Biochem. J. (1982) **207**, 11–19
Printed in Great Britain

Chemical deglycosylation of ovine pituitary lutropin

A study of the reaction conditions and effects on biochemical, biophysical and biological properties of the hormone

P. MANJUNATH,* M. R. SAIRAM* and P. W. SCHILLER†
**Reproduction Research Laboratory and †Laboratory of Chemical Biology and Polypeptides, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec H2W 1R7, Canada*

(*Received 8 February 1982/Accepted 7 June 1982*)

The oligomeric glycoprotein hormone, ovine lutropin was treated with anhydrous HF at 0°C for 30, 60 and 180min and at 23°C for 60 and 180min. The products, designated deglycosylated lutropin 1 (DGLH-1) to deglycosylated lutropin 5 (DGLH-5) respectively, were characterized by gel filtration, concanavalin A–Sepharose binding, disc electrophoresis, amino acid analysis, carbohydrate composition and spectral properties. The preparations were also evaluated for receptor binding activity and immunological activity and bioassayed *in vitro* in collagenase-dispersed rat interstitial cells. In DGLH-1, fucose and galactosamine were removed completely, and there was a 94% decrease in hexoses and 39% decrease in *N*-acetylglucosamine. Reaction with HF at 0°C for 1 or 3h led to removal of all hexoses and additional loss of hexosamines. Reactions at 23°C for either 1 or 3h were not of additional value in deglycosylation and none of the reaction conditions yielded the apohormone. All the five deglycosylated hormone preparations were not retained on immobilized-concanavalin A columns and on Sephadex G-100 they were eluted with an increased V_e/V_0 ratio consistent with the loss of carbohydrate residues. Loss of all but the last of the *N*-acetylglucosamine residues decreased the abnormality of lutropin on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but did not eliminate it. Receptor binding activities of DGLH-1 and DGLH-2 were not different from that of the native hormone, but that of DGLH-3 was slightly decreased and the products obtained at 23°C (DGLH-4 and DGLH-5) had lower activity. Immunoreactivities followed a similar pattern. None of the derivatives had activity in the bioassay *in vitro*. All of the five derivatives inhibited the action of the native hormone in the bioassay *in vitro*. Their hormonal antagonistic activity was consistent with the receptor binding activity, with DGLH-5 being the least potent in this respect. The DGLH-4 and DGLH-5 preparations had undergone conformational changes as revealed by 8-anilinonaphthalene-1-sulphonate fluorescence, but this did not result in loss of quaternary structure.

Ovine pituitary lutropin is an oligomeric glycoprotein hormone containing about 16% carbohydrate attached in the form of *N*-glycosidic linkages at three positions to the polypeptide backbone (Sairam & Papkoff, 1974). The functional significance of these bulky prosthetic groups in the hormone is not understood precisely. In recent studies directed at this problem we reported that treatment of the hormone with anhydrous HF at 0°C for about 60min was effective in removing nearly two-thirds of the carbohydrate residues without affecting the polypeptide moiety of the molecule (Sairam & Schiller, 1979). The treatment has interesting disparate effects on the various facets of the hormone's action. Whereas receptor binding and immunological activities were almost fully retained (Sairam & Schiller, 1979; Sairam & Manjunath, 1982), the derivative had virtually no biological activity

Abbreviations used: DGLH, deglycosylated lutropin; ANS, 8-anilinonaphthalene-1-sulphonic acid; IBMX, isobutylmethylxanthine; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

0306-3275/82/100011-09$01.50/1 © 1982 The Biochemical Society

(Sairam & Schiller, 1979; Sairam & Fleshner, 1981). Furthermore, the partially deglycosylated hormone acted as an antagonist of the native hormone's action. It was further shown that the presence of carbohydrate residues is also not essential for recombination of the α- and β-subunits of the hormone (Sairam, 1980).

As we were interested in examining the properties of the apohormone, we have investigated in the present study the effects of different reaction conditions with anhydrous HF on the extent and efficacy of carbohydrate removal from the native hormone.

Materials and methods

Ovine lutropin was isolated from frozen pituitary glands (Sairam & Schiller, 1979). Bovine serum albumin, cyclic AMP-binding protein and lactoperoxidase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., collagenase and lima-bean trypsin inhibitor from Worthington Biochemicals, IBMX from Aldrich Chemical Co., carrier-free $Na^{125}I$ employed for iodination was purchased from Amersham, Chicago, IL, U.S.A., omnifluor and Triton-X100 were from NEN, Boston, MA, U.S.A., and ANS was obtained from Eastman Chemicals, Rochester, NY, U.S.A. All other chemicals were of reagent grade from Fisher Scientific Co.

