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MOLECULAR ALTERATIONS OF URINARY HCG FROM HYDATIDIFORM MOLE

BY

NAPAPORN KUKIATINANT

(B.Sc. in Medical Technology)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

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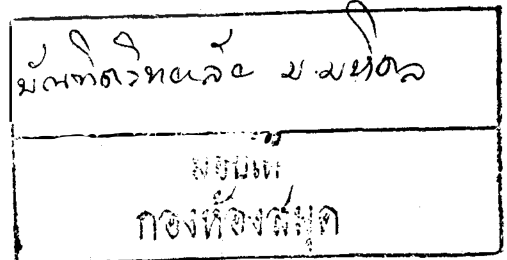
(BIOCHEMISTRY)



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on

November 14, 1984

Napaporn Kukiatinant
....
Napaporn Kukiatinant
Candidate

....
Thanit Kusamran, Ph.D.
Preceptor

Vichai Boonsaeng
....
Vichai Boonsaeng, Ph.D.
Copreceptor

....
Vina Churdboonchard, Ph.D.
Copreceptor

M. Chulasamaya
....
Monthree Chulasamaya, M.D.,Ph.D.
Dean, Faculty of Graduate Studies,
Mahidol University

....
Pairote Prempree, Ph.D.
Dean, Faculty of Science,
Mahidol University

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EVALUATION OF THE FINAL EXAMINATION

THE DEFENSE OF THESIS

We, the members of the Supervisory Graduate Committee

for

NAPAPORN KUKIATINANT

unanimously approve the thesis entitled

MOLECULAR ALTERATIONS OF URINARY HCG

FROM HYDATIDIFORM MOLE

We further agree that she has satisfactorily defended her thesis at the examination given by the Supervisory Committee

on

November 14, 1984

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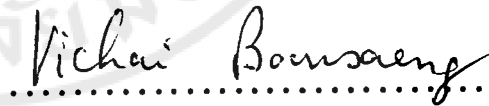
We recommend therefore that

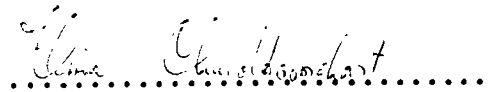
NAPAPORN KUKIATINANT

be awarded the degree of Master of Science in
Basic Medical Science (Biochemistry)
from Mahidol University

Thanit Kusamran, Ph.D.
Chairman



Vichai Boonsaeng, Ph.D.



Vina Churdboonchard, Ph.D.

Monthree Chulasamaya, M.D.,Ph.D.
Dean, Faculty of Graduate Studies,
Mahidol University

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BIOGRAPHY

Name :	NAPAPORN KUKIATINANT
Date of Birth :	May 22, 1958
Place of Birth :	Bangkok, Thailand
Institution Attended:	Strividhya School, Bangkok
	March, 1977
	Certificate of Mathayom Suksa V
	Chulalongkorn University
	Faculty of Medicine, Bangkok
	March, 1981
	Bachelor of Science in Medical Technology

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TO MY PARENTS AND BROTHER

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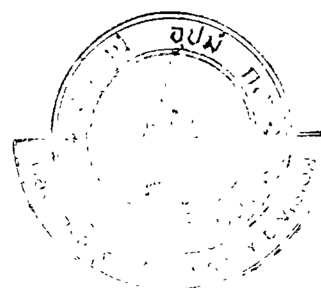

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LIST OF ABBREVIATIONS

Asn	Asparagine
BSA	Bovine serum albumin
^oC	Degree Celcius
CIE	Crossimmunoelectrophoresis
CMP	Cytidine monophosphate
cAMP	Adenosine 3', 5' cyclic monophosphate
cm	Centimeter
DD-H_2O	Double distilled water
DNA	Deoxyribonucleic acid
Fuc	L-Fucose
Gal	D-Galactose
GalNAc	N-Acetyl-D-galactosamine
GNAc	N-Acetyl-D-glucosamine
g	Gram
HCG	Human chorionic gonadotropin
h	Hour
IgG	Immunoglobulin G
IU	International Unit
K	Kilodalton
kg	Kilogram
M	Molar
Man	D-Mannose
mA	Milliampere
mg	Milligram

