Jpn. J. Cancer Res. 80, 968–974, October 1989

# Molecular Forms of Human Chorionic Gonadotropin in Choriocarcinoma Serum and Urine

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**The molecular forms of human chorionic gonadotropin (hCG) were assessed in parallel serum and urine samples from a choriocarcinoma patient by gel chromatography, isoelectric focusing, and Western blot analysis. There were significant qualitative differences in the molecular forms of hCG between the serum and the urine. The serum hCG had a greater molecular weight and a stronger acidic charge than the urinary hCG. These results may be attributed to differences of sialic acid contents. Affinity chromatography using *Ricinus communis* agglutinin-conjugated Sepharose showed that the serum hCG was completely sialylated, while the urinary hCG was partially desialylated. In addition to the complete hCG molecule, two kinds of hCG$\beta$-related fragments were detected in the urine of the patient, but not in the serum. In the Western blot using anti-hCG$\beta$ or anti-hCG$\beta$-CTP under reducing conditions, the urine presented a new band (CTP′) with Mr 23,000 in addition to the $\beta$-subunit, while the serum revealed a single band of the $\beta$-subunit with or without a reducing reagent. The present data revealed striking qualitative differences in the molecular forms of hCG between serum and urine of a patient with choriocarcinoma.**

Key words: Human chorionic gonadotropin — Trophoblastic disease — Glycoprotein — Molecular heterogeneity — Lectin

Human chorionic gonadotropin (hCG) and its $\alpha$- and $\beta$-subunits are normally produced by trophoblast, and can be detected in the blood and urine of women throughout pregnancy or in trophoblastic disease.[1-3] In addition to hCG and free subunits, hCG$\beta$-related fragments with low molecular weight have been demonstrated by many investigators in pregnancy urine[4] and in the urine of patients with trophoblastic[5] and nontrophoblastic[6,7] tumors. Two types of such fragments have been demonstrated. One was recognized by the antiserum directed to the carboxy-terminal peptide (CTP) of hCG$\beta$, and was designated CTP fragment.[5] The other was recognized by the antiserum directed to intact hCG$\beta$ (SB6 type antiserum), and was designated $\beta$-core fragment.[5] Recently, the structure of the $\beta$-core fragment has been determined; it consists of residues 6–40 disulfide-bridged to residues 55–92 of the $\beta$-subunit.[8] It is noteworthy that these fragments were essentially detected in the urine but not in the serum,[4-7] suggesting that they are degradative products of the hCG$\beta$ molecule. Indeed, they appear in urine of normal humans after iv administration of purified $\beta$-subunit of hCG.[9,10] We also found that the $\beta$-subunit of urinary hCG was readily dissociated into two major fragments under reducing conditions,[11] and observed that such fragmentation was much more apparent with hCG from choriocarcinoma urine than with that from normal pregnancy urine.[12] These data indicated that hCG is excreted in the urine after certain proteolytic processing. Therefore, there may be qualitative differences in the hCG molecule between serum and urine.

The present studies were undertaken to assess the molecular forms of hCG in the serum and the urine of a patient with choriocarcinoma.

## MATERIALS AND METHODS

**Patient** A 31-year-old Japanese woman, gravida 3, para 2, presented with vaginal bleeding 6 weeks after a full-term delivery. The patient underwent uterine currettage, a pathological diagnosis of choriocarcinoma was made, and she was referred to Kobe University Hospital. At the time of admission, the patient complained of progressive cough, dyspnea, and poor vision. Chest X-ray revealed multiple discrete lesions in the bilateral lung field and CT scan of the brain showed an intracranial tumor lesion. The concentration of urinary hCG was about 2,000 IU/ml. She responded to intensive chemotherapy and irradiation to the brain, and her hCG titer declined to below 1 mIU/ml in both the urine and the serum. Parallel urine and serum samples were collected from the patient at the same time before the treatment.

**Radioimmunoassay (RIA)** RIAs were carried out employing antisera directed toward the hCG$\beta$ and

hCG$\beta$-CTP antigenic determinants. The anti-hCG$\beta$ (H2) and the anti-hCG$\beta$-CTP (H93) were gifts from Dr. R. O. Hussa (Hybritech, San Diego, CA) and from Dr. H. C. Chen (NIH, Bethesda, MD), respectively. H2 recognizes conformation-specific determinants in regions of hCG$\beta$ not involving the CTP,[13] while H93 is the hCG$\beta$-CTP directed antiserum.[14] RIAs were performed by double antibody RIA,[3] with radiolabeled hCG$\beta$ and with hCG$\beta$ used as a reference preparation.