Chemical deglycosylation and recovery of the product

The freeze-dried hormone (50mg in each experiment) was dried over P_2O_5 *in vacuo* at room temperature for 24h and transferred to the KelF reaction vessel of the HF treatment apparatus. Anisole, which was used as a scavenger in our previous work (Sairam & Schiller, 1979; Sairam, 1980), was omitted in the present series of studies because the hormone lacks tryptophan (Sairam & Papkoff, 1974). The reaction vessel was cooled in a solid CO_2/ethanol bath and 10ml of anhydrous HF was distilled from the reservoir during 3–4min with continuous stirring. The reaction vessel was then warmed up to the desired temperature (0 or 23°C), which was then considered as the starting point of the reaction. At the end of the appropriate reaction time (see the Results section), all visible traces of HF were removed by using first a water aspirator (15–20min) followed by a high-vacuum pump for about 2h. The contents of the reaction vessel, which now appeared sticky, were then dissolved in 0.5ml of ice-cold 0.5M-NaOH to neutralize any remaining trace of HF and the pH, which was between 9 and 10, was re-adjusted to 7.5 by using cold 0.2M-HCl. The solution was then dialysed overnight at room temperature against 0.05M-potassium phosphate buffer, pH7.4, containing 0.02% NaN_3. The small amount of precipitate, obtained under the 3h/0°C and 1h or 3h at 23°C conditions, were removed by centrifugation and the supernatant was chromatographed on a Sephadex G-100 column equilibrated in 0.05M-NH_4HCO_3 at 4°C. Appropriate fractions were pooled and freeze-dried. The deglycosylated hormone preparations were kept at 4°C until use. In control experiments, the native hormone was kept at pH11 and pH12 (0.1M-Na_2CO_3) for 30min at 23°C, desalted on Sephadex G-50 and freeze-dried.

Analytical methods

Polyacrylamide-gel electrophoresis was carried out in a Protean double slab electrophoresis cell (Bio-Rad, Richmond, CA, U.S.A.) at pH4.5 (Reisfeld *et al.*, 1962) and pH8.9 (Davis, 1964). SDS/PAGE under reducing conditions was carried out on 12% and 15% gels as described by Laemmli (1970). The gels were stained with Coomassie Blue.

Amino acids and amino sugars were determined by using an amino acid analyser (Manjunath & Sairam, 1982) after acid hydrolysis of the samples. Neutral hexoses were determined by the phenol/H_2SO_4 reaction with the modification of McKelvey & Lee (1969). Fucose was determined by the cysteine/H_2SO_4 reaction (Winzler, 1955).

The u.v.-absorption spectra of lutropin and deglycosylated derivatives dissolved in 0.05M-potassium phosphate buffer, pH7.4, were recorded at room temperature in a Beckman model 25 recording spectrophotometer.

Fluorescence spectra of aqueous solutions of the hormone and derivatives were examined in the presence of the probe ANS (Ingham & Bolton, 1978) at room temperature. These were recorded in a Hitachi–Perkin–Elmer MPF-3 spectrofluorimeter.

Receptor binding and radioimmunoassays

Receptor binding activity of the hormone and derivatives was assessed by using homogenates of adult rat testes (Sairam & Schiller, 1979) and pooled porcine ovarian granulosa cells. The latter were obtained from the slaughterhouse and collected by aspiration of the fluid from the follicles. After separation of the cells by centrifugation, they were homogenized in 0.025M-Tris/HCl buffer, pH7.4, containing 10mM-$MgCl_2$. The homogenate was centrifuged and washed once and dispersed in the same buffer. Both the testicular and granulosa cell homogenates were stored at −70°C for up to 1 year without any significant loss of hormone specificity or sensitivity. The binding assays were carried out as described previously (Sairam & Schiller, 1979).

Radioimmunoassay was performed by using an antiserum directed against the conformation of the α-subunit (Sairam, 1979). In this assay bound and free hormone were separated by the double-antibody method.

For both radioreceptor assay and radioimmunoassay the native ovine lutropin labelled with ^{125}I by the lactoperoxidase method and having a specific radioactivity of 50–60 Ci/g was employed as the tracer (Sairam & Schiller, 1979). The labelled hormone was usually used within 2 weeks of preparation.

Biological activity in vitro and antagonistic behaviour

The biological characteristics of the hormone before and after deglycosylation were assessed by using adult rat testicular interstitial cells prepared by collagenase digestion and incubated *in vitro* (Ramachandran & Sairam, 1975; Sairam & Fleshner, 1981). About 220000 cells (0.5 ml) were suspended in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin, 0.01% lima-bean trypsin inhibitor and the hormone or derivative (0.1 ml) in the presence of 0.05 mM-IBMX. The incubations were carried out in duplicates in glass tubes (12 mm × 75 mm) at 37°C under O_2/CO_2 (19:1) for 30 min. The reaction was terminated by immersing the tubes in a water bath at 80°C for 10–15 min. The debris was separated by centrifugation and the supernatant was frozen at −20°C until analysed for cyclic AMP by the protein-binding assay (Gilman, 1970; Sairam & Fleshner, 1981).

In experiments to verify the antagonistic activity of the different derivatives, the cells were incubated with a maximum stimulatory concentration of the native hormone (100 ng/0.6 ml) and various concentrations of the deglycosylated samples. Both substances were added to the cells at the same time and the amount of cyclic AMP accumulated was determined as above.

All activity data were expressed on a weight basis and compared with the native hormone, which was taken as 100%.

Results

In the present investigation, five partially deglycosylated derivatives were prepared by exposure of ovine lutropin to anhydrous HF at different temperatures and various time intervals. Their designations are shown in Table 1.