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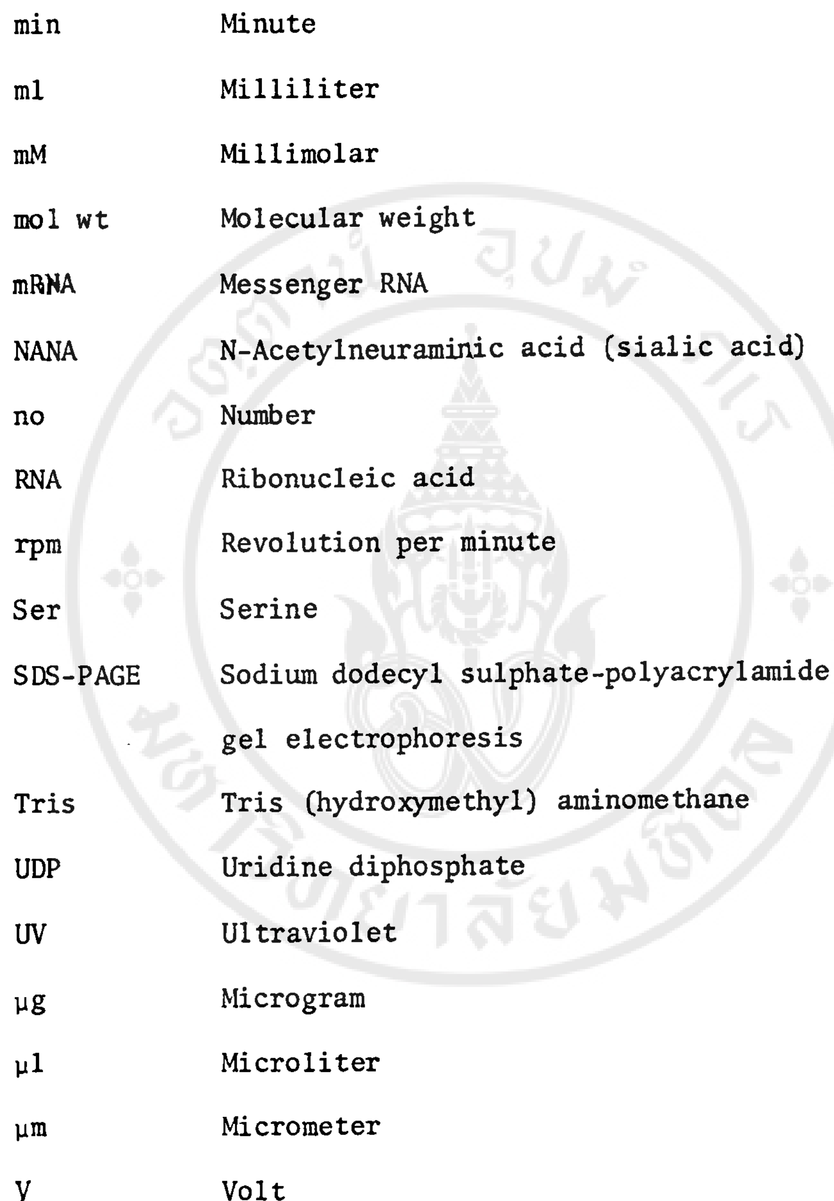

min	Minute
ml	Milliliter
mM	Millimolar
mol wt	Molecular weight
mRNA	Messenger RNA
NANA	N-Acetylneuraminic acid (sialic acid)
no	Number
RNA	Ribonucleic acid
rpm	Revolution per minute
Ser	Serine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine diphosphate
UV	Ultraviolet
μg	Microgram
μl	Microliter
μm	Micrometer
V	Volt

