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Modification of Sialyl Residues of Gonadotropic Hormones by Reductive Amination. Influence on Biological Activity and Circulating Half-life

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The sialic acid residues of human chorionic gonadotropin, human lutropin and human follitropin were quantitatively modified by introduction of an amino compound. In radioreceptor assays, the modified chorionic gonadotropin, lutropin and follitropin saturated the receptors. However, in the low nanogram range, the gonadotropic binding was higher for the control compared to the modified sample.

The hormonal activity of the chorionic gonadotropin was tested *in vitro*. The modified preparations were four- to thirteen-fold less stimulatory compared to the control but elicited the same maximal response. The biological activity of follitropin was determined *in vivo*. In this case, the modified preparations were four- to five-fold less stimulatory than the control. Both the modified chorionic gonadotropin and follitropin preparations were found to act as agonists. Modification of the gonadotropic hormones did not significantly alter the immune recognition of these glycoproteins.

The apparent circulating half-life in rats of the modified chorionic gonadotropin and follitropin was increased six- to nine-fold compared to that of native hormones; this might be a consequence of resistance of the modified sialyl residues to sialidases and the resultant slower exposure of terminal galactosyl residues; the plasma half-life of modified lutropin remained the same as that of the native hormone.

Abbreviations: hCG, human chorionic gonadotropin; hLH; human lutropin or luteinizing hormone; hFSF, human follitropin or follicle stimulating hormone; mala, methyl ester of alanine; hCG(ala, mala, etc.), human chorionic gonadotropin modified on sialic acid by reductive amination with alanine, methyl ester of alanine, etc.; IRP-HMG, intact rat prostrate - human menopausal gonadotropin.

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The glycoprotein hormone family contains four members: chorionic gonadotropin (CG), lutropin or luteinizing hormone (LH), follitropin or follicle stimulating hormone (FSH), and thyrotropin or thyroid stimulating hormone. The pituitary synthesizes three of the glycoprotein hormones, LH, FSH, and thyrotropin, while the fourth, CG, is produced in the placenta by syncytiotrophoblastic cells [1].

The gonadotropic hormones are structurally related. Each hormone consists of two noncovalently interacting subunits (α and β). The α subunit is identical and common to all hormones while the β subunit is unique and hormone specific. Both subunits are glycosylated and all amino sugars are *N*-acetylated [2]. In man, there are two important differences in the oligosaccharides of the pituitary hormones and CG. First, the pituitary hormones may contain a terminal sulfated *N*-acetylgalactosaminyl residue in their *N*-linked oligosaccharide, whereas CG appears to contain the unsulfated sugar residue only as the linking sugar in its *O*-linked oligosaccharides [3, 4]. Second, the gonadotropins contain varying amounts of sialic acid, with CG being maximum, FSH being intermediate and LH having less sialic acid [5, 6]. Bovine and ovine LH contain little or no sialic acid [7].

The carbohydrate portion of the gonadotropic hormones has been shown to be essential for their biological activity in *vivo* but to bear little or no relation to their immunochemical properties [8, 9]. In particular, the removal of sialic acid by sialidase treatment causes a marked reduction of biological activity *in vivo* without altering the immune response or receptor binding properties of hCG [10] and hFSH [11]. A relationship between sialic acid content and *in vivo* biological activity has been observed [8] and in a series of experiments, progressive desialylation of hCG and hFSH [9, 11] results in a decrease in biological activity to extremely low levels [9].

Similarly, the oligosaccharides of the gonadotropic hormones have been shown to be essential for full hormonal function [12, 13]. Generally, a variety of hormone-receptor interactions [14] involve the carbohydrate moieties of glycoproteins and sialic acid in particular.

Sialic acid masks recognition markers and antigenic sites [15, 16], but may also function as a specific ligand. The former role was initially discovered and clearly established by Ashwell and co-workers [15, 17]. They showed that substitution with sialic acid masked the D-galactosyl residues of various serum glycoproteins protecting them from recognition, uptake and degradation by the liver. Modified sialic residues of glycoproteins have been prepared by mild treatment with periodate followed by reduction with sodium borohydride [17]. These glycoproteins retained the same plasma half-life as their native counterpart.

The resultant aldehyde produced from $NaIO_4$ oxidation of sialic acid can also be subjected to a number of other modifications. Thus, the 7-carbon aldehyde analog of sialic acid can be reacted with various amino compounds in the presence of $NaBH_3CN$ to produce reductively aminated sialic acids [18-20]. We have demonstrated that such modified sialyl residues were resistant to various bacterial and viral sialidases [19].

