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Biochemical Studies on Placental Chorionic Gonadotropin. I. Characterization of a High-Molecular-Weight Gonadotropin in Human Chorionic Tissues of the Normal Placenta

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A high-molecular-weight glycoprotein which reacted with a specific antiserum to urinary human chorionic gonadotropin and with the gonadotropin receptor preparation from rat testes was detected in the extract of human chorionic tissues from the first trimester placenta of normal pregnancy and partially purified by ammonium sulfate fractionation followed by chromatographies on DEAE-Sephadex A-50 and Sephadex G-150. Its molecular weight was estimated to be 8.0×10^4 daltons by polyacrylamide gel disc electrophoresis in the presence of sodium dodecyl sulfate. In the presence of 2-mercaptoethanol, the major portion of this preparation was separated into alpha- and beta-subunits of rather high molecular weights compared to those of urinary human chorionic gonadotropin subunits by sodium dodecyl sulfate polyacrylamide gel disc electrophoresis. This preparation was shown to possess 2000 IU/mg of biological activity, and 2200 IU/mg of immunological activity, while its receptor binding capacity was equipotent to that of urinary chorionic gonadotropin. Chemical analyses, including amino acid and carbohydrate, were carried out.

The present study suggests that high-molecular-weight type of human chorionic gonadotropin might be a complex of larger forms of alpha- and beta-subunits. This complex might be an intermediary component in the biosynthetic pathway of human chorionic gonadotropin or a product of posttranslational modification or a degradation product.

Keywords——human chorionic gonadotropin; placental human chorionic gonadotropin; pregnancy; human placenta; glycoprotein hormone; subunit structure

Human chorionic gonadotropin (hCG), a glycoprotein hormone of placentral origin, is secreted normally during pregnancy. In addition, various tumor cells have been shown to possess the capacity to secrete immunoreactive hCG. Recently, immunoreactive hCG was detected in the extracts from a variety of tissues and organs.[1] Moreover, large-molecular forms of a number of polypeptide hormones have been shown to exist in tissues and in plasma, as reviewed by Melani.[2] Several papers have recently appeared regarding the possible existence of large-molecular species of luteinizing hormone (LH),[3] follicle-stimulating hormone (FSH)[4] present in pituitary gland and hCG in placental tissues.[5] These large species of hormones are apparently not mere aggregates of native forms of the hormones, since they are not dissociated into the standard hormone afrer denaturing treatment and refiltration on Sephadex G-100. Nevertheless, evidence that large forms of hCG exist is based on the finding that the meterial obtained from cultured trophoblastic cells was eluted in fractions near the void volume upon gel filtration on Sephadex G-100.[5]

Few workers have attempted to isolate hCG or hCG-like substance directly from

chorionic tissues, the source of secretion.[6] The main reason for this seems to be difficulty in collecting chorionic tissues of the first trimester in sufficient amount.

We believe that isolation of hCG from its tissue source is prerequisite for establishing the physicochemical nature of this hormone and understanding its physiological role. We report here the detection, purification, and characterization of the large-molecular form of hCG from human placentral tissues.

Experimental

The human placental chorionic tissues obtained by artificial abortion in the first trimester of normal pregnancy were immediately irrigated with cold water and then with acetone to remove blood and lipids. The tissues were minced in a homogenizer with acetone at 4 °C. After being washed with the chilled acetone several times, chorionic tissues were filtered off, and allowed to dry overnight at room temperature, then kept at −20 °C until use. By this method, 1 g of acetone powder was obtained from 60 g wet chorionic tissues.