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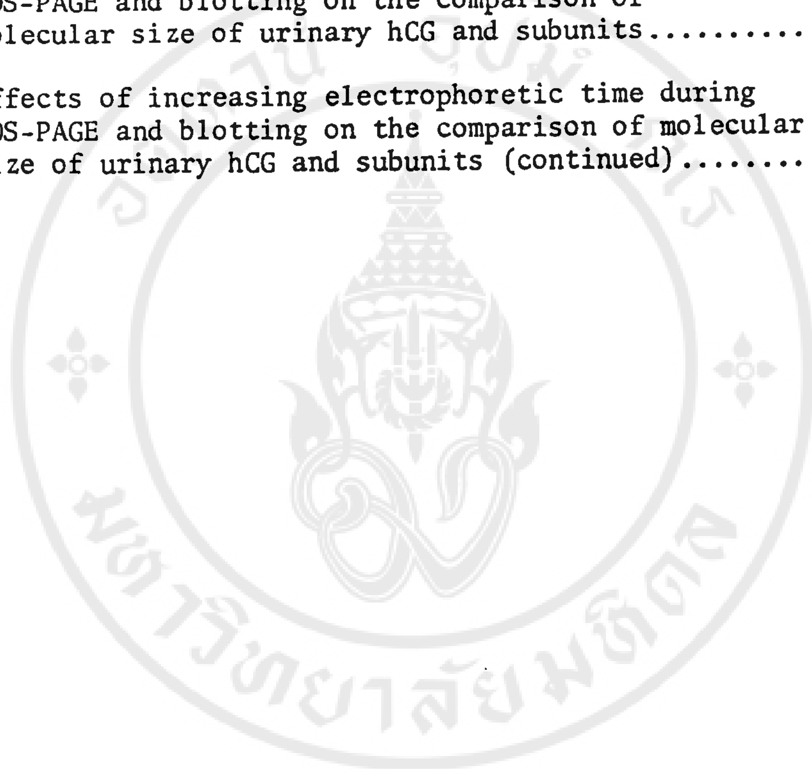

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ABSTRACT

Human chorionic gonadotropin (HCG) is a glycoprotein hormone normally secreted into serum and urine by human placenta during pregnancy. The hormone produced in hydatidiform mole disease, an abnormal proliferation of placental trophoblastic cells, has been found differing from normal hCG in many biochemical properties. The present investigations were undertaken to explore whether the molar hCG would be distinguishable from normal hCG by simple biochemical methods regarding to their differences in molecular charges and sizes. The method may be useful in helping early diagnosis of the disease.

Molecular charges of urinary hCG from normal and molar pregnancies were compared under non-denaturing condition using Tandem-crossimmunoelectrophoresis in Tris-glycine buffer (pH 8.6) and the intact hCG was detected by complementary rabbit anti-hCG. Samples from 10 patients showed significantly slower electrophoretic mobilities of molar hCG than normal hCG indicating less negative charge of the former. However, molar hCG was immunochemically identical to normal hCG. Therefore, the abnormality of this hormone might be due to variation in carbohydrate moiety.

Molecular sizes of normal and molar hCG were also compared by SDS-polyacrylamide gel electrophoresis. The hormones were detected by immunofluorescence or immunoperoxidase after being transferred to a nitrocellulose paper. The molecular size of normal hCG was found to be

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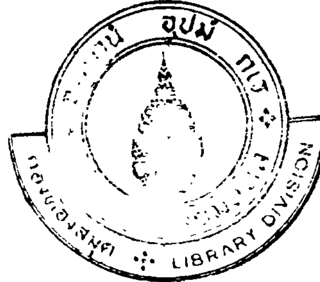

homogeneous (apparent molecular weight 52,000) whereas additional small immunoreactive hCG (apparent molecular weight 43,000) was observed in some hydatidiform mole urines. Moreover, a small molecular size immunoreactive hCG-like substance (apparent molecular weight 17,000) was present in both normal and molar urines. The intact hCG was dissociated into two non-identical subunits (α,β), electrophoresed in SDS-PAGE and determined their molecular sizes separately using specific antiserum corresponding to either subunit. The small hCG from hydatidiform mole possessed identical α subunit to, but smaller β subunit than, that of normal hCG. The present studies suggested that the heterogeneities observed from molar hCG might reside on the β subunit.

