Role of Carbohydrate in Human Chorionic Gonadotropin

EFFECT OF PERIODATE OXIDATION AND REDUCTION ON ITS IN VITRO AND IN VIVO BIOLOGICAL PROPERTIES*

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Sequential removal of the carbohydrate of human chorionic gonadotropin (hCG) results in the impairment of its biological activity in vitro. The in vitro activity of these derivatives, however, could not be investigated due to their rapid removal from circulation by liver. Therefore, asialo-hCG was subjected to controlled oxidation and reduction in order to render it suitable for in vivo studies. Two periodate oxidized-reduced derivatives, designated as PORA-hCG-I and PORA-hCG-II, were obtained. While PORA-hCG-I lost about 85% of the galactosyl residues, in PORA-hCG-II all galactosyl and 40% mannosyl residues were modified. A detailed physico-chemical and immunological characterization of the derivatives established the integrity of the polypeptide chain. The modification did not adversely affect the receptor binding activity of the hormone, rather PORA-hCG-I and -II both bound to the ovarian receptor with about 2.4 times higher affinity ($K_a$, $1.8 \times 10^{-8}$ m$^{-1}$) than hCG ($K_a$, $0.75 \times 10^{-10}$ m$^{-1}$). Furthermore, the oxidation of the terminal galactosyl residues in asialo-hCG resulted in a drastic decrease of about 290-fold in its binding affinity to the liver receptor compared to asialo-hCG ($K_a$, $0.19 \times 10^{-9}$ m$^{-1}$). As a result, the plasma clearance rate of PORA-hCG-I decreased considerably with concomitant increase in its ovarian uptake to a level comparable to hCG. In vitro response in corpus luteal cells showed that, although PORA-hCG-I and PORA-hCG-II stimulated steroidogenesis maximally, they had only 3-5% potency compared to hCG based on the dosage at half-maximal response. On the other hand, in cAMP production, both derivatives stimulated only 20% of the maximal response of hCG at the highest concentration used (1 µg/ml). In both cAMP production and steroidogenesis, PORA-hCG-I inhibited the action of hCG, inhibition being more pronounced in cAMP production (80%) than in steroidogenesis (20-30%). The properties of PORA-hCG-I studied in vivo included the effect on the ovarian ascorbic acid depletion and the trophic effect as indicated by the increase in the mouse uterine weight. This derivative not only showed reduced hormonal activity of 2-5% in these assays, but it also caused a dose-dependent inhibition of the hCG activity. These findings strongly suggest that while the carbohydrate is not involved in the hormone-receptor interaction, it is needed for the expression of the hormonal activity.

Human chorionic gonadotropin is a glycoprotein hormone in which the carbohydrate constitutes about 30% of the molecule. Because of the recent advances in our understanding of the detailed structures of the protein and carbohydrate parts of hCG (1-4) and the vital role it plays in maintaining pregnancy in humans, it has been the target for the development of newer means of fertility regulation. It was reported earlier that sequential removal of monosaccharides from hCG by exoglycosidases resulted in a considerable impairment of biological activity in vitro as judged by steroidogenesis and cAMP production in granulosa and luteal cells (5-8). Furthermore, all the carbohydrate-modified derivatives were able to bind to ovarian and testicular receptors with equal or greater affinity than the native hCG. However, in vivo evaluation of the biological properties of these derivatives could not be undertaken because of their rapid clearance from circulation by the carbohydrate specific receptors on the liver cell surface (9-12). This paper deals with further modification of the nonreducing terminal galactosyl residues in asialo-hCG by controlled periodate oxidation and reduction with the objective of prolonging its plasma half-life. The in vitro and in vivo agonistic and antagonistic properties of the resulting PORA-hCG have been investigated and are the subject of the present. A preliminary report of this work has been made earlier (13).