**Radioreceptor assay (RRA)** The receptor fraction for hCG was prepared from rat testis as described by Catt *et al.*[15] Standard hCG (CR123; 12,800 IU/mg) was generously supplied by the Center for Population Research, NICHHD, NIH.

**Sialidase treatment** A 100 $\mu$l aliquot of the patient's serum or urine was mixed with 50 $\mu$l of 0.1 *M* sodium acetate buffer, pH 4.5, and then incubated with 0.5 unit of neuraminidase (EC 3.2.1, Lot V2H3807, Nakarai Tesque, Kyoto) for 12 h at 37°C. The mixture was subjected to gel chromatography on Sephadex G-100 and isoelectric focusing.

**Gel chromatography on Sephadex G-100** The samples from the patient were applied to a column (2×100 cm) of Sephadex G-100 equilibrated at 4°C with 0.05 *M* sodium phosphate buffer, pH 7.4, containing 0.15 *M* NaCl and 0.02% sodium azide (PBS). The flow rate of the column was adjusted to 10 ml/h and 2.7 ml fractions were collected at 4°C.

**Isoelectric focusing (IEF)** IEF was performed by using a column apparatus of 110 ml capacity as previously described.[16] The urine or serum was introduced into the sucrose gradient from 0 to 50%. The mean concentration of ampholine was 2%. IEF was carried out for 12 h at 400 V, followed by 40 h at 750 V, in a column cooled by a circulating bath. Fractions of 1.5 ml were collected, measured for pH, and subjected to hCG-RIA.

***Ricinus communis* agglutinin 120(RCA)-Sepharose affinity chromatography** RCA-coupled Sepharose 4B was purchased from Honen Oil Company (Tokyo). RCA-Sepharose was packed into a column (0.5×10 cm) and equilibrated with PBS. The sample was applied to the column and eluted with PBS to obtain the unbound fraction, then with PBS containing 0.2 *M* galactose to obtain the bound fraction.

**SDS-PAGE and Western blot analysis** SDS-PAGE was performed in 10% acrylamide slab gels. The samples were dissolved in 3% SDS, 5% glycerol and 100 m*M* dithiothreitol (DTT), and boiled for 2.5 min.

Western blot analysis was performed as previously described.[12] The samples on a gel were transferred to a nitrocellulose membrane (TC119, Bio-Rad Laboratories, Richmond, CA) as described by Towbin *et al.*[17] The membrane was immunoblotted with anti-hCG$\beta$ (H2) or anti-hCG$\beta$-CTP (H93).

## RESULTS

**Gel chromatography on Sephadex G-100** Figure 1 shows the elution profiles of the patient's serum and urine. Immunoreactivities for both anti-hCG$\beta$ and anti-hCG$\beta$-CTP in the serum were eluted just prior to standard hCG (CR123) in a single peak (*K*av=0.10) that was coincident with the activity in the hCG-RRA. On the other hand, in the patient's urine, in addition to the complete hCG (*K*av=0.14), two types of low-molecular-weight materials (fragments 1 and 2) were detected by hCG$\beta$-RIA and/or hCG$\beta$-CTP-RIA. Fragment 1 was recognized by both hCG$\beta$- and hCG$\beta$-CTP-RIAs and was eluted (*K*av=0.40–0.52) near standard hCG$\alpha$ (*K*av =0.46). Fragment 2 exhibited immunoreactivity for anti-hCG$\beta$ but not for anti-hCG$\beta$-CTP and was eluted