A possible mechanism for regulating the activity of sialylated hormones would involve the action of target cell sialidase on the bound hormone. The resulting asialo product could

readily dissociate from its receptor and be rapidly cleared from the circulation by the galactose mediated uptake system in the liver.

This paper reports studies on the modification of the sialyl residues of the gonadotropic hormones, hCG, hLH, and hFSH, and the effect of such on receptor binding, antibody recognition, biological activity (*in vitro* and *in vivo*), and apparent circulating half-life.

Materials and Methods

Materials

Human chorionic gonadotropin and pregnant mare serum gonadotropin were purchased form Behring Diagnostics, San Diego, CA, USA. The pituitary gonadotropins, hLH (AFP-0642B) and hFSH were obtained from the National Hormone and Pituitary Program and the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA.

Aspartic acid and alanine were purchased from Behring Diagnostics and the methyl ester of alanine was obtained from Sigma Chemical Company, St. Louis, MO, USA. 2-(2-Aminoethyl)-1-methyl-pyrrolidine and 1-(2-aminoethyl)-piperidine were supplied by Aldrich Chemical Company, Milwaukee, WI, USA and glycine was purchased from Bio-Rad Laboratories, Richmond, CA, USA. $NaIO_4$ (Matheson Coleman and Bell, Norwood, OH, USA), $NaBH_3CN$ (Aldrich), $NaBH_4$ and ethylene glycol (Fisher Scientific Company, Fairlawn, NJ, USA) were of the highest purity available. The methyl glycoside of neuraminic acid was obtained from Boehringer Mannheim Biochemicals, West Germany. The liquid chromatography column (AMINEX Ion Exclusion HPX-87H, 300 x 7.8 mm) was purchased from Bio-Rad.

Receptor binding properties and antibody responses of the mofidied hCG preparations were examined using commercially available kits, Biocept G and PregnaZyme, obtained from Wampole Laboratories, Cranbury, NJ, USA.

Modification of Glycoproteins and Gonadotropic Hormones

The following compounds were subjected to sialyl modification essentially as described [21]: hCG, hFSH and hLH. Human chorionic gonadotropic was dialyzed against distilled water, lyophilized and dissolved in cold buffer (0.1 M sodium acetate, pH 5.6 containing 0.15 M NaCl), whereas the other hormones were directly dissolved in the acetate buffer. The concentration of all compounds was 2.12 mg/ml. In a typical experiment, freshly prepared ice-cold 0.012 M $NaIO_4$ (a ten-fold excess in relation to the amount of sialic acid present) was added to the hormone solution and the mixture stirred at 0°C for 10 min. The oxidation was terminated by adding an excess of ethylene glycol and the solution was allowed to stir for another 10 min at 4°C. The reaction mixture was dialyzed against distilled water to remove all the formaldehyde (from C-9 of sialic acid and from ethylene glycol) and the oxidized glycoproteins were then lyophilized.

The $NaIO_4$-treated glycoproteins and hormones were resuspended in 0.2 M sodium borate buffer, pH 7.8 and each divided into several equal fractions. To each solution, one of the amino compounds (alanine, methyl ester of alanine, aspartic acid, glycine + 10 µCi of

[^{3}H]glycine, the pyrrolidine or piperidine derivative) was added. A fifty-fold excess of the amino compound was utilized in relation to the amount of sialic acid present in each of the glycoproteins. Immediately following the addition of the amino compound, a ten-fold excess of $NaBH_3CN$ was added. The solutions were stirred initially at room temperature for 30 min and then at 4°C for 90 min. After 10, 20, 30 and 60 min, a ten-fold excess of $NaBH_3CN$ was added and after 90 min, a ten-fold excess of $NaBH_4$ was added. The reduction was terminated after 2 h by neutralizing the solution with 0.04 N acetic acid. The modified glycoproteins were dialyzed agianst 1 M NaCl, distilled water and then lyophilized.

Gel Filtration of the Modified Glycoproteins

The [^{3}H]glycine-modified glycoproteins were chromatographed on a Bio-Gel P-2 column that was equilibrated in 0.1 M pyridinium acetate, pH 5.0. The samples, also in 0.1 M pyridinium acetate were applied to the column (0.9 x 110 cm). Fractions (1 ml) were collected and analyzed for either radioactivity or sialic acid. The recovery from the columns was always 85 to 95%.

Analysis of Radioactivity

Liquid scintillation counting was performed on an Intertechnique Model SL-4000 spectrometer equipped with a disintegrations/min calculating module. Aqueous samples (0.2 or 1.0 ml) were mixed with 2 or 10 ml of ACS II counting scintillant (Amersham) in plastic vials.

Assay for Sialic Acid

Column fractions were screened for sialic acid by using the periodate-resorcinol assay [22].