**EXPERIMENTAL PROCEDURES**

Materials: Commercial preparations of hCG (100 IU/ml) and FSH (10 IU/ml) were obtained from Organon, West Orange, N.J. (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) and NIH (NCI). Products of Sigma Chemical Co., St. Louis, Mo., Fisher Scientific Co., Pittsburgh, Pa., and Becton-Dickinson Lab. (Rutherford, N.J.) were used. Periodate oxidized reduced asialo-hCG was prepared according to (7). Asialo-hCG was purified by gel filtration on Bio-Gel P2 and DEAE-Sephadex. Human chorionic gonadotropin and ovine gonadotropin, were purchased from Nuclear Corp. (Niles, Ill.). Tropine, 1:1 dibutyryl cyclic 3',5'adenosine monophosphate, were from Sigma Chemical Co., St. Louis, Mo. and LKB Waltham, Mass., respectively. Glassware and glassware mill with specific activity of 45 Cl/mmol were purchased from Nuclear Corp. (Niles, Ill.).

1. The abbreviations used are: hCG, human chorionic gonadotropin or chorionic gonadotropin; A-hCG, asialo-hCG; PORA-hCG-I and -II, periodate oxidized reduced asialo-hCG-I and -II; PORA-hCG-I and -II, PORA-hCG-I, OAAD, ovarian ascorbic acid depletion assay; MUW, mouse uterine weight; SDS, sodium dodecyl sulfate; MIX, methyl 1,3, isobutylxanthine; ED$_{50}$, effective dose.

2. Portions of this paper (including "Experimental Procedures" Figs. 1-5, and Tables I and II) are contained in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9630 Rockville Pike, Bethesda, MD 20814. Request Document No. 82 M 901, cite authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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Carbohydrate-modified Analogs of hCG

Preparation and Characterization of PORA-hCG-I and PORA-hCG-II—For the preparation of PORA-hCG-I and II, a highly purified hCG with specific activity of 12,000 IU/mg was used as the starting material (22). Sialic acid was quantitatively removed with neuraminidase treatment. The terminal galactosyl residues were oxidized with periodate under controlled conditions. The optimal experimental conditions for oxidation with respect to time, temperature, and concentration of sodium metaperiodate were established using asialo-fetuin. As is clear from Fig. 1, in 3.5 h 1.8 mol of periodate was consumed per mol of galactose compared to a theoretical value of 2 mol for complete oxidation. Thus, 85% galactosyl residues were destroyed under these conditions without any significant effect on any of hCG and its derivatives to导读 read liver homogenates (25x 10^6 g of liver homogenates in 0.1 M 2-[N-morpholino]ethanesulfonic acid, pH 7.2, containing 1 mM MgCl2) was also performed in the same protocol except that 175 U of neuraminidase were used. Assay of 3H-thymidine incorporation was performed as described earlier (25). In control and treated cultures, the amount of bound and free hormone was derived from the difference between total and non-specific incorporation, corrected for 3H-thymidine uptake in cultures without hormone. The CPM values were converted to nCi using a factor of 27.4 cpm/nCi.

Presentation of Data: The major finding was that 3H-thymidine incorporation into DNA was increased in response to hormone treatment. The data were presented as a percentage of the control values. The results were expressed as the mean ± SEM. The statistical significance was assessed using the Student’s t-test and the analysis of variance (ANOVA).

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other carbohydrate. It is worth pointing out that for NaBH₄ reduction, the pH should be strictly controlled and kept below 7.0. Even a brief exposure of the derivative to alkaline conditions may lead to reduction of the disulfide bonds and thereby inactivation of the hormone. The PORA-hCG-I thus obtained was further treated with periodate for an additional period of 4 h to oxidize completely the galactosyl residues. The second preparation was designated as PORA-hCG-II. The recovery of the derivatives was about 80–85%.

