueous solution of preparation CP1 (20 mg.), cooled to 4°, was treated with NaIO₄ to give a final concentration of 29 mM-IO₄⁻ (final vol. 5.6 ml.). After oxidation at the same temperature in total darkness for 28 hr., the solution was checked for the presence of excess of periodate. A sample (20 µl.) was removed and treated with ethylene glycol diluted with water (30 µg. in 250 µl.) and after 20 min. the colorimetric pentane-2,4-dione procedure (Nash, 1953) was employed to detect any formaldehyde released.

Reduction procedure. After oxidation of preparation CP1, excess of periodate was destroyed by the addition of ethylene glycol (19.6 mg., twice the theoretical amount), and after 20 min. NaBH₄ (15.7 mg., five times the theoretical amount) was added over 2 hr. Excess of borohydride was then destroyed by the dropwise addition of 0.2 M-HCl to pH 4.

Gas-phase analysis. Dry samples were dissolved in pyridine containing 0.2% (w/v) of LiClO₄·3H₂O (250 µl.), and warmed at 37° for 2 hr. Trimethylsilyl chloride (25 µl.) and hexamethyldisilazane (50 µl.) were added and the mixture was maintained at 37° for 10 min. After dilution to 1.0 ml. with *n*-hexane, samples were analysed by g.l.c. with silicone ester 30 (10% on Celite) as stationary phase and N₂ as carrier gas. For C₆ sugars runs were made isothermally at 210°, whereas for smaller fragments the temperature programme 70°×20 min., 75°–210° at 4°/min., 210°×20 min., was used. A flame-ionization detection system was employed.

Amino acid analysis. Samples were hydrolysed in 5.7 M-HCl at 108° for 30 hr. and, after removal of the acid as before, the amino acids were determined in a Technicon AutoAnalyzer.

RESULTS

Component analysis of preparation CP1. The carbohydrate and amino acid analyses of preparation CP1 are shown in Tables 1 and 4 respectively. Unhydrolysed FSH did not give a response in the galactose oxidase assay (Avigad *et al.* 1962).

Oxidation-reduction of preparation CP1. The solution of preparation CP1 after oxidation in excess of periodate and reduction was concentrated to 2 ml. by rotary evaporation at 20° and was then fractionated by gel filtration on a column (bed volume 20 ml.) of the cross-linked polyacrylamide Bio-Gel P-2 with water as eluent. The extinctions (at 280 nm.) of the fractions are shown in Fig. 1; ethylene glycol, sodium chloride and boric acid were each eluted in the same position as formaldehyde. Fractions containing the high-molecular-weight material were pooled and examined by the

Table 1. *Carbohydrate components of preparation CP1*

The carbohydrates were determined by the methods described in the text.

Component	Composition (nmoles/mg. of prep. CP1)
L-Fucose	39
D-Galactose	125
D-Mannose	394
N-Acetylneuraminic acid	155
2-Amino-2-deoxy-D-galactose	29
2-Amino-2-deoxy-D-glucose	824

Fig. 1. Fractionation on Bio-Gel P-2 of preparation CP1 after oxidation and reduction. —, E_{280} ; ···· E_{410} (formaldehyde).

radioimmunoassay and bioassay for FSH: > 70% and < 2.3%, respectively, of the original activity of preparation CP1 were retained in this fraction. A sample was examined, after hydrolysis in 0.1 M-hydrochloric acid at 80° for 1 hr., by the Warren (1959) procedure, and gave a value 24.1% of the original Warren positive value.

Samples of the pooled fraction were hydrolysed in sealed tubes in 0.5 M-sulphuric acid at 100° for 3 hr. or in 2 M-hydrochloric acid at 100° for 13 hr. The sulphuric acid was removed by precipitation with barium carbonate, and the hydrochloric acid was removed by rotary evaporation. G.l.c. showed that apparently more hexose was liberated by the milder hydrolytic conditions, but only 72% of the 2-amino-2-deoxyhexose was liberated, compared with the stronger conditions.

To avoid losses of hexose, and in particular of fucose and galactose, the milder conditions were selected, and the remainder of the solution of oxidized-reduced preparation CP1 was hydrolysed and neutralized to pH 9. The combined supernatant and washings from centrifugation were concen-

trated to 2ml. by rotary evaporation at 25°, and then transferred to a column (bed volume 1.25ml.) of AG50W resin (X8; H⁺ form; 200–400 mesh). The neutral products were eluted with water (10ml.) and the basic products with 2M-hydrochloric acid (5ml.).