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INTRODUCTION

During pregnancy, the human placenta produces large variety of proteins which are not normally detected in serum and urine of non-pregnant women. Among various placental specific proteins, human chorionic gonadotropin (hCG) is an important marker of pregnancy because it is secreted in large quantities into serum and urine at the early stage of pregnancy (Braunstein *et al.*, 1976; Hiranyavasit, 1978). The level of hCG reaches maximum between 8-12 weeks of gestation, then decreases in the second trimester (3-6 months) and remains constant at a basal level throughout the rest of gestation. In hydatidiform mole, a disease of an abnormal proliferation of placental trophoblastic cells, the hormone level markedly increases higher than normal level and has been used as a criteria for prognosis.

Properties of hCG

HCG is a glycoprotein hormone consisting of two dissimilar, noncovalently bound subunits, designated α and β. The subunits can be dissociated and separated from each other by ion-exchange chromatography in urea-containing buffers (Swaminathan & Bahl, 1970). The individual subunits are biologically inactive but the reconstituted molecule is almost fully active. The molecular weight of hCG calculated from its chemical composition is 38,000 (Bahl, 1977). It is composed approximately 30% by weight of carbohydrate which contains six different monosaccharides: D-mannose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine,

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L-fucose and sialic acid. The first four of which are internally linked and the last two are located at terminal. The structure of hCG is closely related to those of the pituitary glycoprotein hormones: follicle stimulating hormone, lutenizing hormone and thyroid stimulating hormone (Ashitaka *et al.*, 1970; Ross, 1977). The α subunit of all four human glycoprotein hormones are nearly identical but the β subunit is hormone specific.

The α subunit of hCG has molecular weight of 14,700, approximately 10,000 for protein and 4,700 for carbohydrate. The peptide chain consists of 92 amino acid residues and five disulfide bonds. It has two oligosaccharide side chains attached by glycosylamine linkages between N-acetyl-D-glucosamine of the oligosaccharide and the amide nitrogen of asparagine residues at positions 52 and 78 (Morgan *et al.*, 1975) as shown in Fig.1.

The β subunit of hCG has molecular weight of 23,000, approximately 16,000 for protein and 7,000 for carbohydrate. It has six disulfide bonds and 147 amino acids. The carboxy-terminal sequence of 30 amino acids is unique. In the immunization, this extra C-terminal portion is responsible for the production of antiserum which is specific for detection of hCG (Vaitukaitis *et al.*, 1972; Birken *et al.*, 1982). It is composed of six oligosaccharide side chains, two of them are attached to asparagine residues at positions 13 and 30, the other four side chains are attached by glycosidic bonds between N-acetyl-D-galactosamine and the hydroxyl group of serine residues at 121, 127, 132 and 138 (Morgan *et al.*, 1975) as shown in Fig.2.

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Figure 1 The asparagine-linked carbohydrate chains of the α-hCG subunit (Morgan *et al.*, 1975).

```
                                                      COOH
                                                      /
Man  -  Man  -  Man  -  Man  -  GNAc  -  Asn78
 |       |       |                        (
GNAc    GNAc    GNAc                    52Asn  -  GNAc  -  Man  -  Man  -  Man  -  Man
 |       |                                 )                        |        |        |
Gal     Gal                                                        GNAc     GNAc     GNAc
 |       |                                                                   |        |
NANA    NANA                                                                Gal      Gal
                                                                             |        |
                                                                            NANA     NANA

                                          NH2
```

Figure 2 The asparagine-linked and serine-linked carbohydrate chains of the β-hCG subunit (Morgan *et al.*, 1975).

```
Fuc    NANA  NANA
 |      |     |
Gal    Gal   Gal
 |      |     |
GNAc   GNAc  GNAc  GNAc
 |      |     |     |
Man-   Man-  Man-  Man-  GNAc-Asn30

                                  121 Ser - GalNAc - Gal - NANA
                                  127 Ser - GalNAc - Gal - NANA
                                  132 Ser - GalNAc - Gal - NANA
Man-   Man-  Man-  Man-  GNAc-Asn13   138 Ser - GalNAc - Gal - NANA
 |      |     |     |
GNAc   GNAc  GNAc  GNAc
 |      |     |
Gal    Gal   Gal
 |      |     |
Fuc    NANA  NANA            NH2          COOH
```