Chromatographic characteristics

The elution patterns of the five deglycosylated derivatives of ovine lutropin are shown in Fig. 1. DGLH-1 and -2 were each eluted in a single peak, and DGLH-3, -4 and -5 were each eluted in one major peak with a small but discernible shoulder corresponding to deglycosylated α-subunit. The deglycosylated β-subunit, which was insoluble in these instances, was recovered as a precipitate before gel filtration (Sairam, 1980). Only the major fractions of DGLH-3, -4 and -5 were analysed. All deglycosylated derivatives were retarded (V_e 310–321 ml) compared with the native hormone, which was eluted with significantly lower elution volume (268 ml). These results are consistent with the

Table 1. *Designation of deglycosylated ovine lutropin preparations*

	Conditions of HF solvolysis	Designation of derivative
1	0°C, 30 min	DGLH-1
2	0°C, 1 h	DGLH-2
3	0°C, 3 h	DGLH-3*
4	23°C, 1 h	DGLH-4*
5	23°C, 3 h	DGLH-5*

* Major fraction in Sephadex G-100 filtration (see Fig. 1).

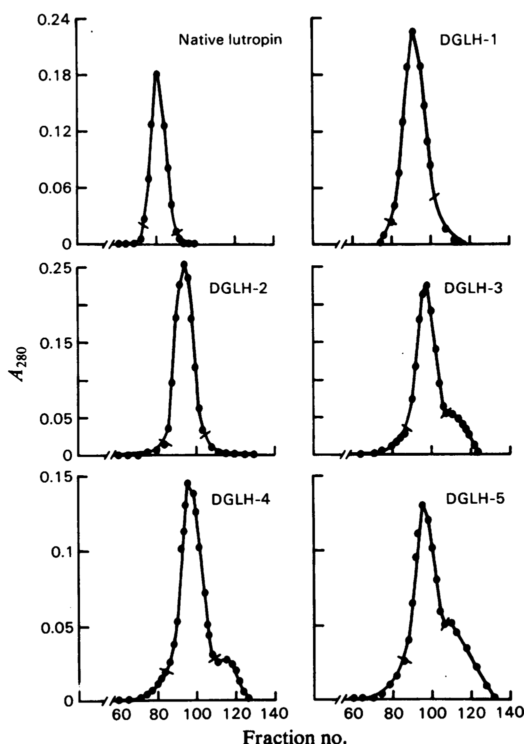


Fig. 1. *Gel filtration pattern of lutropin and its deglycosylated preparations*

Sephadex G-100 column (2.5 cm × 106 cm) was previously equilibrated in 0.05 M-NH_4HCO_3. Lutropin or DGLH preparation (50 mg) was loaded on the column and eluted with the same eluent. Fractions (3.8 ml) were collected at a rate of 35 ml/h. Fractions were pooled as indicated by the cut-off marks in the Figure. Recovery was: DGLH-1, 37 mg; DGLH-2, 35 mg; DGLH-3, 32 mg; DGLH-4, 28 mg; DGLH-5, 27 mg.

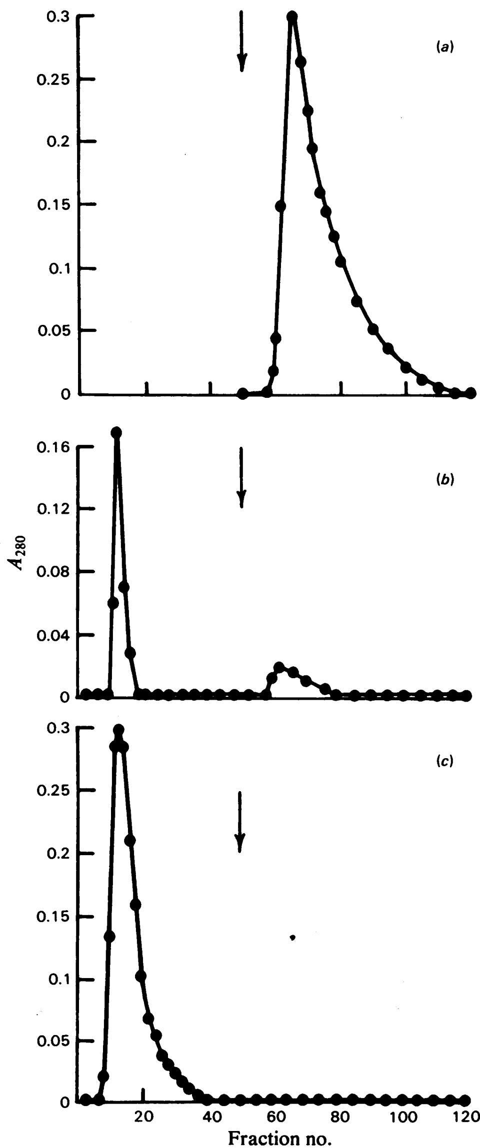


Fig. 2. *Concanavalin A–Sepharose chromatographic patterns of lutropin, DGLH-1 and DGLH-2*

The column (1.5 cm × 22 cm) containing concanavalin A–Sepharose was equilibrated in the concanavalin A buffer (25 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-$MgCl_2$, 10 mM-$CaCl_2$, 0.5 M-NaCl and 0.02% NaN_3). The samples were loaded in 3 ml of equilibrating buffer and elution was carried out as shown. Fractions (3.8 ml) were collected at a rate of 45 ml/h (patterns obtained for DGLH-3, -4 and -5 were identical with that for DGLH-2). In all cases the column flow was temporarily stopped for 30 min after application of the sample to allow for effective interaction with the lectin. The arrow indicates the time of addition of the competing glucoside, methyl glucoside, in concanavalin A buffer. (*a*) Native lutropin; (*b*) DGLH-1; (*c*) DGLH-2.

decrease in carbohydrate content of the hormone on treatment with anhydrous HF.