In order to assure that the chemical treatment used in the preparation of hCG derivative produced only the desired modification of the carbohydrate, a detailed physico-chemical characterization of PORA-hCG-I and its subunits was undertaken. The separation of PORA-hCG-I subunits, after dissociation in 8 M urea, was achieved by gel filtration on Sephadex G-75 (Fig. 2). The two subunits of PORA-hCG-I, α and β, eluted as sharp peaks with elution coefficients \( K_{av} \) of 0.41 and 0.22 compared with 0.35 and 0.14, respectively, for native hCG subunits. Tables I and II show the carbohydrate and amino acid compositions of the derivatives, respectively. While 85% of the exposed galactosyl residues were destroyed in PORA-hCG-I, almost all galactose was oxidized in PORA-hCG-II. In addition, the latter also lost 40% of mannose. The bulk of 15% galactosyl residues still remaining in PORA-hCG-I were found to be in the β-subunit. These were further located in the COOH-terminal chymotryptic peptide (Table I), βC-19 (residues 109-145) which contains all O-glycosidically-linked sugar chains (2, 4). The periodate treatment did not affect the hexosamine content in both preparations. The amino acid analyses of both PORA-hCG preparations did not show any significant change from that of hCG. It should be noted that cystinyl and methioninyl residues which are quite susceptible to oxidation did not undergo any loss, suggesting that the protein backbone was essentially unaffected during oxidation. The NH₂-terminal amino acids of α and β subunits of PORA-hCG-I were found by dansyl method to be alanine and serine, respectively. In addition, a small amount of valine was observed in PORA-hCG-I-α, consistent with the microheterogeneity in the NH₂-terminal amino acid sequence of hCG-α as reported earlier (1).

Both preparations of PORA-hCG were homogeneous when examined by polyacrylamide gel electrophoresis in acidic and alkaline buffer systems (Fig. 3). At pH 8.3, while hCG with highly negative charge due to 14–16 sialic acid residues (3, 4) was able to penetrate the gel (RF, 0.21), A-hCG and both preparations of PORA-hCG did not migrate appreciably. Conversely, at pH 4.5, while hCG failed to enter the gels, its derivatives migrated with identical RF values of about 0.26. When the above derivatives were examined by SDS-polyacrylamide gel electrophoresis under completely denaturing condition, their α and β-subunits moved with identical RF values of 0.50 and 0.42, respectively. Under similar conditions, the
Comparison of binding affinities of hCG and its derivatives to ovarian and liver receptors

The binding affinities of hCG and its derivatives for ovarian and liver receptors were determined from the Scatchard plots prepared from the competition binding data in Figs. 6 and 9, respectively. $K_a$ is the apparent equilibrium binding constant.

| Hormone       | Ovarian receptor $K_a \times 10^{10} \text{M}^{-1}$ (mean $\pm$ S.E., $n = 3$) | Relative affinity $^*$ | Liver receptor $K_a$ (Relative affinity$^*$) |
|---------------|---------------------------------------------------------------------------------|------------------------|-------------------------------------------|
| hCG           | 0.75 $\pm$ 0.17 100                                                             | 0.93 $\times 10^6$     | 0.05                                      |
| A-hCG         | 1.99 $\pm$ 0.20 266                                                             | 0.19 $\times 10^6$     | 100                                       |
| PORA-hCG-I    | 1.78 $\pm$ 0.29 237                                                             | 6.54 $\times 10^6$     | 0.35                                      |
| PORA-hCG-II   | 1.80 $\pm$ 0.11 240                                                             | N.D.$^*$               |                                           |

$^*$ $K_a$ values for hCG and A-hCG were assumed 100% in ovarian and liver receptor binding assays, respectively.  
$^*$ Not determined.