High Performance Liquid Chromatography

Chromatographic separations were carried out utilizing an AMINEX ion exclusion HPX-87H column (300 x 7.8 mm). The solvent was 0.006 N H_2SO_4 and the flow rate was maintained at 0.1 ml/min. The solvent and the samples were filtered through a 0.45 nm Millipore filter, before use. Products were detected by absorbance at 206 nm.

Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done according to the methods described by Laemmli [23] and Hass and Kennett [24]. Gels were run in the presence of 2-mercaptoethanol. Molecular weight markers were not included for gels used for autoradiography.

Iodination of Gonadotropic Hormones and α_1-Anti-trypsin

Iodination of the glycoproteins was carried out using $^{125}I_2$ by the chloramine-T method [25].

Circulation Studies

The plasma half-life of the gonadotropic hormones was studied using Sprague-Dawley female rats weighing approximately 200 g as described by Morell *et al.* [17]. The iodinated

glycoprotein (3×10^6 cpm) was injected into the rat's tail vein which had been previously dilated using xylene and warm water. At indicated time intervals, the rats were bled through the tail (200 µl) into tubes containing heparin (10 units). The blood samples were centrifuged and the plasma was treated with trichloroacetic acid to determine the per cent of ^{125}I-radiolabel associated with proteins. The reason for the apparent increase in some of the early data points is not clear but none of these differences is statistically significant; injection variables are one possible explanation.

Receptor Binding Studies

Ovaries from adult female rats were isolated and the *in vitro* bioassays of hCG and hLH were performed as described by Dufau and co-workers [26]. In addition, ^{125}I-labeled native and modified hCGs were examined for receptor binding [27]. Data from these experiments were also analyzed by Scatchard plots.

Immunological Studies

Antibody recognition of native and modified hCGs was determined by a commercial monoclonal enzyme immunoassay, PregnaZyme [28]. Antibody recognition of native and modified hFSH preparations was determined by radioimmunoassay using polyclonal antibodies specific for hFSH.

In Vitro Bioassay

Hormonal activity of hCG was tested *in vitro* using a sensitive bioassay that measures the testosterone response of collagenase-dispersed rat Leydig cells to gonadotropic stimulation [29]. These assays were kindly done by Dr. M. Dufau at the National Institutes of Health, Bethesda, MD, USA.

In Vivo Bioassay

The *in vivo* bioassays for hFSH were kindly performed by Dr. Albert Parlow at Harbor-UCLA Medical Center, California [30] by the ovarian ascorbic acid depletion assay.

Results

The sialic acid residues of the gonadotropic hormones were modified by the described procedure [21] and the glycoprotein products fractionated on a Bio-Gel P-2 column to separate free amino compound and reducing agent that might still remain undialyzed. The specificity of the conversion to the modified product and the structural identity of the derivatized sialic acids are detailed elsewhere [20, 21]. Combined results from hydrolysis studies, amino acid analysis, thin-layer and high performance liquid chromatography showed that the modification was essentially quantitative.

In the present study the reactions were routinely monitored by HPLC. The modified gonadotropic hormones contained neither native sialic acid nor any of the C7 analog; it can

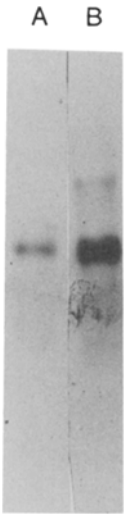

Figure 1. Polyacrylamide gel electrophoresis of [^{14}C]glycine-modified hCG (lane A) and [^{14}C]glycine-modified hFSH (lane B). The samples (about 60 000 cpm) were electrophoresed on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate and the bands detected by fluorography.

be estimated that much less than 0.1% of the native glycoprotein is present. In all cases, the per cent incorporation of amino compound into the above glycoproteins was between 90 and 95% based on sialic acid.

Gel electrophoresis was performed to determine if any polymers of the glycoproteins had formed by intermolecular cross-linking since intra- and intermolecular reactions could occur between the sialyl aldehydes and the protein amino groups [21]. The hormones were labeled on their sialyl residues using [^{14}C]glycine and, as seen in Fig. 1, one major band was detected by fluorography for the modified hCG and modified hFSH; estimates of possible cross-linking indicated values of 1-3%. Similar data were obtained in a model study with α_1-acid glycoprotein [21]. Iodination was carried out with hLH before and after modification and the products were examined for cross-linking by SDS-PAGE gels followed by autoradiography. There was no change in apparent molecular weight between the native and modified hLH. In this case, no high molecular weight bands were detected indicating that little if any intermolecular cross-linking had occurred.