Chemical deglycosylation markedly altered the binding of the hormone to the affinity column containing concanavalin A–Sepharose gel. Fig. 2 shows the pattern obtained for the native lutropin and derivatives. Native lutropin was strongly adsorbed on concanavalin A–Sepharose and could be eluted by the competing glycoside (Fig. 2*a*). On the other hand the pattern obtained for deglycosylated derivatives was different. For instance, under identical conditions of the experiment, a major portion (approx. 80%) of DGLH-1 was eluted unadsorbed from the concanavalin A–Sepharose with a small fraction (approx. 20%) of it being

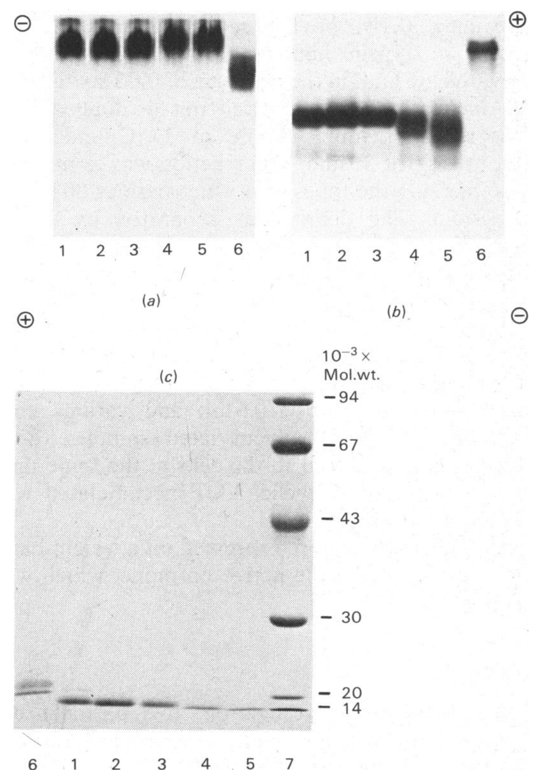


Fig. 3. *SDS/PAGE pattern of lutropin and deglycosylated preparations*

(*a*) 7.5% gel, pH 8.9; (*b*) 10% gel, pH 4.5; (*c*) 12% gel in the presence of sodium dodecyl sulphate; 50 μg of protein per slot in (*a*) and (*b*), and 25 μg per slot in (*c*) were loaded. The gels were stained with Coomassie Brilliant Blue-R250. 1, DGLH-1; 2, DGLH-2; 3, DGLH-3; 4, DGLH-4; 5, DGLH-5; 6, lutropin; 7, protein standards (phosphorylase *b*, mol.wt. 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 20000; lactalbumin, 14000). Similar results were obtained by using 15% gel (results not shown).

adsorbed (Fig. 2*b*). The adsorbed fraction could be eluted with 0.3M-methyl glucoside in a manner similar to native lutropin. However, it is unlikely to represent unreacted native hormone because its elution volume on Sephadex G-100 is distinctly different. As it represented only a small percentage, it was not analysed in detail. The data given in this article for DGLH-1 are derived from the use of the unabsorbed fraction from the concanavalin A–Sepharose column (see Fig. 2*b*), after Sephadex G-100 chromatography. These results indicate that a portion of hormone in DGLH-1 still contains substantial amounts of carbohydrate in a configuration that could interact and bind to the lectin. On the other hand, the other deglycosylated preparations (Fig. 2*c*), DGLH-2, -3, -4 and -5 were not retained on the concanavalin A–Sepharose column indicating the lack of mannose residues, which are apparently necessary for binding of glycoproteins to the lectin concanavalin A.

Electrophoretic properties (*Fig. 3*)

In SDS/PAGE at pH 8.9 the mobility of the deglycosylated derivatives towards the anode was greatly decreased compared with the native hormone and at pH 4.5 deglycosylated hormones migrated more rapidly than the native hormone towards the cathode. Thus the removal of carbohydrate rendered the hormone less acidic in character.

In SDS/PAGE, using 12% or 15% gels (results not shown), lutropin gave two bands corresponding to the α- and β-subunits, whose molecular weights were estimated to be 17000 and 20000 respectively. All deglycosylated preparations gave a single band with an apparent molecular weight of about 13000. It is clear that both subunits of the deglycosylated hormone appear to have the same molecular weight. The molecular weights obtained for the subunits in the native hormone are substantially higher. This is partly due to the anomalous behaviour of the glycoprotein in SDS gels. This anomaly is considerably decreased on deglycosylation.