In order to assess further the effect of modification of carbohydrates of A-hCG on binding to the ovarian receptor, radio-ligand receptor assay was performed with crude ovarian plasma membranes (Fig. 9). The equilibrium binding constants ($K_a$) listed in Table III were determined from the Scatchard plots of the competitive binding curves shown in Fig. 6. Desialylation of hCG resulted in about 2.6-fold increase in its binding affinity ($K_a$, 1.99 $\times 10^{10}$ M$^{-1}$). The $K_a$ value for native hCG was found to be 0.75 $\times 10^{10}$ M$^{-1}$. These results are in close agreement with those reported by Moyle et al. (8), and Tsuruhara et al. (34) for rat testicular and ovarian membranes, respectively. Further oxidation of the terminal galactosyl residues resulted in a slight decrease in the binding affinity as compared to A-hCG, but it was still 2.4 times higher than hCG. Similar results were obtained when testicular plasma membranes were used in the assay. The above data, thus, suggests that higher ovarian uptake of PORA-hCG-I in vivo was probably due to its higher affinity for the receptor (see below).

**Stimulation of cAMP Production and Steroidogenesis in Corpora Luteal Cells**—The dose response curves of cAMP production and steroidogenesis obtained with varying amounts of hormone is shown in Fig. 7. Although both preparations of PORA-hCG were able to stimulate steroidogenesis maximally, they had only 3-5% hCG activity calculated on the basis of $ED_{50}$ (see "Experimental Procedures"). Half-maximal stimulation of steroidogenesis was obtained at a concentration of about 1 ng/ml of PORA-hCG compared with a value of 0.03 ng/ml obtained with the native hormone. In case of cAMP production, both preparations of PORA-hCG stimulated only 20% response of that of hCG at the highest concentration used (1 $\mu$g/ml). A-hCG produced about 50% of the maximal response of hCG at 1 $\mu$g/ml. Thus, PORA-hCG acted as a partial

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**Table III**

Comparison of binding affinities of hCG and its derivatives to ovarian and liver receptors

**Fig. 6. In vitro binding of hCG and derivatives to rat ovarian homogenates.** 100,000 cpm of 125I-hCG was incubated with ovarian homogenate in the presence of different concentrations of serially diluted hCG or derivatives. In the absence of hCG, 30,000 cpm were bound and assumed as 100% binding. Specific binding was calculated by subtracting the nonspecific binding, i.e., in the presence of 10 $\mu$g hCG, which was 2,500 cpm. The per cent binding, displayed as logit transform, was plotted against the log of the dose. ---, hCG; - - A-hCG; - - PORA-hCG-I; - - - - PORA-hCG-II.

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**Fig. 4. Ultraviolet absorption spectra of hCG and its derivatives.** The samples were dissolved in 0.01 M potassium phosphate, pH 7.4 at concentration of 3 n mole. The amount of protein was determined from the absorbance at 280 nm and the proteins were almost identical in their absorption with molar extinction coefficients at 278 nm of 19,880, 17,950, and 17,840, respectively. These studies suggest the absence of any detectable denaturation in hCG on oxidation. This is further supported by the identical immunological activity of hCG and its derivatives (91-102%) as determined by radioimmunoassay using 125I-hCG and anti-hCG antiserum (Fig. 5). Furthermore, hCG and the derivatives yielded parallel dose response curves, again confirming the integrity of the polypeptide chains.

**Binding of hCG and Its Derivatives to Ovarian Receptor**—In order to assess further the effect of modification of carbohydrates of A-hCG on binding to the ovarian receptor, radio-ligand receptor assay was performed with crude ovarian plasma membranes (Fig. 9). The equilibrium binding constants ($K_a$) listed in Table III were determined from the Scatchard plots of the competitive binding curves shown in Fig. 6. Desialylation of hCG resulted in about 2.6-fold increase in its binding affinity ($K_a$, 1.99 $\times 10^{10}$ M$^{-1}$). The $K_a$ value for native hCG was found to be 0.75 $\times 10^{10}$ M$^{-1}$. These results are in close agreement with those reported by Moyle et al. (8), and Tsuruhara et al. (34) for rat testicular and ovarian membrane receptor, respectively. Further oxidation of the terminal galactosyl residues resulted in a slight decrease in the binding affinity as compared to A-hCG, but it was still 2.4 times higher than hCG. Similar results were obtained when testicular plasma membranes were used in the assay. The above data, thus, suggests that higher ovarian uptake of PORA-hCG-I in vivo was probably due to its higher affinity for the receptor (see below).
agonist of hCG. Consequently, the ability of PORA-hCG-I to inhibit hCG action in the two assays was determined. As shown in Fig. 8, equal or higher amount of PORA-hCG-I was able to neutralize about 80% of the activity of 10 ng and 100 ng of hCG in cAMP assay. In steroidogenesis, PORA-hCG-I neutralized the activity of hCG only partially (20–30%). Similar results were obtained previously with the glycosidase-treated hCG derivatives in Leydig cells (8).