In separate experiments, comparable modifications were carried out on bovine serum albumin and bovine LH (which lacks sialic acid). In neither case was any radioactivity incorporated into the macromolecule nor were amino acid compositions affected (data not shown).

Biological Properties of the Modified Gonadotropic Hormones

The first set of experiments examined the circulating half-lives of the modified ^{125}I-labeled hormones. Examination of the half-life curves obtained for hCG, hFSH, and hLH, shown in

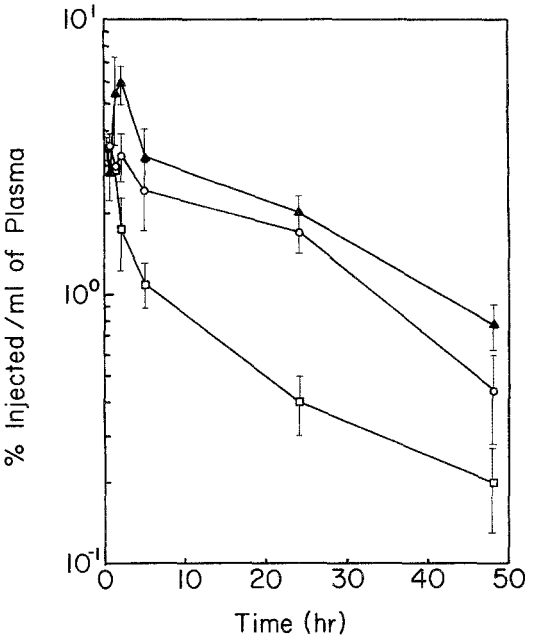

Figure 2. Plasma survival time of native and modified hCG in the rat.
The preparation of hCG was labeled with ^{125}I. Each injection (0.5 ml) contained 2.5-3.0 x 10^6 cpm. Injections were made in the tail vein of Sprague-Dawley female rats weighing about 200 g. Blood was collected in heparinized tubes at various time intervals, centrifuged, and the radioactivity in the plasma determined. ^{125}I-hCG (native, □; ^{125}I-hCG(ala), ○; and ^{125}I-hCG(mala), ▲. Half-lives were calculated from the curve by determining the time required for half of the initial hormone to disappear from the circulation. Each point was an average value from three rats. Per cent injected/ml of plasma = [(cpm of hCG in 1 ml of plasma/total radioactivity injected) x 100%].
Note that the data are expressed per ml of plasma. The average blood volume for the animals used in this study is 8 ml. Accordingly, the earliest time points account for up to 80% of the initial dose.

Figs. 2-4, respectively, indicated that the removal of these hormones from the circulation was multiphasic. There are several possible explanations for this, including the existence of two or more populations of the hormone with varying clearance times, possibly arising from heterogeneity in the saccharide moiety. The chemical data indicate that all sialyl residues were modified, however, and the cause is not known. Therefore, the clearance time was calculated from an *operational* half-life, defined as the time required for half of the initial hormone dose to disappear from the circulation. This does not resolve the problem of alternate degradative pathways but permits an overall comparison between the native hormone and the various derivatives. Thus, the obtained values represent apparent half-lives as defined.

Data for hCG shown in Fig. 2 show no difference in $T_{1/2}$ between hCG(ala) and hCG(mala) but a striking difference between the modified hormone and the control was observed, with the former showing a six- to eight-fold increase in the apparent circulating half-life. Similar results were obtained for hFSH. In this case, the differences from control were six- to nine-fold (Fig. 3). However, in the case of hLH, there was no significant difference in the apparent

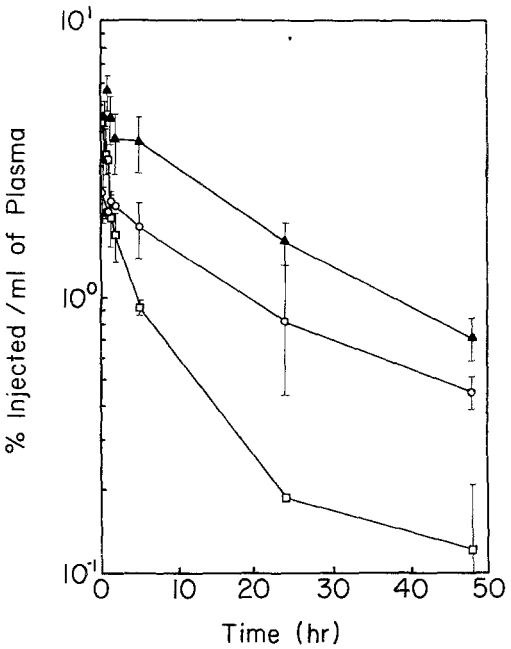

Figure 3. Plasma survival time of native and modified hFSH in the rat. The details of this experiment were similar to those described in Fig. 2 with the exception that 2.5-3.0 x 10^6 cpm of ^{125}I-hFSH were injected. ^{125}I-hFSH(native), □; ^{125}I-hFSH(ala), ▲; and ^{125}I-hFSH(mala), ○.