The biological gonadotropic activity was determined by measuring the weight response of both uterus and ovary of immature female mice on subcutaneous injection of the hormone, *i.e.*, the modified Aschheim–Zondek test.[7] IRC mice, 14—16 days old and weighing 6.0—8.0 g were used. Immunological gonadotropic activity of the eluate from a DEAE-Sephadex A-50 column was estimated by radioimmunoassay employing rabbit anti-urinary human chorionic gonadotropin (anti-u-hCG) antibody and anti-placental human chorionic gonadotropin (anti-p-hCG) antibody (in the later stage of the study) and ^{125}I-u-hCG as described earlier.[8] Immunological activity of the final product was assayed with the hCG RIA kit (Green Cross Co., Japan: HCGK-PR, Lot 62860). Radioligand receptor assay (RRA) was performed by the method described by Catt *et al.*[9] Plasma membrane fraction of rat testis (as a receptor preparation) and ^{125}I-u-hCG were used in this assay. A detailed study on the interaction of u-hCG and p-hCG and gonadotropin receptor will be described in the succeeding paper.[10] In all three assays employed, the standard hormone preparation of hCG (National Institute of Hygienic Sciences, Tokyo, Japan) was included and the activities were expressed as IU/mg of hormone.

Biologically and immunologically active fractions obtained by purification were subjected to electrophoresis in 7.5% polyacrylamide gel (PAGE) containing 0.1% sodium dodecyl sulfate (SDS). SDS-PAGE was performed as described by Weber and Osborn.[11] The gels were stained with 0.2% Coomassie Brilliant Blue.

The amino acid composition of the final product was determined by hydrolyzing it with 6 N hydrochloric acid at 110 °C for 24 h *in vacuo* and analyzing the hydrolysate with a JEOL 6AH amino acid analyzer. Sugars were analyzed by the phenol–sulfuric acid method for neutral sugars,[12] by the use of the amino acid analyzer for hexosamines after hydrolysis with 4 N hydrochloric acid at 105 °C for 4 h, and by the thiobarbituric acid method for sialic acid.[13]

DEAE-Sephadex A-50 and Sephadex G-150 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Carrier ampholyte used for isoelectric focusing separation of u-hCG from commercial hCG (Mochida Pharmaceutical Co., Japan) was the product of LKB Produkter, AB, Sweden.

Results

Extraction of p-hCG

After 10 g of acetone powder of the chorionic tissues had been ground with 30 ml of chilled acetone in a mortar, 500 ml of 2% potassium chloride solution was added and the mixture was adjusted to pH 8.6 with 1 N potassium hydroxide solution. Extraction was performed under gentle stirring overnight at 4 °C. The suspension was centrifuged at 9000 rpm for 10 min, and the supernatant was collected. The pellet was re-extracted with 300 ml of 2% potassium chloride solution at pH 8.6 overnight, then centrifuged and the supernatant was combined with the first supernatant. The combined supernatant solution was designated as the placental extract. The biological gonadotropic activity of the extract was estimated to be 1—2 IU/mg.

Detection of a Large-Molecular Form of hCG

When the placental extract was fractionated by molecular sieving through a Sephadex G-150 column, two immunologically active peaks, one at the void volume and the major one at almost the same volume as that at which bovine serum albumin (BSA) is eluted were detected (Fig. 1). The major immunoreactive peak, 80—90% of the activity present in the extract, was