Analysis of the basic carbohydrate products is given in Table 2. The total 2-amino-2-deoxyhexose was identified and determined by the Elson–Morgan method (Svennerholm, 1956). G.l.c. indicated that the major component corresponded in retention time to 2-amino-2-deoxy-D-glucose, and chromatography on AG50W (X8) was used to determine separately the individual 2-amino-2-deoxyhexoses.

The neutral products were fractionated by g.l.c. and the following were identified: propane-1,2-diol, glycerol or glycolaldehyde, or both, mannose, galactose. Since the positions of glycerol and glycolaldehyde dimer coincided, a further sample of the neutral eluate was reduced with sodium borohydride at 20° for 30 min. The sulphonic acid exchange group resin Dowex 50 (X8; H⁺ form; 20–50 mesh) was added, and boric acid was removed, after filtration, by co-distillation with methanol. The residue was trimethylsilylated and ethylene glycol, propane-1,2-diol, glycerol and hexitol were identified by g.l.c.

Another portion of the neutral eluate was diluted with ethanol to give a final concentration of 86% (v/v) ethanol, and chromatographed on the quaternary ammonium exchange group resin AG-1 (X8; SO₄²⁻ form; 200–400 mesh) at 50° with 86% (v/v) ethanol as eluent, as described by Barker, How, Peplow & Somers (1968). The column eluate was continuously monitored automatically by the pentane-2,4-dione method, excess of periodate in this case being destroyed with butane-2,3-diol.

Comparison with the elution positions of standard compounds showed the following to be present: propane-1,2-diol, glycerol, glycolaldehyde, hexose. Since the positions of propane-1,2-diol and glycolaldehyde overlap, a further sample was reduced as before: ethylene glycol, propane-1,2-diol and hexitol were identified.

Quantitative measurement of each component

Table 3. *Neutral carbohydrate components of preparation CP1 after oxidation and reduction*

Neutral components were determined, after hydrolysis in 0.5M-H₂SO₄ at 100° for 3 hr., by g.l.c. and column chromatography on AG-1 resin (X8; SO₄²⁻ form) by the method of Barker *et al.* (1969). These determinations were also made after a further reduction of the hydrolysate with NaBH₄.

Component	Composition
	(nmoles/mg. of original prep. CP1)
Ethylene glycol (≡ glycolaldehyde)	87.0
Propane-1,2-diol	44.1
Glycerol	93.4
Tetritol	0.0
L-Fucose	0.0
D-Galactose	51.6
D-Mannose	79.2

Table 4. *Amino acid composition of preparation CP1 before and after oxidation and reduction*

Amino acids were determined, after hydrolysis in 5.7M-HCl at 108° for 30 hr., in a Technicon AutoAnalyzer.

Component	Composition (nmoles/mg. of original prep. CP1)	
	Before	After
Cysteic acid	0	142
Aspartic acid	312	242
Threonine	222	182
Serine	223	176
Glutamic acid	409	395
Proline	225	168
Alanine	218	183
Valine	154	161
Methionine	46	27
Isoleucine	96	73
Leucine	229	156
Tyrosine	118	54
Phenylalanine	131	108
Lysine	220	111
Histidine	67	67
Arginine	159	98
Glycine	199	185
Cystine (half)	224	148

Table 2. *Basic carbohydrate components of preparation CP1 after oxidation and reduction*

The total 2-amino-2-deoxyhexose was determined, after hydrolysis, by the Elson–Morgan reaction. The individual components were determined, after hydrolysis, by column chromatography on AG 50W resin (X8; H⁺ form) by the method of Cho Tun *et al.* (1969). Hydrolyses were performed in 0.5M-H₂SO₄ at 100° for 3 hr., to avoid losses of hexose. The results were corrected to allow for incomplete liberations of 2-amino-2-deoxyhexoses under these conditions.

Component	Composition (nmoles/mg. of original prep. CP1)	
	Uncorrected	Corrected
2-Amino-2-deoxyhexose (total)	78.6	—
2-Amino-2-deoxy-D-galactose	4.1	5.7
2-Amino-2-deoxy-D-glucose	78.9	109.6

was obtained by integration of the areas under the peaks. In the g.l.c. experiment, the C₁-C₃ fragments could not be determined because of loss by volatilization during preparation of the sample, and in the ion-exchange chromatography D-galactose and D-mannose, and galactitol and mannitol, were eluted together. The quantities determined are given in Table 3.

The amino acid analysis of the high-molecular-weight material remaining after oxidation and reduction is shown in Table 4, and is compared with the analysis of the original material.