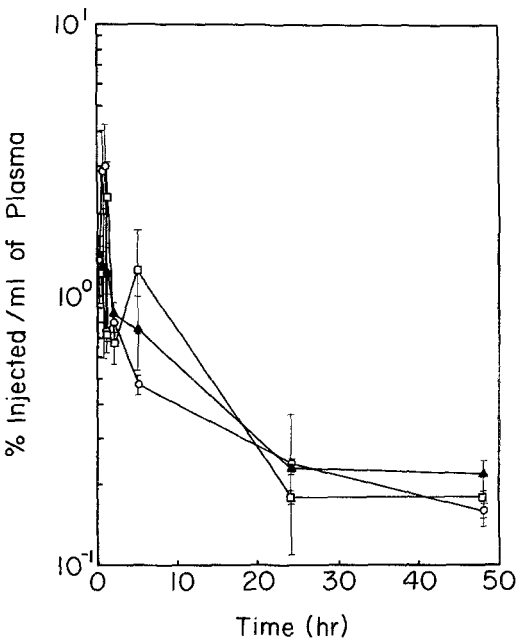

Figure 4. Survival time of native and modified hLH in the rat.
The details of this experiment were similar to those described in Fig. 2 with the exception that 2.5-3.0 x 10^6 cpm of ^{125}I-hLH were injected. ^{125}I-hLH(native), □; ^{125}I-hLH(ala), ○; and ^{125}I-hLH(mala), ▲.

Table 1. Circulating half-lives (hours) of native and sialyl modified gonadotropic hormones in the rat.

Hormone	Native	Sialic acids modified with:	
		alanine	methyl ester of alanine
hCG	2.5 ± 0.48[a]	21.7 ± 0.9	15.2 ± 0.8
hFSH	2.0 ± 0.2	19.0 ± 0.8	12.0 ± 0.9
hLH	1.5 ± 0.2	1.7 ± 0.1	1.7 ± 0.2

[a] calculated from error bars on graph.

circulating half-life as shown in Fig. 4. The circulating half-life data are summarized in Table 1. Analysis of the data indicated a highly significant difference in the apparent half-lives between the native hCG and the modified hCGs [hCG(ala), hCG(mala), hCG(asp) and hCG(piperidine)] and between hFSH(control) and hFSH(ala) or hFSH(mala).

No significant difference was seen between native and modified hLH [hLH(ala) and hLH(mala)]. From the half-life curves, it appears that hLH was cleared faster, accounting for the lower percentage of radioactivity after the first bleeding (20 min), than either hFSH or hCG. The fact that modification of sialic acid residues in hLH had no effect on the apparent circulating half-life suggests that other saccharide residues present on the unique subunit may be involved in clearance. This is consistent with other data in the literature which suggest that the sialic acid residues of glycoproteins are important for the masking of recognition markers and for *in vivo* biological activity [31].

It was of interest to examine the effect of R groups of differing charge introduced into sialyl residues on the plasma half-life. There was no apparent effect on the circulating half-life by hCG modified utilizing R = asp[-2] or R = pyrrolidine [+2], which suggests that the charge of the modified saccharide moiety has no effect on the plasma half-life.

The modified preparations of hCG and hLH were examined for their receptor binding properties using ovarian receptors [26]. The results are illustrated in Fig. 5 and 6. It is clear that the receptors can be saturated by all of the modified hormones; however, the capacity of native and modified hCG to compete with ^{125}I-hCG for binding to the receptor is clearly higher for the native hormone. In both cases, ten times more unlabelled modified hormone was required to displace 50% of the ^{125}I-hCG hormone. Scatchard analysis of the data confirmed this interpretation and indicated a single class of high affinity sites for the native and mala-modified hormones with the former having a fourteen times greater affinity (data not shown).