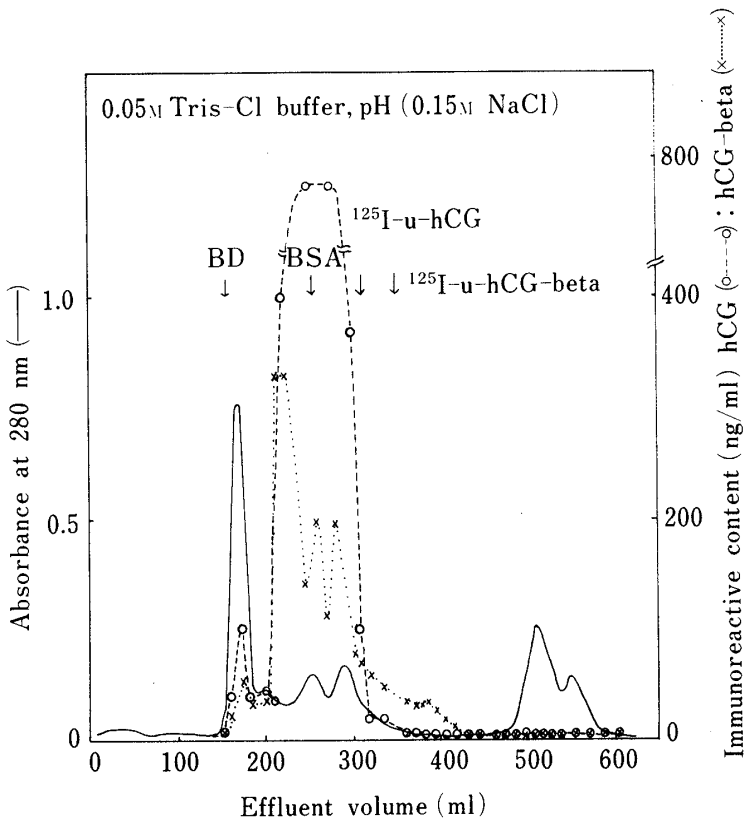

Fig. 1. Detection of Large Forms of hCG in the Placental Extract

The chorionic villi taken at early pregnancy were immediately extracted with phosphate-buffered saline, pH 7.4, and the extract was chromatographed in the presence of inhibitors of proteases (0.4 mM diisopropylfluorophosphoridate, 200 IU/ml kallikrein inhibitor, 20 mM EDTA, and 20 mM sodium tetrathionate). The column (2.6 × 90 cm) was eluted with 0.05 M Tris-HCl buffer containing 0.15 M sodium chloride and 1% *n*-butanol, and the eluate was analyzed by specific RIA for immunoreactive hCG and hCG-beta-subunit, respectively. The arrows indicate the positions at which authentic protein markers were eluted in a separate run in the same column: BD, blue dextran (void volume); BSA, bovine serum albumin (molecular weight = 68000); ^{125}I-u-hCG (47000) and ^{125}I-u-hCG-beta-subunit (30000).

apparently eluted faster than ^{125}I-u-hCG and ^{125}I-u-hCG-beta-subunit, indicating that an hCG-like substance in placenta (p-hCG) appears to be different in molecular size from u-hCG.

Purification of p-hCG

All steps were carried out at 4 °C. The protein fraction (NP-40) precipitated from the placental extract by 40% saturation with solid ammonium sulfate was removed by centrifugation at 9000 rpm for 10 min. The supernatant was fractionated by increasing the concentration of ammonium sulfate to 70%. The resulting precipitate (NP-70) was collected by centrifugation, and the supernatant solution was finally saturated to 100% giving a trace of precipitate (NP-100). The precipitates were dissolved and dialyzed against dist. water until free of sulfate ion, then lyophilized. The biological activities of NP-40, NP-70 and NP-100 were determined to be 1—2, 4—10, and 1—2 IU/mg, respectively. By this salting-out fractionation, more than 60% of the biological activity was recovered in NP-70, 16% in NP-40, and 5% in NP-100. The NP-70 fraction was dissolved and dialyzed against 0.01 M phosphate buffer, pH 6.6, and applied to a column of DEAE-Sephadex A-50 which had been equilibrated with the same buffer. Several fractions designated as 1 to 8 were isolated as shown in Fig. 2 (the lower figure). Most of the biological activity was recovered in fraction 3, which was eluted with 0.01 M phosphate buffer containing 0.1 M sodium chloride, pH 6.6,