Amino acid composition

Amino acid analysis of native lutropin, control lutropin and deglycosylated derivatives did not

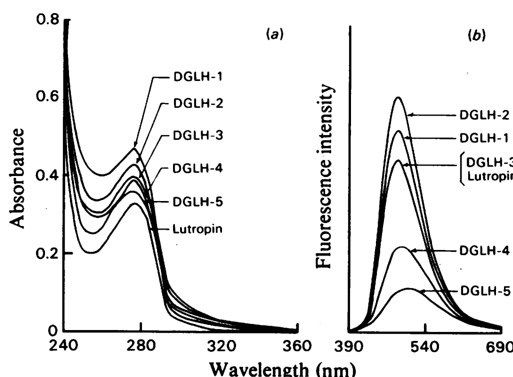


Fig. 4. *Spectral characteristics of lutropin and DGLH preparations*

(*a*) shows u.v.-absorbance spectra of lutropin before and after deglycosylation. The proteins (200 μg/ml) dissolved in 0.05M-potassium phosphate buffer, pH 7.4, were used for recording the spectrum at room temperature. (*b*) shows fluorescence spectra of lutropin and deglycosylated derivatives in the presence of ANS; 950 μl of a 300 μM solution of ANS in 20 mM-potassium phosphate buffer, pH 7.4, containing 100 mM-KCl and 100 μg of protein in 50 μl of the above buffer were mixed. After incubation for 1 h at room temperature, the emission spectra were recorded with excitation wavelength set at 360 nm.

Table 2. *Carbohydrate composition of ovine lutropin and deglycosylated lutropin preparations*

Values in parentheses represent numbers of sugar residues left after HF solvolysis and are calculated by using the formula:

$$\text{Number of residues remaining after deglycosylation} = \frac{\text{Proportion of sugar in DGLH (\%)}}{\text{Proportion of sugar in lutropin (\%)}} \times \text{initial number of residues in lutropin}$$

	Ovine lutropin		Content (g/100 g of glycoprotein)				
Component	(g/100 g of glycoprotein)	(mol/mol of lutropin)*	DGLH-1	DGLH-2	DGLH-3	DGLH-4	DGLH-5
Hexoses	7.2	11.3	0.45 (0.7)	Nil	Nil	Nil	Nil
Fucose	1.5	2.6	Nil	Nil	Nil	Nil	Nil
Glucosamine	5.9	9.3	3.6 (5.7)	3.0 (4.7)	3.0 (4.7)	1.99 (3.1)	1.9 (3)
Galactosamine	2.0	3.2	Nil	Nil	Nil	Nil	Nil

* Molecular weight of ovine lutropin assumed to be 28300.

reveal significant differences (results not shown). The numbers of each amino acid residue in native lutropin and in all deglycosylated preparations correspond well to the values computed for lutropin from structural determinations (Sairam & Papkoff, 1974).

Carbohydrate composition

Treatment of ovine lutropin with anhydrous HF at two different temperatures and for various periods had a significant effect on its oligosaccharide moiety as revealed by the carbohydrate analyses (Table 2). Reaction with HF for 30 min at 0°C resulted in complete loss of fucose and *N*-acetylgalactosamine, and 94% loss of neutral hexose and a decrease of *N*-acetylglucosamine content by 39% Treatment for 1 h at 0°C further removed the neutral sugars completely with an additional loss of a residue of *N*-acetylglucosamine. Longer exposure (3 h) at 0°C did not result in any additional cleavage. Exposure of the hormone at 23°C for 1 h resulted in complete loss of neutral hexoses, fucose, *N*-acetylgalactosamine and 6.2 residues of *N*-acetylglucosamine. Prolonging the exposure to HF as in DGLH-5 did not result in any additional cleavage of the sugars. Thus in ovine lutropin at least three residues of *N*-acetylglucosamine are resistant to HF action even at elevated temperature for prolonged periods.

Spectral characteristics

The u.v.-absorption spectra of the deglycosylated derivatives showed a significant enhancement in absorbance at 278 nm (Fig. 4*a*). The fluorescence probe ANS has been very useful for the analysis of the conformational differences between native lutropin and α- and β-subunits and in recombination studies. Exposure of the hormone to HF at 0°C at all reaction times studied did not decrease the enhancement of fluorescence of ANS (Fig. 4*b*). But the reaction at 23°C resulted in a significant loss of the enhancement of ANS fluorescence and induced a progressive red shift in the maximum of the emission spectrum.

Receptor binding and immunological activities

Partial deglycosylation of lutropin for 30 or 60 min at 0°C did not alter the receptor binding (Fig. 5) and immunoreactivities (Fig. 6). However, treatment with HF for longer periods or at higher temperature resulted in substantial loss of both these activities. Treatment with HF at 0°C for 3 h resulted in some loss of receptor binding and immunoreactivities. Increase in temperature (0°C to 23°C) resulted in almost 70% loss of activity. Increase of both temperature (0°C to 23°C) and period of exposure (1 h to 3 h) resulted in virtually complete loss of receptor binding activity and approx. 80% immunoreactivity (Table 3).