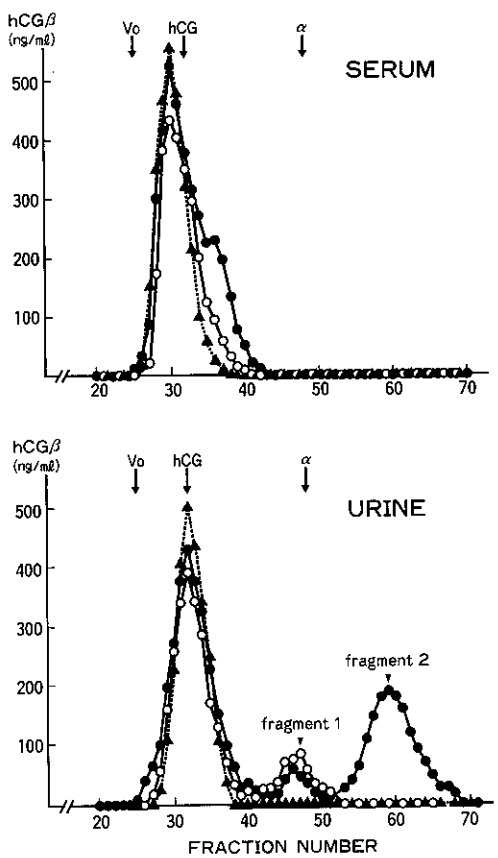


Fig. 1. Gel chromatography on Sephadex G-100 of serum and urine of the patient with choriocarcinoma. One ml of the sample was applied to a column (2×100 cm) of Sephadex G-100, and eluted with PBS. Fractions of 2.7 ml were collected and aliquots were assayed by hCG$\beta$ (●) and hCG$\beta$-CTP (○) RIAs, and RRA (▲).

($K$av = 0.64–0.78) significantly later than standard hCG$\alpha$. The immunoreactivity and the $K$av value of fragment 2 indicated that it corresponded with the so-called $\beta$-core fragment.[4-7, 18] On the other hand, the immunoreactivity and the molecular size of fragment 1 were quite similar to those of CTP′, which could be detected in Western blot analysis of the patient's urine under reducing conditions (Figs. 5 and 6).

Neither fragment showed any binding activity to hCG receptor, indicating that they had no biological activity.

After sialidase treatment, the peak of the serum hCG was shifted to the position of $K$av 0.26 (Fig. 2). The peak of the complete hCG in the urine was also shifted to the same position as that of the desialylated serum hCG after the sialidase treatment (Fig. 2). Interestingly, the sialidase treatment significantly reduced the molecular weight of fragment 1, but did not reduce that of fragment 2.

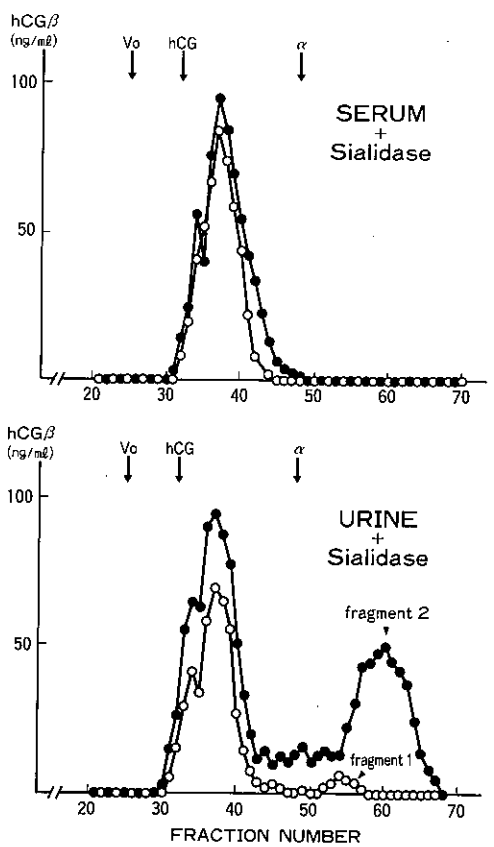


Fig. 2. Gel chromatography on Sephadex G-100 of the sialidase-treated serum and urine of the patient with choriocarcinoma. After treatment with sialidase, the sample was applied to the column and eluted as described in Fig. 1. Assayed by hCGβ-RIA (●) and hCGβ-CTP-RIA (○).

**IEF** Figure 3 shows the IEF profiles of hCG in the serum and the urine of the patient. Most of the serum hCG was distributed in the more acidic regions (pH 3.0–4.5) than standard hCG (pH 4.0–5.0). On the other hand, major components of the urinary hCG were also distributed in strongly acidic regions (pH 3.0–4.0); minor but distinct components were observed in the neutral regions (pH 6.0–7.5).