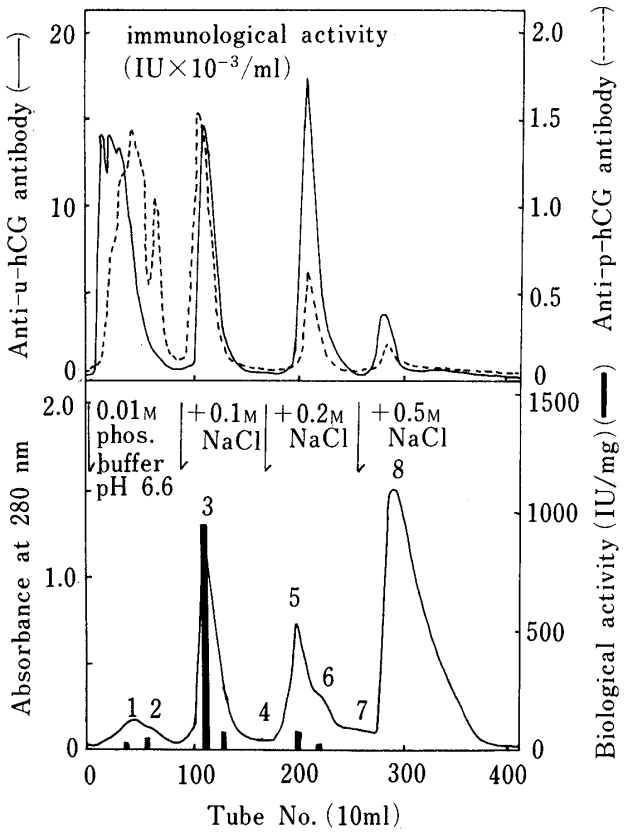

Fig. 2. Ion-Exchange Chromatography of NP-70 on DEAE-Sephadex A-50 by Stepwise Elution

A 1.2 g sample of NP-70 in 25 ml of 0.01 M phosphate buffer, pH 6.60, was applied to a column (2.6×98 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was eluted stepwise as indicated in the figure. Fractions of 10 ml were collected. Aliquots were withdrawn from every other tube for the determination of immunological gonadotropic activity, which was carried out by RIA employing specific anti-u-hCG and anti-p-hCG antibodies; the results are shown in the upper figure. Fractions designated as Nos. 1 to 8 were pooled, dialyzed against 0.01 M ammonium bicarbonate buffer, pH 6.60 and lyophilized. Biological activities of the pooled fractions were determined as described in the text (horizontal bars in the lower figure).

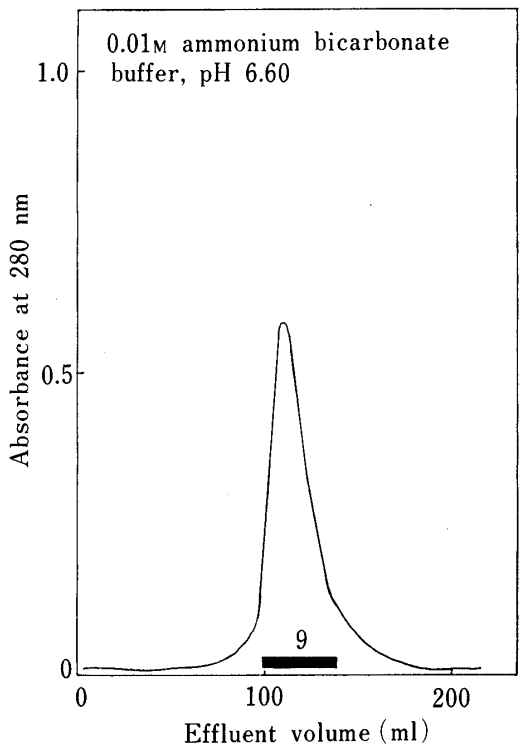

Fig. 3. Final Purification of p-hCG through Sephadex G-150

After fraction 3 in Fig. 2 had been rechromatographed on DEAE-Sephadex A-50 by linear gradient elution, the active fractions were pooled and dialyzed against 0.01 M ammonium bicarbonate buffer, pH 6.60, and lyophilized. This fraction was dissolved in the same buffer, applied to Sephadex G-150 (1.5×95 cm) and eluted with the same buffer. The active fractions from the first gel filtration were pooled, and the pooled fractions were gel-filtered through the same column under the same conditions as described above. A single and symmetrical peak was obtained and designated as fraction 9. Fraction 9 was pooled, lyophilized, and employed as p-hCG. preparation.

although weak activity was seen in other fractions (fractions 1, 2, 5, and 6). It should be noted that almost all the fractions including fraction 3 were found to be immunologically positive against anti-u- and anti-p-hCG antibodies as shown in Fig. 2 (the upper figure). The biological activity of fraction 3 was found to be 600—1000 IU/mg.