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Biosynthesis of hCG

HCG is synthesized by syncytiotrophoblast of the placenta (Midgley & Pierce, 1962; Dreskin *et al.*, 1970). The biosynthetic mechanism of the hormone in normal placenta has been studied both *in vivo* and *in vitro* experimental systems. The α and β subunits of hCG are translated from separate mRNAs. The production of these two subunits are often unbalanced, the α subunit is synthesized in larger quantity than the β (Daniels-McQueen *et al.*, 1978; Quigley *et al.*, 1980). This is consistent with the observation that the level of the α subunit mRNA is more than that of the β (Boothby *et al.*, 1983). Though the synthesis of the β subunit appears to be a rate-limiting step in the production of hCG.

In recent years, studies have been focused on the glycosylation of hCG. When placental mRNAs coding for the α and β subunits of hCG are translated in membrane-depleted cell-free systems, two *de novo* polypeptides (apparent molecular weights of 13,000 and 16,000) were precipitated with anti-αhCG and anti-βhCG, respectively (Fiddes & Goodman, 1979). The membrane-dependent glycosylation in cell-free system of pre-α subunit and pre-β subunit results to the products of molecular weights 14,000 and 18,000, respectively (Bielinska & Boime, 1978). The products are specifically bound to concanavalin A column, and sensitive to the action of a variety of glycosidases. Radioactive α and β subunits of hCG isolated from cultures of human choriocarcinoma (JAR cell line) by immunoprecipitation and analyzed on SDS-polyacrylamide gel (Ruddon *et al.*, 1979, 1980) are partially glycosylated which contain

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high mannose core. Both radioactive subunits lack terminal carbohydrate sequences, as judged by sensitivity to endoglycosidase H(cleave mannose oligosaccharide units from asparagine residues specific for high mannose oligosaccharides) but resistance to endoglycosidase D (cleave side chain-free complex-type glycopeptides but not high mannose glycopeptides). These core oligosaccharides are transferred by an activated lipid carrier (dolichol phosphate) to the polypeptide chain and processed by the action of specific glycosidases and glycotransferases. Further processing of the hCG subunits probably occurs in the Golgi apparatus where an α-mannosidase removes extra mannose residues (Henner *et al.*, 1983) followed by the terminal glycosylation by the sequential addition of UDP-N-acetylglucosamine, UDP-galactose and CMP-neuraminidate to the core oligosaccharides. This mechanism would be analogous to the processing pathway of other glycoproteins such as vesicular stomatitis virus G protein (Hunt, *et al.*, 1978; Lodish & Rothman, 1979), immunoglobulin G heavy chain (Tabas *et al.*, 1978), and pituitary glycoprotein hormones (Giudice & Weintraub, 1979; Kourides *et al.*, 1979; Landefeld,1979).

Vaitukaitis and Ebersole (1973, 1976) have pointed out that the different forms of hCG synthesized by tumors may be due to relative imbalances of synthetic and hydrolytic enzymes (e.g. in the case of choriocarcinoma cell lines, perhaps deficiency in mannosidase or transferase activity). Therefore, the tumors are unable to synthesize complete carbohydrate portion of the molecule. As a result, the tumor hCG varies widely in binding to concanavalin A (Yoshimoto *et al.*, 1979;

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Mann & Karl, 1983). In addition, its *in vivo* biological activity is also lower than normal presumably due to rapid metabolic clearance (Van Hall *et al.*, 1971; Dufau *et al.*, 1972).