It is clear from the data that the two preparations of PORA-hCG were indistinguishable in their physico-chemical, immunological, and in vitro biological properties. Therefore, further in vitro as well as in vivo studies were conducted with only PORA-hCG-I.

Binding of hCG and Its Derivatives to Liver Membrane Receptors—In order to assess the effect of oxidation of terminal galactosyl residues of A-hCG on its binding to the liver Gal/Glu receptor, competitive binding assay was performed using 125I-A-hCG and crude rat liver membranes. As may be seen from Fig. 9, the oxidation of terminal galactosyl residues led to a greatly diminished ability of A-hCG to bind to the liver membrane receptor. While 20 ng of A-hCG was able to cause 50% inhibition of the 125I-A-hCG binding, ED50 for PORA-hCG-I was 12,000 ng. At the highest level tested (25 μg), hCG produced only 20% inhibition. Scatchard analyses of the above data showed that, while A-hCG had very high affinity for Gal/Glu receptor ($K_a = 0.19 \times 10^{-9}$ M$^{-1}$), PORA-hCG-I possessed 290-fold less affinity, with $K_a = 6.54 \times 10^{-8}$ M$^{-1}$ (Table III). The association constant $K_a$ for hCG was 0.93 × 10$^{-8}$ M$^{-1}$. Thus, the increased plasma survival time of PORA-hCG-I was probably due to its decreased binding to liver Gal/Glu receptor (see below).

Plasma Clearance of hCG and Its Analogs—The plasma survival curves of 125I-labeled hCG, A-hCG, and PORA-hCG-I are shown in Fig. 10. As expected, the desialylation of hCG led to a dramatic increase in its plasma clearance rate with
t\_n/2 of less than 6.5 min as a result of the exposed galactosyl residues. The plasma half-life of the oxidized derivative was substantially increased (t\_n/2, 15 min), though still less than that of native hormone (t\_n/2, 48 min). During the duration of these experiments (30 min), no phosphotungstic acid-soluble counts were detectable in the plasma, indicating that little or no degradation of hCG and its analogs had occurred.

Ovarian Uptake of hCG and Its Derivatives in Immature Superovulated Rats—Although the mature female rats provided a good system to examine the plasma clearance of hCG and its derivatives, it was a poor model for the comparison of their ovarian uptake because of the low levels of uptake, i.e. less than 1% of the injected dose. Moreover, the corpora lutea of mature rats contain few receptors and remain nonfunctional unless activated by mating or other stimuli (35). Therefore, to study the effect of oxidation of A-hCG on ovarian uptake, immature, superovulated rats were used. The results of these studies are shown in Fig. 11. While A-hCG did not accumulate in the ovaries to a significant extent, PORA-hCG-I was taken up by the ovaries at a level comparable to hCG.

Biopotency of PORA-hCG-I—Since PORA-hCG-I binds to the ovarian or testicular receptors in vivo or in vitro equally or better than hCG, it was possible to assay its biological activity in vivo. Two bioassays, ovarian ascobic acid depletion and mouse uterine weight assay were performed to determine the specific activity of PORA-hCG-I, the former measures hCG/LH activity and the latter total gonadotropin activity. The

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**Fig. 10. Plasma survival curves of hCG and derivatives in rats.** Mature female rats in diestrous phase were cannulated through femoral vein and artery. About 0.5 x 10^6 cpm of ^125I-labeled hCG or derivatives were injected through the vein and blood samples were collected from the artery at the indicated time intervals in heparinized tubes. The radioactivity in 100 μl of plasma were counted and expressed as per cent of the injected cpm/ml of plasma. Each point is the average from three animals and vertical bars extend to the limit of S.E.