Further purification of fraction 3 was attempted by rechromatography on DEAE-Sephadex A-50 equilibrated with 0.01 M phosphate buffer, pH 6.6, by linear gradient elution with sodium chloride (0—0.5 M). However, this step only effected the removal of the component which was eluted with the starting buffer, and the biologically active fraction was eluted as a broad peak with the buffer containing 0.08—0.1 M sodium chloride. The resulting active fraction was finally subjected to gel filtration on Sephadex G-150. Since the separation was incomplete, a second gel filtration was necessary to obtain a single and symmetrical peak (Fig. 3). Fraction 9 was referred to as p-hCG.

Properties of p-hCG

Gonadotropic activity of p-hCG was determined by bioassay, RIA, and RRA, and the results are summarized in Table I. p-hCG was shown to behave as a single band in SDS-PAGE without 2-mercaptoethanol, while it gave two polypeptide chains after treatment with

TABLE I. Gonadotropic Activity of p-hCG[a]

Activity	Biological[b] (IU/mg)	Immunological[c] (IU/mg)	Receptor binding[d] (ng)
p-hCG	2000 ± 800	2800 ± 400	10 ± 1
u-hCG-4.3[e]	12000 ± 2000	6000 ± 940	10 ± 1

a) Gonadotropic activity was determined with three different assay methods. In each assay, activity for p-hCG was measured in parallel with that of the purified u-hCG. Each value is the mean of duplicate determinations.
b) The biological gonadotropic activity was determined by measuring the weight response of both uterus and ovary of immature female mice on subcutaneous injection of the hormone, *i.e.*, the modified Aschheim–Zondek test.[7] Groups of 4—6 IRC mice, 14—16 days old and weighing 6.0—8.0 g, were used.
c) The immunological gonadotropic activity was estimated by radioimmunoassay with the HCG·I-125·kit (Green Cross Co., Japan, HCGK-PR, Lot 62860), in which specific anti-hCG antibody and highly purified hCG for labeling with ^{125}I were employed. The supplier noted that the cross reactivity of this kit was 0.66% for LH, 0.21% for TSH and 0.01% for FSH.
d) Values represent the amount necessary for 50% inhibition in the competitive binding assay system where plasma membrane fraction from rat testis (as a receptor preparation) and ^{125}I-u-hCG (as a tracer) were utilized.
e) The purified u-hCG (u-hCG-4.3) was prepared from commercial hCG preparation (Mochida Pharmaceutical Co., Japan: 1000 IU/mg biological activity) by electrofocusing and gel filtration.