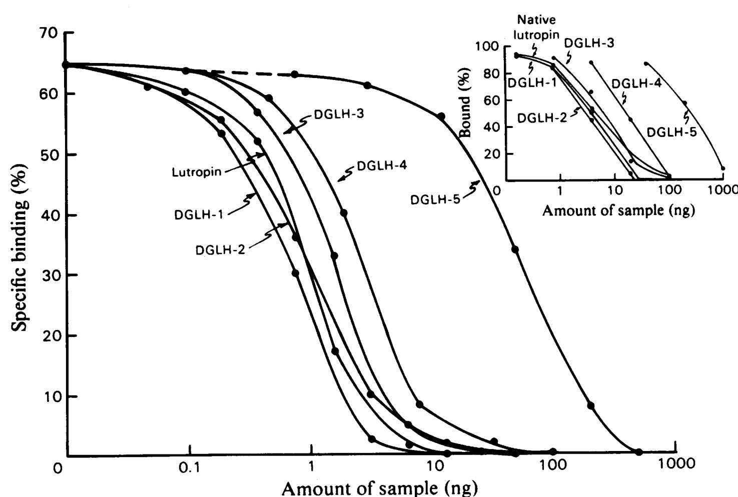


Fig. 5. *Determination of the receptor binding activity of lutropin and its derivatives*
About 50000 c.p.m. of ^{125}I-labelled lutropin was incubated with 25 mg wet wt. equivalent of rat testicular homogenate for 2 h at 37°C in a total volume of 0.5 ml. The total binding in the absence of the unlabelled hormone was 20% of added radioactivity. Non-specific binding in the presence of 1 μg of lutropin was 4.5%. Labelled hormone and unlabelled samples were added at the same time. The inset shows displacement curves obtained by using porcine granulosa cells as the source of lutropin receptor. Details are similar to those given above. In this assay 24% of the added radioactivity was specifically bound in 2 h. This was set as 100% binding.

Hormonal activity (Fig. 7)

Unlike the native hormone, all deglycosylated preparations failed to stimulate rat interstitial cells incubated *in vitro*, despite being present at high concentrations (Fig. 7*a*). Thus there was a complete loss of the ability of the hormone to initiate response, even though good receptor binding activity was evident in three of the five deglycosylated preparations. We have previously shown (Sairam & Fleshner, 1981) that the loss of the ability of the deglycosylated lutropin (DGLH-2) to induce cyclic AMP accumulation is due to neither activation of phosphodiesterase nor altered kinetics.

In view of the differential effects of deglycosylation on receptor binding and cell response, their effect on cyclic AMP accumulation induced by the native hormone was tested. As shown in Fig. 7(*b*), the amount of cyclic AMP accumulated in response to 100ng of native lutropin was gradually decreased by the concomitant presence of increasing concentrations of the deglycosylated preparations. All the five DGLH preparations were effective antagonists and were able to block the action of the hormone. Their antagonistic potency was more or less consistent with receptor binding activity. The DGLH-5 preparation, which was least potent in competing with ^{125}I-labelled lutropin for binding sites, was also the least active in terms of antagonistic behaviour.

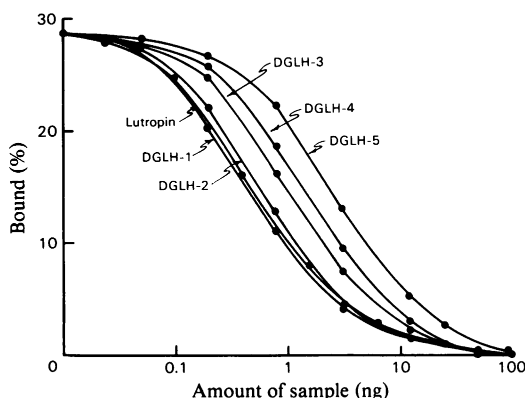


Fig. 6. *Radioimmunological activity of lutropin and its derivatives in a subunit conformation-oriented radioimmunoassay*

^{125}I-labelled lutropin (50000c.p.m.) and antiserum were incubated in a total volume of 0.3ml. After 24h incubation at room temperature antibody-bound hormone was separated by using the second antibody method.

Discussion

Recently we reported that lutropin could be effectively deglycosylated by brief exposure of the hormone to anhydrous liquid HF in the presence of a scavenger (Sairam & Schiller, 1979) resulting in a 70% loss of the carbohydrate moiety without any evidence of peptide bond cleavage. However, the treatment resulted in partial dissociation of deglycosylated hormone into subunits. It was suspected that the partial dissociation into subunits could be due to the method of processing of the deglycosylated hormone rather than a consequence of carbohydrate removal. Despite the application of high vacuum, the traces of HF left adsorbed on the protein resulted in acidic conditions (pH below 4) on addition of aqueous solvents. This condition is adequate to induce significant dissociation of lutropin even at 4°C. This problem can be greatly decreased by employing the modified procedure, including the immediate neutralization of remaining HF by the addition of NaOH. Although the use of 0.5M-NaOH might appear to be a drastic step, creating high-alkaline conditions, the resultant pH in the reaction vessel did not rise above 10. A control experiment in which ovine lutropin was deliberately exposed to pH 11 or pH 12 in 0.1M-Na_2CO_3 at 23°C for 30min showed no decrease in biological activity, including the ability to initiate cell response (results not shown). Ellis (1961) has already shown that

Table 3. *Receptor-binding, immunological, biological and antagonistic activity of ovine lutropin and DGLH preparations*

Numbers in parentheses indicate the numbers of determinations. Results are means ± S.D.