**Fig. 11. In vivo ovarian uptake of the derivatives.** Immature superovulated female rats 22-24 days old were injected with 0.5 x 10^6 cpm of ^125I-labeled hCG and derivatives through tail vein. Two rats were killed at each indicated time intervals and ovaries were removed, freed of connective tissue, dried on filter paper, weighed, and counted. The results are expressed as per cent of injected counts found per pair of ovaries. 

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Table IV

| Bioassay | Specific activity IU/mg | ±S.D. |
|----------|-------------------------|-------|
| OAAD     | 499                     |      |
| MUW      | 181                     |      |

*Calculated from the data in Fig. 12.

* Number of independent observations.

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**Table V**

Ability of PORA-hCG-I to neutralize the biological activity of hCG in ovarian ascobic acid depletion assay

For details of the assays see legend to Table IV.

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Results of these bioassays are shown in Fig. 12 and Table IV. While a mean specific activity of 499 IU/mg (369-629, 95% confidence limit) was obtained with OAAD assay, a lower value of 181 IU/mg (146-216, 95% confidence limit) was estimated by MUW. The average specific activity obtained from the two assay systems was 357 ± 68.9 IU/mg (222-492, 95% confidence limit). Thus, oxidation of galactosyl residues re-
sulted in a derivative which had only 2-5% agonist activity as compared with hCG.

**Ability of PORA-hCG-I to Neutralize the Biological Activity of hCG in Vivo**—Since PORA-hCG-I binds to the ovarian receptor in vitro as well as in vivo, it was examined for its ability to neutralize the effect of hCG in the above two bioassay systems. Tables V and VI show the results of these studies. As may be seen from Table V, 0.25 µg and 0.5 µg of PORA-hCG-I almost completely neutralized the effect of 6 IU of hCG (p < 0.001) in OAAD assay. Similarly, in the MUW assay (Table VI), it is obvious that PORA-hCG-I significantly reduced the effect of the hCG to about 40% (II:IV, p < 0.001; VI:VIII, p < 0.001).

**DISCUSSION**

The role of carbohydrate in the function of glycoproteins has attracted a great deal of attention in recent years. Since the classical findings of Ashwell and Morel of the presence of galactose/glucose receptor on hepatocytes (36), several other receptors specific for sugars such as mannose, fucose and N-acetylgalcosamine, and mannose-6-phosphate have been found in reticuloendothelial system and skin fibroblast, respectively (11, 12, 37-39). Although the physiological significance of the receptors in the metabolism of glycoproteins remains to be clarified, these studies do establish the function of carbohydrates in cellular recognition. As far as the role of carbohydrate in the mechanism of action of glycoproteins of biological significance is concerned, only a limited number of such studies have been reported thus far (5-8). We first initiated studies on the role of carbohydrate in the biological and immunological function of hCG in vitro (8). The sequential removal of sugar residues from hCG resulted not only in the loss of hormonal activity but also imparted antagonistic properties to the hormone. However, these in vitro studies could not be extended to in vivo situation in the whole organism because of the removal of the derivatives from plasma by the specific receptors for sugars on the liver cell surface. This prompted the present investigation, in which further modification of A-hCG was necessary in order to prolong its plasma half-life by inhibiting the binding to the liver glycoprotein receptors without affecting its ability to bind to the ovarian receptor. This was achieved by the controlled peridate oxidation and reduction of the terminal galactosyl residues in A-hCG. In order to ensure that the polypeptide chain was unaffected by the modification, and that any change in the biological properties of the hormone was due to the carbohydrate modification, a detailed physicochemical and immunological characterization of the derivative was undertaken. The data on the amino acid composition, NH₂-terminal amino acid analysis, polyacrylamide gel electrophoresis, and gel filtration chromatography failed to show any detectable alteration in the polypeptide backbone. Also, there seems to be no apparent denaturation of the derivative as indicated by the UV absorption spectrum (Fig. 4) and its immunological activity determined by radioimmunoassay (Fig. 5). The retention of the receptor binding activity as measured by radioreceptor assay further substantiates the above conclusion. In fact, PORA-hCG has 2.4 times higher affinity for the receptor than hCG (Fig. 6, Table III).