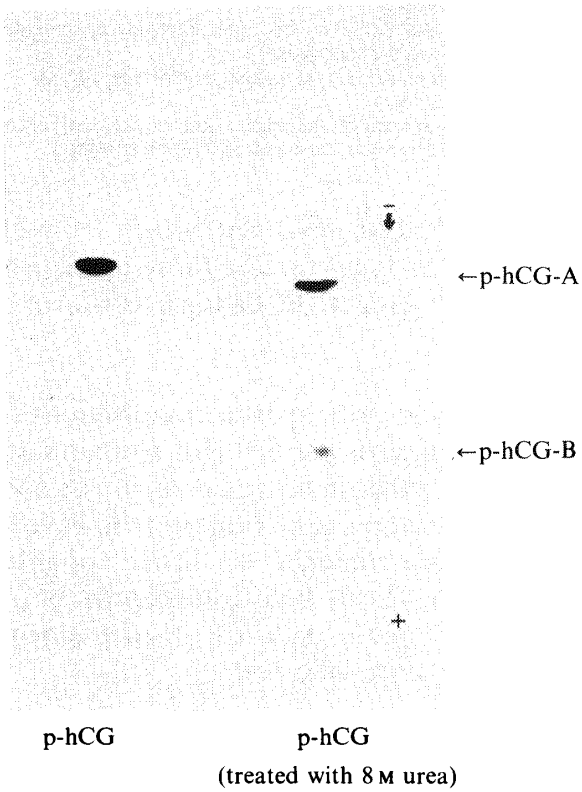

Fig. 4. SDS-PAGE of p-hCG

In order to confirm the homogeneity of p-hCG, fraction 9 was subjected to SDS-PAGE in the absence of 2-mercaptoethanol (the left column) and after treatment with 8 M urea, SDS, and 2-mercaptoethanol (the right column). SDS-PAGE was performed according to the standard method described by Weber and Osborn.[11]

TABLE II. Chemical Composition[a] of hCG

Amino acid[b]	p-hCG	Purified u-hCG[c]	u-hCG[d]
Lys	6.15	4.78	5.15
His	2.48	1.22	2.40
Arg	3.50	6.41	9.93
Asp	9.59	8.56	8.46
Thr	6.45	6.96	7.20
Ser	8.39	7.02	7.26
Glu	10.11	8.21	9.79
Pro	6.01	11.65	11.16
Gly	10.27	6.48	2.90
Ala	7.28	6.20	3.69
1/2 Cys	4.08	6.83	8.28
Val	6.22	9.17	6.88
Met	1.62	1.80	2.06
Ile	3.13	3.09	2.32
Leu	8.37	7.21	6.93
Tyr	2.88	1.44	4.28
Phe	3.47	2.94	3.61
Carbohydrate[e]			
Neutral hexoses	11.1	13.5	11.2
Total hexosamines	7.9	14.1	11.0
Sialic acid	0.3	9.5	9.0
Total carbohydrate	19.3	37.1	31.2

a) Amino acids are expressed as moles of amino acid per 100 mol of amino acid residues and carbohydrates as g/100 g protein, respectively.
b) Hydrolysis was carried out for 24 h only and therefore no correction was made for destruction. Tryptophan was not determined.
c) u-hCG-4.3 prepared by isoelectric focusing from commercial u-hCG.
d) Data taken from reference 15.
e) Neutral hexoses were determined by the phenol–sulfuric acid method, hexosamines by the use of the amino acid analyzer and sialic acid by Warren's method.

8 M urea and 2-mercaptoethanol. The molecular weight of p-hCG was calculated to be 8.0×10^4 daltons without the reducing agent, and those of the new polypeptide chains were 6.0×10^4 (p-hCG-A) and 2.0×10^4 (p-hCG-B) daltons, as shown in Fig. 4.

Chemical Compositions

Table II lists the amino acid and carbohydrate compositions of p-hCG. Amino acid data were calculated as moles of amino acids per 100 mol of amino acid residues. The weight of polypeptide moiety calculated from the amino acid content was about 85% which agreed with the value obtained by the biuret reaction. p-hCG appears to have higher lysine, aspartic acid, glutamic acid, glycine, and alanine contents, but lower arginine, proline and half-cystine contents as compared with u-hCG. The carbohydrate content of p-hCG was about 20%. The sugar moiety was composed of mannose, galactose, fucose, glucosamine and galactosamine (both are probably *N*-acetylated), and sialic acid.

Discussion

Recently in the field of polypeptide hormone research, many unusually large-molecular weight hormones have been isolated and characterized as prohormones or preprohormones.[2] With respect to gonadotropins, Prentice and Ryan[3] noted the existence in human pituitary extract of a large non-dissociable molecule that reacted with antisera to hLH, hLH-

alpha- and hLH-beta-subunits. They further found that this molecule contained more alpha-reactive material than did native LH, and postulated the existence of an alpha : alpha-like : beta prohormone complex which, after cleavage, gave rise to native LH and excess free alpha-like subunit. A large species of FSH has also been noted in the crude pituitary extract,[4] and a large molecular form of hCG in urine and in cultures of trophoblastic cells.[5] In these studies, however, no detailed physicochemical characteristics of the large-molecular forms of hCG have been reported since only trace amounts of the materials were available.