The immunological reactivity of the modified hormones was tested to examine whether any major alterations had occurred in the molecule after sialyl modification. Data for hCG showed that the native and modified hCGs react identically over a fifty-fold range with monoclonal antibodies specific for the β-subunit. Similar results were obtained for hFSH by radioimmunoassay utilizing a polyclonal antiserum specific for hFSH. Since the immune recognition of these modified hormones was apparently not changed, major alterations in

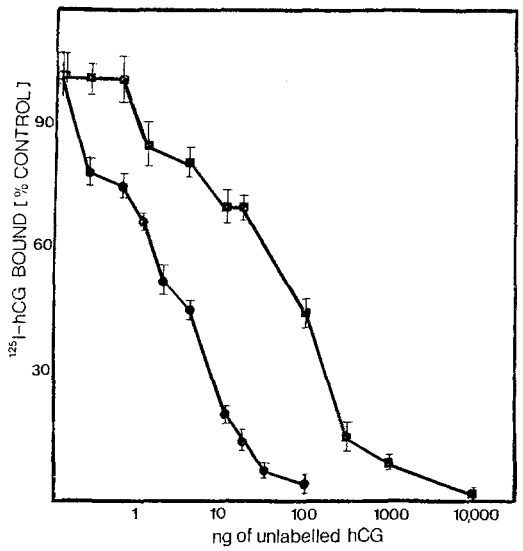

Figure 5. Radioreceptor assay of native and modified hCG.
The radioreceptor assay is based on the competition between hCG (native and modified) and ^{125}I-labeled hCG for ovarian receptors. The receptors were incubated with hCG (native and modified) and ^{125}I-labeled hCG overnight at room temperature. The reaction mixtures were centrifuged and pellets assayed for radioactivity in a gamma counter to determine per cent binding. hCG (native), ●, hCG(ala), ■; hCG(mala), ■.

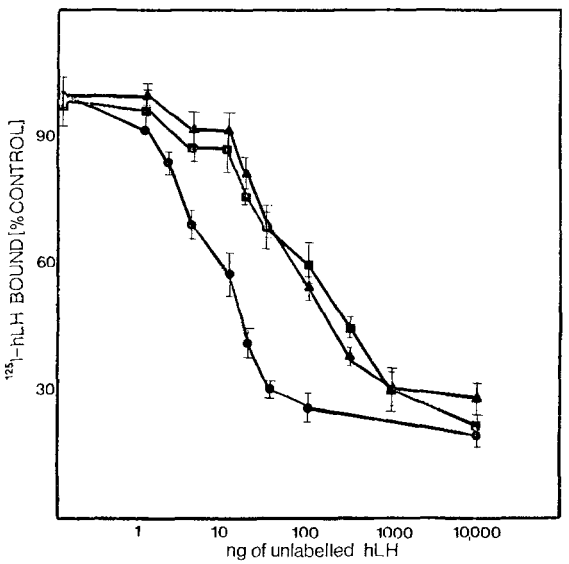

Figure 6. Radioreceptor assay of native and modified hLH.
The details of this experiment were identical to those in Fig. 5. hLH (native), ● ; hLH(ala), ■ ; hLH(mala), ▲

Table 2. Immuno-potency and bio-potency of modified preparations of human FSH.

	Immuno-potency[a]			Bio-potency[b]		
Sample	(IU/mg)	95% limits	lambda	(IU/mg)	95% limits	lambda
hFSH(control)	3916	3700-4143	0.041	1008	675-1508	0.209
hFSH(ala)	1798	1690-1910	0.045	230	119-352	0.140
hFSH(mala)	1218	1146-1293	0.036	277	143-434	0.199

[a] In terms of 2nd intact prostrate-human menopausal gonadotropin. (IRP-HMG).
[b] In terms of 2nd intact rat prostate-human menopausal gonadotropin. These experiments were carried out by Dr. A. Parlow.

the conformational features or in the structures required for antibody binding are not likely to have occurred as a result of the modification. However, a separate set of immunoassays with the modified preparations of hFSH showed decreased antibody recognition (Table 2). Bahl reported that immunological activity is unaffected by desialylation and to some degree, sequential deglycosylation of the inner sugars [12]. Immunological data from hFSH (data not shown) is not complete in the 1/500 to 1/100 dilution range, and it is possible that within this range a difference in antibody recognition could exist. Another explanation for this observed discrepancy in the hFSH results is a difference in antibody specificity.

Key experiments involved determining the *in vitro* biological activity of hCG and the *in vivo* bio-activity of hFSH. The modified hCGs were four- to thirteen-fold less stimulatory than the control but were agonists and elicited the same maximal response (Fig. 7). The effective doses yielding 50% stimulation (ED_{50}) were determined to be 60, 210, and 800 pg/tube for hCG(control), hCG(mala) and hCG(ala), respectively. Similar results were obtained with hFSH (Table 2). Here the bio-potency for the modified preparations of hFSH was determined to be four- to five-fold less than that of the control but they acted as agonists *in vivo*.

Discussion

The gonadotropic hormones, hCG, hLH and hFSH have been modified by introduction of an amino compound into their sialyl residues [20, 21]. These modified sialic acids are resistant to various bacterial and viral sialidases [21].