DISCUSSION

Certain conclusions about the nature of the hormone can be drawn from the results obtained on this more available preparation, CP1, although it is not completely homogeneous.

Essentially all the immunological activity of FSH was retained after oxidation and reduction, a process known to destroy biological activity (Butt, Crooke, Cunningham & Evans, 1961). The present preparation lost virtually all of its biological activity on oxidation and reduction. Since a large portion of the remaining carbohydrate moiety is also destroyed during oxidation, it appears that the protein rather than the carbohydrate moiety is associated with immunological activity.

The fucose units in this preparation, and therefore in FSH, cannot be linked in the 1- and 3-positions, since none was recovered from the oxidized and reduced products. Further, since the amount of propane-1,2-diol recovered after oxidation, reduction and hydrolysis was approximately equivalent to the original content of fucose, all the fucose units must be linked in the 1- and 2-positions or occur as terminal non-reducing units.

Of the galactose units, 41% were recovered intact after oxidation and reduction, and these are thus linked in the 1- and 3-positions, or are involved in branch points. Since no threitol was identified in the polyol analysis, it appears that the susceptible galactose units are linked in the 1- and 2-positions or the 1- and 6-positions, or occur as terminal non-reducing chain ends. The removal of galactose by galactosidase has been reported (Butt, Jenkins & Somers, 1967), suggesting that the galactose units are occupying terminal non-reducing positions, but the interpretation of the evidence obtained is now open to question. Furthermore, D-galactose that is substituted only at C-1 responds to the enzyme assay (Schlegel, Gerbeck & Montgomery, 1968), and since unhydrolysed FSH gave a negligible response it is suggested either that none of the galactose units is situated at the terminal non-reducing ends of the carbohydrate chains or that the presence of *N*-acetylneuraminic

acid units at the ends of some of the carbohydrate chains prevents susceptibility of any terminal non-reducing galactose units.

Of the mannose units 80% were oxidized, and since no erythritol was identified at the end of the oxidation-reduction sequence it appears that these units are not 1- and 3- or 1- and 4-substituted, but are 1- and 2- or 1- and 6-substituted or terminal non-reducing units. It is unlikely that they all exist as terminal units, on account of the high proportion of mannose in FSH. The oxidation-resistant mannose units would be linked in the 1- and 3-positions, or occur at branch points.

Of the 2-amino-2-deoxy-D-glucose units 87% were oxidized, and unless some occur as end groups it seems that these units are 1- and 6-substituted. Assuming that in the parent polymer all the 2-amino-2-deoxy-D-glucose units are *N*-acetylated, then linkage involving C-2 would not occur, and neither would oxidation of the 2- and 3-positions. Hence the 2-amino-2-deoxy-D-glucose units that do remain intact would be 1- and 3- or 1- and 4-substituted, or occur at branch points. Similar reasoning applies to the 2-amino-2-deoxy-D-galactose unit(s), of which 80% were oxidized.

It has been shown that the *N*-acetylneuraminic acid of FSH can be liberated by neuraminidase with concomitant complete loss of biological activity (Butt *et al.* 1967), and it seems on this basis that such units are situated at the terminal non-reducing ends of the chains. If the *N*-acetylneuraminic acid were terminal, non-reducing and C-2-linked, the periodate oxidation would cleave the C-7-C-8 bond, leaving *N*-acetylheptulosaminic acid as the terminal non-reducing unit. *N*-Acetylheptulosaminic acid liberated by subsequent hydrolysis would give a positive reaction in the Warren assay. The present result is consistent with a C-2-linked terminal non-reducing structure, since the oxidized-reduced material gave on analysis 24% of the original Warren positive value. It has been shown (Kuhn & Gauhe, 1965) that when acidic periodate is used in the similar thiobarbituric acid assay (Aminoff, 1961) the molar extinction coefficient of *N*-acetylheptulosaminic acid is considerably less than that of *N*-acetylneuraminic acid.

From the results of the amino acid analyses before and after oxidation for 28 hr. it may be calculated that 82% of the polymeric protein is retained. This is in contrast with the behaviour of interstitial-cell-stimulating hormone, in which, under identical conditions, the amino acids are destroyed to the same degree within 1 hr. and cysteine is completely destroyed (Gan, Papkoff & Li, 1968). It is noteworthy that the protein portion remaining intact possesses essentially (within experimental limits) the same specific immunological activity as the original preparation.

Further studies of this nature, with the radioimmunoassay and bioassay in parallel, should be of use in further structural investigations of the FSH molecule.

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