The importance of the carbohydrate portion of hCG

During the first few months of pregnancy, the function of hCG is to prevent the degeneration of the corpus luteum in order to continue the synthesis of steroid hormones (Savard *et al.*, 1965; Langman, 1975). Many studies have pointed out that the carbohydrate part may play an important role in the mechanism of action of this hormone. The biological and immunological roles of sialic acid in the hCG molecule are investigated *in vitro* by treated with neuraminidase (Dufau *et al.*, 1971; Tsuruhara *et al.*, 1972). These studies show that the desialylation of hCG results to a marked reduction of biological activity without affecting immunological activity. Similar results were obtained from *in vivo* studies carried out with chemically modified analogs of asialo-hCG (Madnick *et al.*, 1981; Kalyan *et al.*, 1982). Besides, sialic acid residues are important for determination of the half-life of the circulating gonadotropic hormones. The plasma half-life of hCG drops from 20 to 1 min after complete desialylation and this may be the major cause of its loss of biological activity *in vivo* (Van Hall *et al.*, 1971). Sequential removals of the sialic acid, galactose, N-acetyl-glucosamine and mannose residues by a series of exoglycosidase digestion do not impair the *in vitro* receptor binding properties of hCG. However, the ability to stimulate cAMP and steroidogenesis in rat Leydig cells and granulosa cells by the hormone is considerably reduced (Moyle *et al.*,

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1975; Bahl, 1977; Channing *et al.*, 1978). Further study by Chen and co-workers (1982) show that chemical deglycosylation of purified hCG by treatment with hydrogen fluoride (HF) in the presence of anisole displays 2.5 times higher binding activity than intact hCG using rat testis luteinizing hormone receptor assay. In contrast, the *in vitro* biological activities of HF-hCG upon stimulation of testosterone and cAMP production in Leydig cells and activation of adenylate cyclase in interstitial membranes are at least 100 folds less active than hCG. The removal of almost all carbohydrate from hCG has been attempted by the application of trifluoromethane sulfonic acid to the subunits followed by the reconstitution of the deglycosylated subunits (Kalyan & Bahl, 1983). This further demonstrates that the carbohydrate is not involved in the association of the α and β subunits, the antibody, or the receptor binding activity of the hormone but it is required for the expression of the hormone activity.

Abnormal production of hCG in hydatidiform mole

Hydatidiform mole is a disease of abnormal pregnancy in women. The word "hydatidiform mole" is derived from Latin Language: hydatid means watery vesicle, and mole means mass. Hydatidiform mole is a condition in which chorionic villi become enormously overdistended with fluid, appearing as translucent grapelike vesicles which may vary in size from a few millimeters to more than 2 cm in diameter. The conglomerate mass of vesicles grows rapidly in the first week of pregnancy, filling and distending the uterine cavity, usually in the absence of the fetus (Novak *et al.*, 1981; Hilgers & Lewis, 1982).

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Without treatment, the course of hydatidiform mole is rapidly progress. It can develop to be the invasive mole (chorioadenoma destruens; invasive of trophoblastic cells in the myometrium) and choriocarcinoma which is a malignant tumor.

In patient with hydatidiform mole, the neoplasm retains some characteristics of the normal placenta, such as the ability to produce hCG which is a placental specific protein. However, marked increase of hCG produced correlates directly with the amount of viable trophoblastic tissue present. Therefore, persistent or rising titers without declining to the basal level of hCG has been routinely used as an indication of hydatidiform mole.

Biochemical distinction between normal and molar hCG

HCG from normal pregnant women and from patients with hydatidiform mole has been purified and characterized by many investigators. Purified molar hCG has shown to be biologically active but heterogeneous in its molecular form on moving-boundary electrophoresis (Reisfeld & Hertz, 1960). Hiranyavasit *et al.*, (1979) have demonstrated that urinary hCG purified from patients with molar pregnancy exhibits 2 forms of molecular size as analyzed on SDS-PAGE. One form is equal to the normal hCG while the other is smaller. The heterogeneity of molar hCG which has been speculated might be related to the carbohydrate moiety. Choy *et al.*, (1979) have found that there is no difference in amino acid composition between normal and molar hCG. However, the latter is lower in sugar composition, especially, hexosamine, mannose and sialic acid. Yoshimoto *et al.* (1979) extract an immunoreactive