After IEF of the urinary hCG, the acidic (A) and the neutral (B) fractions were separately pooled, and subjected to RCA-Sepharose affinity chromatography. Only

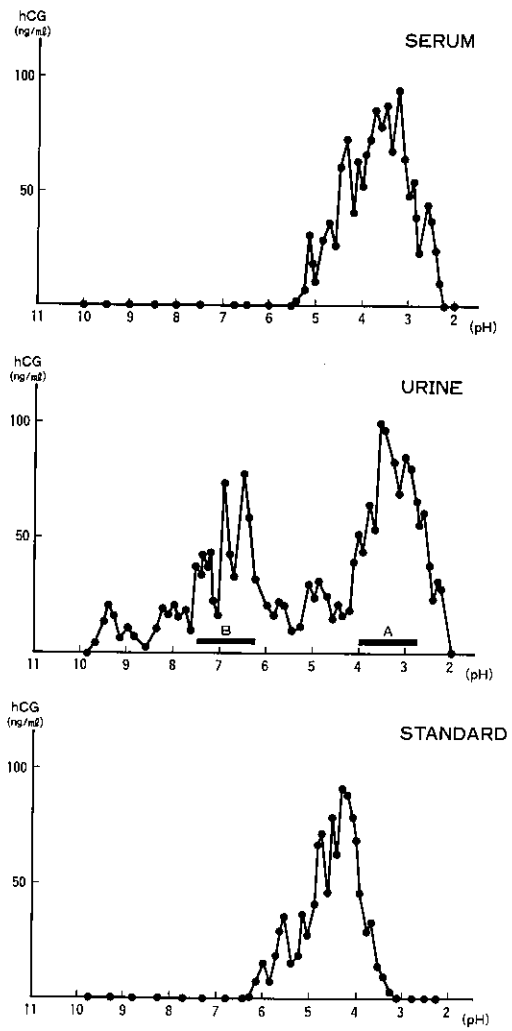


Fig. 3. Isoelectric focusing patterns of hCG in the serum and urine of the patient with choriocarcinoma, and standard hCG (CR123). All the fractions were assayed by hCG-RIA. In IEF of the urine, the acidic (A) and the neutral (B) fractions were pooled separately, and subjected to RCA-Sepharose affinity chromatography.

about 7% of fraction A bound to RCA, compared to 82% of fraction B.

All of the hCG immunoreactivities in both serum and urine were shifted to the basic regions (pH 10–11) after sialidase treatment (data not shown).

**RCA-Sepharose affinity chromatography** The patient's serum and urine were applied to a column of RCA-Sepharose. In the serum, essentially none of the hCG immunoreactivity applied was adsorbed on the column, whereas, in the urine, about 30% of the hCG immunoreactivity was adsorbed on the column and was eluted with 0.2 *M* galactose (Fig. 4). These results indicated that all of the serum hCG molecules were completely sialylated while some of the urinary hCG molecules were devoid of sialic acid, since RCA binds specifically the terminal carbohydrate structure of desialylated galactose-N-acetylglucosamine.[19]

**Western blot analysis** Figure 5 presents the immunoblots of standard hCG and the patient's samples with anti-hCG$\beta$ (H2). Under nonreducing conditions, the serum contained a single immunoreactive band with Mr 34,500, which is slightly greater than that of standard hCG$\beta$ (Mr 34,000), whereas the urine revealed one intense band of the $\beta$-subunit with Mr 34,000 and another weak band with Mr 16,500. Based on its molecular size and immunoreactivity, this low-molecular-weight material may represent the $\beta$-core fragment. Under reducing conditions, the serum revealed a broad immunoreactive band with Mr 34,000–35,000, whereas the urine revealed two major bands with Mr 34,000 and 23,000. We have previ-

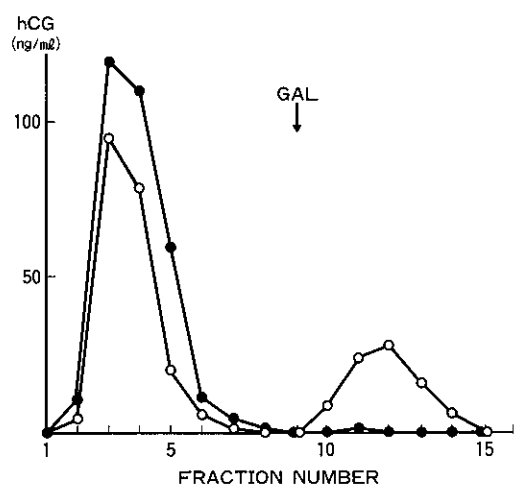


Fig. 4. RCA-Sepharose affinity chromatography of hCG in serum and urine of the patient with choriocarcinoma. An aliquot of serum or urine was applied to a small column, and 1 ml fractions were collected before and after elution with 0.2 *M* galactose. Each fraction was assayed by hCG-RIA.