Prior to evaluating the efficacy of PORA-hCG in vivo, initial studies were carried out in vitro. The data on the cellular response of PORA-hCG show that the modification markedly impaired the ability of the hormone to stimulate cAMP production and steroidogenesis without affecting the receptor binding activity. While the derivative stimulated maximal progesterone production at higher dosage (about 40 times), it could produce only 20% of the maximal level of cAMP stimulated by hCG. These results agree with those of previously reported by Moyle et al. (8) in Leydig cells. While the carbohydrate modification resulted in the loss of the binding of the derivative to the liver receptor (Fig. 9), the binding to the target tissue was maintained or even enhanced. In agreement with the in vitro activity of PORA-hCG, the in vitro bioassays showed that it had 2-5% biopotency of hCG; 5% as measured by OOAD assay, a value significantly higher than that of 2% determined by MUW assay. Further carbohydrate modification of PORA-hCG was found to be more effective in inhibiting the activity of hCG in OOAD than MUW assay. No definitive explanation can be given for the above discrepancy except that the time course of experiment varied considerably in the two assays. While OOAD assay required 4 h, MUW assay was performed over a period of 4 days. The plasma half-life of PORA-hCG-I (15 min) was less than that of hCG (48 min) because of its increased removal by the kidney (22). However, it had higher binding affinity (Kₐ = 1.78 × 10⁶ M⁻¹) for the receptor than hCG (Kₐ = 0.75 × 10⁶ M⁻¹).

The present data strongly indicate that the carbohydrate plays a crucial role in the function of the hormone, although the precise molecular site of its action has not been established. Since the derivative does bind to the receptor, the lesion must lie in the post receptor binding event(s). Based upon the currently available model of polypeptide hormone action, several possibilities can be considered for the molecular site of action of the carbohydrate. It is conceivable that the carbohydrate modification may prevent the aggregation of hormone-receptor complex essential for the expression of hormonal response (40, 41). This may occur due to a change in the fluidity of the hormone-receptor complex in the membrane. A recent report by Amsterdam et al. (40) suggests that small clusters of hCG/LH receptors in granulosa cells may play a role in the cellular responsiveness. Similarly, another report by Schechter et al. (41) shows that while a CNBr-cleaved analog of epidermal growth factor is devoid of biological activity, it retains sufficient receptor binding activity, but cannot form cell surface clusters in fibroblast. Addition of bivalent, but not monovalent, antibody of epidermal growth factor restores the biological response as well as the patch formation. It is also possible that the modification may affect the conformation of the hormone-receptor complex and thereby its fluidity in the membrane and interaction with the regulatory subunit of adenylate cyclase. Whether the carbohydrate binding component is an independent entity or a part of the adenylate cyclase system is not known. It is conceivable, however, that one of the subunits of the guanine nucleotide binding protein (G) may be involved in the interaction with the carbohydrate. The G protein from rat liver and turkey erythrocyte membrane has been found to be oligomeric in
nature (42, 43). If this is the case, the presence of such a subunit may represent an additional step in the regulation of glycoprotein hormone action. Similar interaction between the target tissue gangliosides and cholera toxin have been implicated in the binding of ligand to the receptor (44-46).

In short, the in vivo studies on the role of carbohydrate in hCG were made possible by the modification of Carbohydrate 21. Gray, W. (1970) Methods Enzymol. 1263-1273. Madnick, H. W., Kalyan, N. K., Segal, H. L., and Bahl, O. P. (1981) Arch. Biochem. Biophys. 212, 432-442

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