	Radioreceptor activity (%)*	Radioimmunoactivity (%)	Biological activity (%)	Antagonistic activity†
Ovine lutropin	100	100	100 (3)	—
DGLH-1	130 ± 10 (3)	110 ± 5 (2)	Nil (3)	67
DGLH-2	99 ± 7 (14)	90 ± 2 (13)	Nil (3)	100
DGLH-3	62 ± 7 (5)	60 ± 5 (4)	Nil (3)	83
DGLH-4	33 ± 7 (3)	32 ± 4 (2)	Nil (3)	21
DGLH-5	1.5 ± 0.4 (3)	22 ± 3 (2)	Nil (3)	1.8

* Pooled data from assays using testicular or porcine granulosa cell membranes (Fig. 5).

† This was calculated from data in Fig. 7(*b*) based on the amount of the sample necessary to cause 50% inhibition of hormone response.

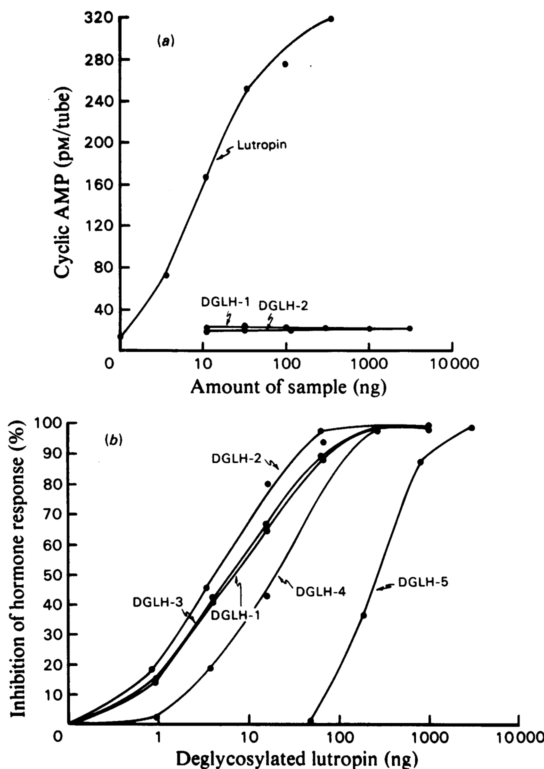
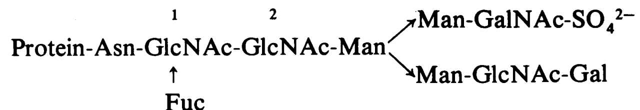


Fig. 7. *Biological activity of lutropin and DGLH preparations (a) and hormonal antagonistic activity of the deglycosylated lutropin preparations (b)*

(*a*) The effect of lutropin and DGLH derivatives on collagenase dispersed rat testicular interstitial cells *in vitro* was evaluated. Approx. 220000 cells were incubated for 30min at 37°C. The incubation consisted of adding 0.5ml of cell suspension in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin, 0.01% lima-bean trypsin inhibitor and 0.05mM-IBMX to glass tubes (12mm x 75mm) containing the samples in 0.1 ml of potassium phosphate buffer, pH7.5, with 0.1% bovine serum albumin. The Figure shows results from one of three typical experiments. Each point is a mean of duplicate incubations with less than 5% variation. Cyclic AMP was measured by protein-binding assay after termination of reaction at 80°C. (*b*) The bioassay *in vitro* of lutropin was carried out as above. The net accumulation of cyclic AMP (303 ± 2.1 pM, $n=6$) in the presence of 100ng of native ovine lutropin was considered as 100% hormone response (i.e. no inhibition). The amount of cyclic AMP accumulated in the presence of 100ng of lutropin and various concentrations of the deglycosylated hormone preparations was determined. The native hormone and the DGLH preparations were added to the cells at the same time. For comparison of the relative antagonistic activity of the preparations, the inhibitory activity shown by DGLH-2 was set as 100% (see Table 3).

ovine lutropin can survive incubation at pH 11 for 24h at room temperature without losing activity. This modification has resulted in higher yields (80%) with almost no dissociation into subunits when applied for the milder reaction conditions, as in DGLH-1 and DGLH-2. Exposure of lutropin for longer periods, as in DGLH-3, and at higher temperature (23°C), as in DGLH-4 and -5, led to some dissociation into subunits even after following the modified procedure.

As we were further interested to know if this approach of chemical deglycosylation could be used to obtain the apoprotein, the effect of HF on carbohydrate cleavage at different temperatures and various periods of time were investigated in the current studies. Interestingly, most of the accessible sugars, including the sulphated hexosamines, were completely removed from the hormone after a brief exposure for 30min at 0°C. The loss of sugars (Table 2) was quite consistent with the proposed structure for the three glycopeptides in lutropin (Parsons & Pierce, 1980; Bahl *et al.*, 1980) as shown below.