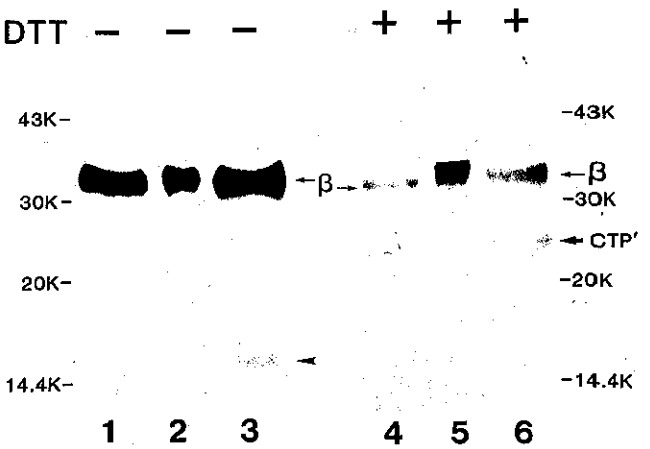


Fig. 5. Western blots of standard hCG (lanes 1 and 4), and the patient's serum (lanes 2 and 5) and urine (lanes 3 and 6). After electrophoresis under nonreducing or reducing conditions with DTT, proteins on a gel were electrotransferred to a nitrocellulose membrane and stained with anti-hCG$\beta$ (H2). The band of $\beta$-core fragment was weakly observed under nonreducing conditions (◄ in lane 3), but not under reducing conditions. Note that anti-hCG$\beta$ stained the CTP′ with equal intensity to the $\beta$-subunit in the urine under reducing conditions (lane 6), but no CTP′ was found in the serum.

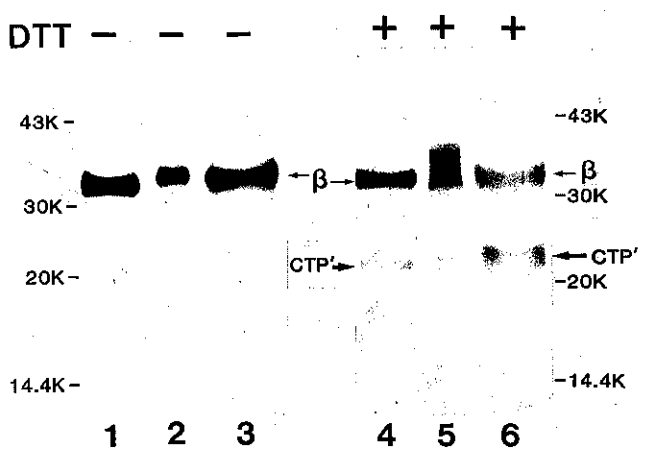


Fig. 6. Western blots of standard hCG (lanes 1 and 4) and the patient's serum (lanes 2 and 4) and urine (lanes 3 and 6). After electrophoresis under nonreducing and reducing conditions with DTT, proteins on a gel were electrotransferred to a nitrocellulose membrane and stained with anti-hCG$\beta$-CTP (H93). Note that anti-hCG$\beta$-CTP stained the CTP′ with equal intensity to the $\beta$-subunit in the urine under reducing conditions (lane 6), but no CTP′ was found in the serum.

ously demonstrated that the β-subunit of urinary hCG was dissociated into two fragments under reducing conditions, and that the carboxy-terminal side fragment (designated CTP′) could be observed as a band with Mr 22,000 in SDS-PAGE followed by Western blotting using antisera for hCGβ and hCGβ-CTP.[12]

In the urine of the patient examined here, the CTP′ was clearly observed as a band with Mr 23,000 as shown in lane 6 of Figs. 5 and 6. However, it is noteworthy that CTP′ could not be detected in the patient's serum under either nonreducing or reducing conditions.

Figure 6 shows the immunoblots of standard hCG and the patient's samples with anti-hCGβ-CTP. Under nonreducing conditions, all the samples gave a single band, and their molecular weights were 34,000, 35,000 and 34,000 for standard hCG (CR123), serum and urine, respectively. No β-core fragment was found in the serum or the urine by anti-hCGβ-CTP. Under reducing conditions, the serum revealed a broad band with Mr 34,000–36,000, and the urine showed two distinct bands with Mr 34,000 and 23,000.

## DISCUSSION

The present study showed striking qualitative differences of the molecular forms of hCG between serum and urine of the patient with choriocarcinoma.