The present investigation was aimed at isolating a possible large molecular form of hCG from the chorionic villi and characterizing it as a presumed precursor form of this glycoprotein hormone. For the first time, we were able to isolate a glycoprotein (designated as p-hCG) with biological and immunological gonadotropic activities from placental tissues of early pregnancy. About 10% of the gonadotropic activity in the crude extract was recovered in p-hCG. p-hCG thus obtained was shown to possess biological activity of 1000—2000 IU/mg, which is about 1/10—1/6 of that of purified u-hCG (10000—12000 IU/mg for u-hCG-4.3). The finding that p-hCG possesses lower activity than u-hCG is consistent with previous reports.[6,14]

The p-hCG preparation described above appears to be a larger-molecular form of hCG, since p-hCG was eluted faster than ^{125}I-u-hCG in gel filtration on a Sephadex G-150 column as shown in Fig. 1. The molecular weight of p-hCG was estimated to be 8.0×10^4 daltons by SDS-PAGE, a value is higher than that reported for u-hCG. Bahl *et al.*[15] estimated the molecular weight of hCG from urine of pregnant women to be 2.7×10^4 daltons as determined from the chemical composition, 4.7×10^4 daltons by the sedimentation equilibrium method and 5.9×10^4 daltons by gel filtration. Morgan and Canfield, on the other hand, reported that u-hCG is composed of alpha-(1.8×10^4 daltons) and beta-(3.0×10^4 daltons) subunits as demonstrated by SDS-PAGE.[16] When p-hCG was treated with 8 M urea, SDS and 2-mercaptoethanol, it was dissociated into two new polypeptide chains with molecular weights of 2.0×10^4 (p-hCG-B) and 6.0×10^4 (p-hCG-A) daltons in SDS-PAGE. Maruo *et al.*[5] also reported the presence of a large-molecular species of hCG in chorionic tissues cultivated for a short period, and estimated its molecular weight to be 9.0×10^4 daltons; upon treatment with SDS and 2-mercaptoethanol, it was dissociated into three polypeptide chains.

The amino acid composition of p-hCG is distinct from that of u-hCG (Table II). The characteristically high proline content of u-hCG was not found in p-hCG. The carbohydrate content of p-hCG is as low as 19.3%, compared to 31.2% for u-hCG. In particular, the amount of sialic acid, a carbohydrate moiety necessary for the biological activity but not essential for the receptor binding activity,[17] in p-hCG is remarkably less than that of u-hCG. This fact may partly explain the lower biological activity and the equipotent affinity to gonadotropin receptor of p-hCG (Table I).

p-hCG appears to exist in several forms: while most of the biological activity was eluted in one peak (fraction 3) in DEAE-Sephadex A-50 column chromatography, the immunological activity could be detected in almost all the peaks eluted (Fig. 2). These results indicate the presence of multiple forms of hCG in the chorionic villi, each possessing different activity.

It is also apparent that p-hCG and u-hCG are physicochemically different. In addition, since p-hCG is about twice as large as u-hCG in particle size, and since the biological activity of p-hCG is lower than that of u-hCG even though the immunological and receptor binding activities are almost equipotent, p-hCG may be a prohormone of u-hCG. Alternatively, p-hCG might be an intermediary complex in the biosynthetic pathway of a large protein containing hCG or a product of posttranslational modification or a degradation product.

Finally, the present results suggest that the large immunological species of hCG could be the predominant form of the hormone in the chorionic tissues, while the authentic form or a

u-hCG-like form predominates in blood and urine. However, hCG has not been purified and characterized from human serum. In the placenta, the immunoreactive large hCG might be synthesized initially and converted to the authentic form of u-hCG by enzymatic processing.

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