The apparent half-life data for hCG showed no difference between the hCG(ala) and hCG(mala), but a striking difference was observed between them and the hCG(control) in that the modified compounds had a six- to eight-fold increse in the apparent circulating half-life. Data using various R groups suggests that incorporation of different charge moieties does not further alter the circulating half-life.

Schengrund *et al.* [32] have shown that a sialidase activity is localized in the plasma membrane of rat liver cells. The increased apparent half-life observed here for the modified preparations of hCG may reflect the sialidase resistance of the modified sialyl residues, although we have not directly tested this. Modification is sufficient to alter the plasma half-life, but clearly structural features related to the amino terminal peptide sequence [33] must

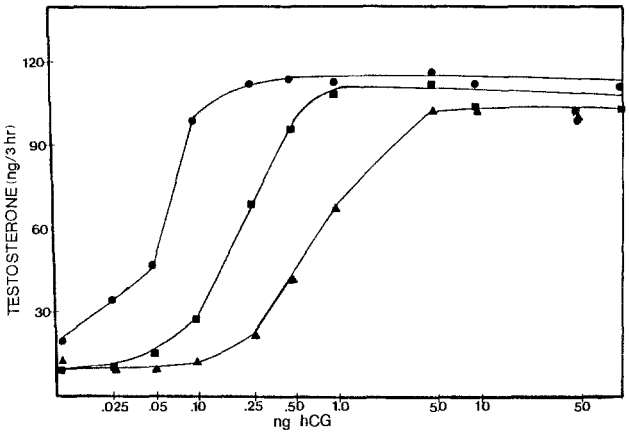

Figure 7. *In vitro* bio-assay of native and modified hCG.
Rat Leydig cells were mixed with various concentrations of native and modified hCG and incubated for 2 h at 37°C in an atmosphere of 5% CO_2 and 95% O_2. Acetic acid was added to terminate the reaction and the production of testosterone was determined by radioimmunoassay. hCG(native),○, hCG(ala), ▲; hCG(mala), ■. Performed by Dr. M. Dufau.

also play a role since the half-life does not extend to a period comparable to that for albumin. Endoglycosidase action [34] and intracellular trafficking wherein both mannosyl and *N*-acetylglucosaminyl residues are recognition elements [35, 36], may also be relevant. It appears that the clearance of glycoproteins may involve both saccharide and peptide moieites.

Similar results were obtained for hFSH. In this case, the differences from the control were six- to nine-fold (Fig. 3). However, there was no significant change in the apparent circulating half-life of hLH as shown in Fig. 4. The saccharide residues in hLH are more sulfated and less sialylated than those on hFSH by a factor of 10 or more [7]. The observed differences suggest that residues on the unique (β) subunit are involved in both clearance and recognition. These findings are consistent with previous reports on the possible role of sialic acid in these hormones [17]. Other workers found human α_1-protease inhibitor modified on sialic acid with ethanolamine to an extent of 78% to be cleared from rat blood circulation at the same rate as the native inhibitor [37].

Immunological studies done with hCG clearly indicated that the immune response remained unaltered after modification. The retention of immunological reactivity suggested that the major conformational features required for antibody binding were still preserved. These results are consistent with our findings that both native and modified α_1-acid glycoprotein gave single bands of identity utilizing anti-α_1-acid glycoprotein antisera [21]. In contrast, one immunoassay of modified hFSH showed that reactivity decreased after modification (Table 2), suggesting that recognition by the antibodies employed involved the carbohydrate moieties and that the modification of sialyl residues altered antibody binding.

The biological activity of the modified preparations of hLH and hCG was initially examined by radio-ligand receptor assays. The results obtained for hCG and hLH demonstrated that the receptors can be saturated by both native and modified preparations. However, the ability of the modified and the control hCG to compete with ^{125}I-hCG for the receptor was clearly higher for the control than for the modified sample. Although the sialyl residues of hCG and hLH are not essential for receptor binding [38], the oligosaccharide chains in the β-subunit apparently play a critical role in coupling the hormone-receptor complex to the adenylate cyclase system [39]. It seems likely that the altered saccharide units, although still able to bind to the receptor, do so less effectively. It should be noted that the individual subunits are biologically inactive when measured by radioligand receptor assays [12] suggesting that conformational sites result from subunit interactions [40].

The biological activity of hCG was determined using an *in vitro* bioassay which measures the testosterone response of collagenase-dispersed rat Leydig cells to gonadotropic stimulation. The modified preparations of the hCG were four- to thirteen-fold less stimulatory when compared to the control but were able to elicit the same maximal response. As previously observed for deglycosylated hCG preparations, the modified preparations were agonists [31]. The biological activity of hFSH determined *in vivo* by the ovarian ascorbic depletion assay system gave similar results. The modified preparations were four-to five-fold less stimulatory than that of the control but also acted as agonists.