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hCG-like substance from tumor tissue and serum. They find that the substance binds to concanavalin A (con A) at varying extent from 3-90% whereas the binding of normal hCG to con A is always over 90%. The findings indicate that tumor hCG may lack carbohydrate moieties at various extent. Results from other investigators indicate that the incompleteness of sugar may occur during biosynthesis. Molar hCG from chorionic placental tissues, the source of this hormone, was purified and characterized by Imamura (1980). This molar hCG has lower contents of hexosamine and sialic acid. Vesicular fluid from molar placenta, the most appropriate model *in vivo*, was studied by using Tandem-crossimmunoelectrophoresis (Rajatasriprasert, 1982). Vesicular hCG shows the same immunochemical property as that of the normal, but its electrophoretic mobility is slower. This implies the less negative charge of hCG in the vesicular fluid. From these evidences, the abnormality of molar hCG might be due to the defective biosynthesis, especially in glycosylation, and led to the loss of negative-charged sialic acid of this hormone.

Statement of the problems

In patients with hydatidiform mole, the level of hCG is elevated several folds higher than that in normal pregnant women. Therefore, the quantitative determination of this hormone has generally been used as a marker for diagnosis of this disease. Since the amount of hCG secreted correlates with the mole formation, at the time of the abnormality of hCG observed the hydatidiform mole usually develops and metastasizes becoming very dangerous. Therefore, it should be other

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alternative methods which could differentiate hydatidiform mole from the normal at the early stage of pregnancy.

It has been established that the lack of some carbohydrate moieties of molar hCG occurs during biosynthesis. This incompleteness of carbohydrate might be resulted to the variation of molecular charges and sizes. Previous evidences from Hiranyavasit *et al.* (1979) find that SDS-polyacrylamide patterns of molar and normal hCG are different but this observation is still unclear. In order to confirm the result, another possible approach is to separate the urine directly by size using SDS-PAGE followed by specific immunodetection. This approach can be achieved by using electrophoretic blotting technique. By this method, several problems involved with the separation and purification of hCG were overcome.

Electrophoretic transfer or blotting has become a well-known technique for detection of biologically interesting macromolecules. The process involves transferring of macromolecules, e.g. DNA, RNA and protein, from the gel to an immobilized matrix. These immobilized macromolecules can be detected by staining (Burnette, 1981; Wojtkowiak *et al.*, 1983), specific fluorescence (Towbin *et al.*, 1979; Schmidt *et al.*, 1982), enzyme-substrate (Glynn *et al.*, 1982; Palfree & Elliott, 1982; Clegg, 1982) or autoradiograph (Vaessen *et al.*, 1981; Swanson *et al.*, 1982). Using blotting technique, the high resolution obtained from the gel is maintained and the transferred molecules which located at the surface of an immobilized matrix become accessible to analysis. One important point to be concerned is that blotting is not useful for

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all kinds of macromolecules, only transferred molecules which still retain their immunochemical property can be analyzed by this technique.

Southern (1975) described a technique for the capillary transfer of DNA from agarose gels to nitrocellulose sheets. Since that time, blotting has been developed and applied to RNA and protein. It was popularized when Towbin (1979) described the transfer of ribosomal proteins from polyacrylamide-urea gel to nitrocellulose sheets. Many kinds of glycoproteins could be efficiently transferred and detected (Erlich *et al.*, 1979; Glass *et al.*, 1981; Clegg, 1982; Palfree & Elliott, 1982). Therefore, this method is chosen in the present studies to investigate the heterogeneity of hCG from molar pregnancy.

The aims of the thesis

1. To study the molecular charge of urinary hCG from normal pregnant women and from patients with hydatidiform mole by Tandem-crossimmunoelectrophoresis.

2. To study the molecular size of urinary hCG and its subunits obtained from normal and molar pregnancies by SDS-PAGE and blotting. Unlike many previous methods, blotting does not require purification which is time-consuming and may cause incomplete recovery due to the heterogeneity of molar hCG.