It is most likely that, except for two *N*-acetylglucosamine residues numbered 1 and 2, as shown above, all the remaining sugars were completely cleaved by 1h at 0°C. Prolonging the reaction to 3h at 0°C or 3h at 23°C may only cleave an additional residue of *N*-acetylglucosamine (residue 2). It can also be argued that this could be due to a differential (50%) loss of -GlcNAc-GlcNAc- at about half of the carbohydrate attachment sites.

Although prolonged treatment and/or exposure at higher temperatures did not cause significant additional cleavage of remaining sugars, it resulted in substantial damage to the deglycosylated hormone, as indicated by an alteration in some of its properties. The hormone dissociated into subunits to the extent of about 5–10% as indicated by the amount of material recovered in the α-subunit region and solubility was slightly decreased. The decreased enhancement in fluorescence of the probe ANS indicates some change in conformation in DGLH-4 and DGLH-5. As there was no decrease in A_{278} of all DGLH preparations (Fig. 4*a*), it is reasonable to conclude that the aromatic residues such as tyrosine have not been altered. This is further substantiated by amino acid analysis.

The molecular weight of lutropin estimated by SDS/PAGE (37000 for the sum of α- and β-subunits) is much higher than the value of 28300 calculated from structural determinations (Sairam & Papkoff, 1974). This anomaly was decreased upon

deglycosylation. The apparent molecular weight of DGLH preparations by SDS/PAGE (26000 for sum of the deglycosylated α- and β-subunits) is very close to the value of 23900–24500 expected from the loss of carbohydrate (Table 2).

The effect of carbohydrate removal induced by HF reaction of 0°C (DGLH-1, -2 and -3) on receptor binding and immunological activities (Figs. 5 and 6 and Table 3) are minimal, confirming our most recent data (Sairam & Manjunath, 1982). However, similar removal obtained by the reaction at 23°C has more drastic and deleterious effects on receptor binding activity as well as immunological activity, the latter being affected to a lesser degree in DGLH-4. This loss must be due to changes secondary to carbohydrate removal and caused by conformational alterations induced by the elevated temperature of the reaction. The available data (amino acid composition and SDS/PAGE analysis, disc electrophoresis) suggest that these changes are unlikely to be due to peptide bond cleavage.

It was of interest to observe that even the 30min/0°C deglycosylated product (DGLH-1) lost all of its ability to evoke a response in cells, as seen by the failure to accumulate cyclic AMP in the medium (Fig. 7). The hormonal antagonistic activity of all the derivatives were quite consistent with their receptor binding activities (Fig. 7).

Based on data from the present studies the optimum conditions for chemical deglycosylation of ovine lutropin appear to be the exposure of the hormone to HF at 0°C for 30–60min. This results in almost 90% cleavage of all susceptible sugars. The recovery of the deglycosylated hormone was high (over 80%) and the derivative was easily soluble in aqueous buffers and was stable in the freeze-dried form. In 3 years, we have not observed significant changes in the receptor-binding biological, immunological and antagonistic properties of DGLH-2. None of the reaction conditions investigated in the present study were suitable to achieve complete deglycosylation; thus the apohormone could not be obtained.

The assistance of R. Carrière in these studies is appreciated. This investigation was supported in part by the M.R.C. of Canada and the W.H.O Special Programme in Reproduction.

References

Bahl, O. P., Reddy, M. S. & Bedi, G. S. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1192–1199
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Ellis, S. (1961) in *Human Pituitary Gonadotropins* (Albert, A., ed.), pp. 378–380, Charles C. Thomas, Springfield, Illinois
Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305–312
Ingham, K. C. & Bolton, C. (1978) *Arch. Biochem. Biophys.* **191**, 134–145
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Manjunath, P. & Sairam, M. R. (1982) *J. Biol. Chem.* **257**, 7109–7115
McKelvey, J. F. & Lee, Y. C. (1969) *Arch. Biochem. Biophys.* **132**, 99–110
Parsons, T. F. & Pierce, J. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7089–7093
Ramachandran, J. & Sairam, M. R. (1975) *Arch. Biochem. Biophys.* **167**, 294–300
Reisfeld, R. A., Lewis, W. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283
Sairam, M. R. (1979) *J. Endocrinol.* **83**, 149–155
Sairam, M. R. (1980) *Arch. Biochem. Biophys.* **204**, 199–206
Sairam, M. R. & Fleshner, P. (1981) *Mol. Cell. Endocrinol.* **22**, 41–54
Sairam, M. R. & Manjunath, P. (1982) *Int. J. Protein Peptide Res.* **19**, 315–320
Sairam, M. R. & Papkoff, H. (1974) *Handb. Physiol. Sect. 7: Endocrinol.* **4**, 111–131
Sairam, M. R. & Schiller, P. W. (1979) *Arch. Biochem. Biophys.* **197**, 294–301
Winzler, R. (1955) *Methods Biochem. Anal.* **2**, 279–311