The described modifications of hCG and hFSH produce agonists with increased circulating half-life; further modifications to yield antagonists may be possible. It should be emphasized that the observed activity of the modified hormones cannot be due to residual, unreacted starting material and represents that of the structurally altered derivative. It is not known at this time if the modified hormones elicit an antibody response.

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References

1 Fiddes JC, Talmadge K (1984) Recent Prog Horm Res 40:43-49.
2 Ward DN, Coffee J (1964) Biochemistry 3:1575-77.
3 Parsons TF, Pierce JG (1980) Proc Natl Acad Sci USA 77:7089-93.
4 Tolvo A, Fujiki Y, Bhavanandan VP, Rathnam P, Saxena BB (1982) Biochim Biphys Acta 719:1-10.
5 Jutisz M, Teergrin-Clary C (1974) Curr Top Exp Endocrinol 2:195-246.
6 Stockell-Hartree A (1972) Pituitary Glycoprotein Hormones, ed. M Jutisz, Proc INSERM Colloq 71-82.
7 Green ED, Baenziger JU (1988) J Biol Chem 263:36-44.
8 Tsuruhara T, van Hall EV, Dufau ML, Catt KJ (1972) Endocrinology 91:463-69.

9 Tsuruhara T, Dufau ML, Hickman J, Catt KJ (1972) Endocrinology 91:296-301.
10 Schuurs AH, Dejager E, Homan JD (1968) Acta Endocrinol 59:120-38.
11 Manjunath P, Sairam MR, Sairam J (1982) Mol Cell Endocrinol 28:124-38.
12 Bahl OP (1977) Fed Proc 36:2119-27.
13 Keutmann HT, McIlroy PJ, Bergert RJ, Ryan R (1983) Biochemistry 22:3067-72.
14 Fishmann PH, Brady RO (1976) Science 194:906-15.
15 Ashwell G, Morrell AG (1974) Adv Enzymol 41:99-128.
16 Springer GF (1984) Science 224:198-206.
17 Morrell AG, Gregoriadis G, Scheinberg IH, Hickman J, Ashwell G (1971) J Biol Chem 246:1461-67.
18 Itaya K, Gahmberg CG, Hakomori S (1975) Biochem Biophys Res Commun 64:1028-35.
19 Barsoum AL, Davidson EA (1981) Mol Immunol 5:367-72.
20 Murray MC, Bhavanandan VP, Davidson EA (1988) Carbohydr Res, in press.
21 Bhavanandan VP, Murray MC, Davidson EA (1988) Glycoconjugate J 5:467-84.
22 Jourdian G, Dean L, Rosemans S (1971) J Biol Chem 246:430-35.
23 Laemmli JK (1970) Nature 277:680-85.
24 Hass JB, Kennett RH (1979) in Monoclonal Antibodies, eds. Kennet RH, McKear RT, Bechtol KB, Plenum Press, New Ypok p 407-11.
25 Hunter WM, Greenwood FC (1962) Nature 194:495-96.
26 Mendelson C, Dufau ML, Catt KJ (1975) J Biol Chem 250:8818-23.
27 Sekiya T, Furuhashi Y, Goto S (1981) Acta Endocrinol 97:562-68.
29 Dufau ML, Catt KJ, Tsuruhara T (1972) Endocrinology 90:1032-40.
30 Parlow AF, Reichert LE (1963) Endocrinology 73:740-43.
31 Bahl OP, Shah RH (1977) in Glycoconjugates, Vol 1, eds. Horowitz MI, Pigman W, Academic Press, New York, p. 385-410.
32 Schengrund C-L, Jensen DS, Rosenberg A (1972) J Biol Chem 247:2742-46.
33 Bachmair A, Furley D, Varsharsky A (1986) Science 234:179-86.
34 Maury P (1971) Biochim Biophys Acta 252:4857.
35 Schlesinger PH, Doebber TW, Mandell BF, White R, DeSchryver C, Rodman JS, Miller MJ, Stahl P (1978) Biochem J 176:103-9.
36 Achord DT, Brot FE, Sly WS (1977) Biochem Biophys Res Commun 77:409-15.
37 Omichi K, Savic G, Yoshida A (1983) Int J Biochem 15:1345-51.
38 Catt KJ, Dufau ML, Tsuruhara T (1972) J Clin Endocrinol 34:123-32.
39 Sairam MR, Bhargavi GN (1985) Science 229:65-67.
40 Bahl OP (1973) in Hormonal Proteins and Peptides, ed. Li CH, Academic Press, New York, p. 171-99.