In IEF, hCG in the patient's serum exhibited a greater acidic charge than standard hCG. On the other hand, although the major hCG immunoreactivities in the urine also exhibited strong acidic charge, about 35% of the urinary hCG exhibited neutral charge. The excessive acidic charge of choriocarcinoma hCG has been previously observed by Yazaki *et al.*[20] It is due to the appearance of unusual triantennary asparagine-linked sugar chains with peripheral sialic acids that carry the strong acidic charge, as demonstrated by our previous study.[21] Conversely, the deletion of sialic acid residues converted the electric charge of hCG from acidic to basic. Therefore, the neutral components of the urinary hCG found in IEF (Fig. 3, pool B) possibly represent a desialylated form. This was further supported by the evidence that more than 80% of the neutral components bound to the RCA-Sepharose.

The molecular size of hCG was different between serum and urine. The apparent molecular weight of hCG in the patient's serum was significantly greater than that in the urine as determined by both gel chromatography and SDS-PAGE. The molecular size difference can also be explained by relative sialic acid content, since both of the hCG immunoreactivities in the serum and urine were eluted in exactly the same position on gel chromatography after the removal of sialic acid residues. Thus, the present results indicate that the secreted hCG was completely sialylated in the circulation but was excreted in the urine as a partially desialylated form in this patient. We[22, 23] and others[5] have previously reported that incompletely sialylated forms of hCG often occur in the urine of patients with choriocarcinoma. However, desialylation is not a commonly observed structural change in choriocarcinoma urine hCG, and it is not observed in normal pregnancy as previously described.[21]

In addition to the complete hCG molecule, two types of hCGβ-related fragments were present in the patient's urine. The first type, fragment 1, contained a part of the β-core structure and the β-CTP portion, since it could be recognized by both anti-hCGβ and anti-hCGβ-CTP. It is of interest that the immunoreactivity and the molecular weight of fragment 1 were quite similar to those of the CTP′ observed in SDS-PAGE under reducing conditions (Figs. 5 and 6). The second type, fragment 2, seemed to represent the so-called β-core fragment that has previously been demonstrated,[4–7, 18] since it exhibited exactly the same immunoreactivity and molecular size as the β-core fragment. The β-core fragment was initially found in the urine of pregnant women by Franchimont *et al.*[4] and was also found in the urine of patients with trophoblastic disease[5] and with nontrophoblastic malignant diseases.[6, 7] Recently, structural studies on the β-core fragment from normal pregnancy urine have been performed. Birken *et al.*[8] showed that the β-core fragment consists of two polypeptide chains composed of residues β-(6–40) disulfide-bridged to residues β-(55–92). The β-core fragment retains the β-core conformational immunological determinant recognized by the SB6 type of antiserum, but has lost the carboxy-terminal immunological determinant. Blithe *et al.*[24] characterized the carbohydrate moiety of the β-core fragment and found that it is almost free from sialic acid and galactose residues, but has retained the Con A-binding site and most of the core fucose. In our present study too, sialidase treatment did not reduce the molecular weight of the β-core fragment (fragment 2). Although the origin of the β-core fragment is unclear, it has been detected exclusively in the urine but not in the serum.[4–7] Therefore, it is likely that the β-core fragment found in urine is a degradative product of the β-subunit of hCG.

We[12] have previously found that a part of the β-subunit of urinary hCG was dissociated into two parts under reducing conditions, and that the fragment of the carboxy-terminal side (CTP′) could be visualized by Western blotting using either anti-hCGβ (H2) or anti-hCGβ-CTP (H93). Since the β-subunit was readily dissociated simply by reduction with DTT, its peptide chain had been already cleaved but was still connected through disulfide bonds in the urine. In the patient with choriocarcinoma examined in this study, the fragmentation of the β-subunit was also clearly observed with the urinary

hCG under reducing conditions. In contrast to urinary hCG, no fragmentation was observed in the patient's serum hCG with or without DTT, indicating that the DTT cleavage site as found in the urinary hCG is not present in the $\beta$-subunit of the serum hCG. Therefore, the peptide cleavage of the $\beta$-subunit occurs during excretion.

The present study has demonstrated that secreted hCG is metabolized not only in its carbohydrate but also in its protein moiety and it is suggested that such proteolytic degradation may result in the appearance of the urinary fragments.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Robert O. Hussa (Hybritech Inc., La Jolla, CA, USA) for a critical review of the manuscript and for invaluable advice. The authors are grateful to Miss Mika Ida for her excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (No.63570780) from the Ministry of Education, Science and Culture, Japan.

*(Received June 12, 1989/Accepted August 11, 1989)*

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