In the present studies, hCG from urines of normal and molar pregnancies was characterized in two aspects; differences in molecular charges and in molecular sizes. The findings could be briefly summarized in the followings :-

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ห้องสมุด

1. The electrophoretic mobility of hCG from both sources was compared by Tandem-crossimmunoelectrophoresis. Molar hCG was similar in immunochemical property to the normal but electrophoretic mobility of the former was slower which implied less negative charge of the molar hCG.

2. HCG and its subunits from both sources were analyzed by SDS-PAGE, electrophoretic transfer to nitrocellulose papers and incubated with rabbit antisera against intact hCG, βhCG and αhCG. The immune complexes were detected with FITC-conjugated anti-rabbit IgG or peroxidase-conjugated anti-rabbit IgG. The molar hCG showed heterogeneity in its molecular form. Moreover, the immunoreactive hCG-like substance, which contained the small molecular size, was found in both normal and molar urines. Some portions of β subunit of 6 from 10 molar patients were found to be smaller in molecular size than the normal βhCG. No difference in molecular size of α subunit was found.

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MATERIALS AND METHODS

Materials

1. Biological samples

Urines were individually collected from women with either normal pregnancy (total of 4, 8-12 weeks of gestation) or clinical diagnosis of hydatidiform mole (total of 10) at the Department of Obstetrics and Gynecology, Siriraj Hospital, Bangkok. The samples were stored at -20°C with 0.02% sodium azide as a preservative until use.

Negative control urine (no hCG) was collected from normal healthy nonpregnant women and negative serum was collected from normal healthy men.

2. Animals

Female New Zealand white rabbits weighing about 2 kg were obtained from the Central Animal Facility, Faculty of Science, Mahidol University, Bangkok. Each rabbit was individually kept in a cage and conditioned in the animal room of the animal centre.

3. Antisera

Rabbit anti-hCG was either provided by Dr. Watcharee Hiranyavasit or purchased from Dakopatts (Denmark) which will be notified in the text as anti-hCG_W and anti-hCG_D, respectively. Rabbit anti-βhCG was provided by Dr. Watcharee Hiranyavasit and the rabbit anti-αhCG was prepared during this study.

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Fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin was purchased from Dakopatts.

Peroxidase-conjugated goat anti-rabbit IgG was the product of Sigma Chemical Company.

4. Chemicals

Purified hCG used as standard calibration in Ouchterlony double immunodiffusion was provided by Miss Rachanee Kongsayreepong. Crude hCG (specific activity 3,360 IU/mg) from normal pregnant urines used as a marker in both polyacrylamide gel electrophoresis and Ouchterlony double immunodiffusion was purchased from Sigma Chemical Company. Purified α and β hCG used for determination of antiserum specificity were provided by Dr. Kavi Ratanabanangkoon and Dr. Watcharee Hiranyavasit, respectively. Other protein markers were purchased from Sigma Chemical Company.

Chemicals for polyacrylamide gel electrophoresis are as follows: acrylamide, glycine, Trizma-base, sodium dodecyl sulfate (SDS), Coomassie brilliant blue R, bromophenol blue, ethylene diamine tetraacetic acid (EDTA) and 2-mercaptoethanol were purchased from Sigma Chemical Company. N,N'-bis-methylene acrylamide and N,N,N',N'-tetra-methylethylenediamine (TEMED) were obtained from Eastman Kodak Company. Ammonium persulfate was purchased from May & Baker Ltd.

Chemicals for immunodiffusion, immunoelectrophoresis and crossimmunoelectrophoresis are as follows: special nobel agar was purchased from Difco Laboratories. Agarose was the product of Kallestad. Coomassie brilliant blue R-250 was obtained from Serva.

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Sodium barbital was purchased from Merck and sodium azide was from Riedel-De Haen.

Chemicals for blotting are as follows: bovine serum albumin (BSA, fraction V), amido black and 3,3'-diaminobenzidine (DAB) were purchased from Sigma Chemical Company. Nitrocellulose (BA 85, 0.45 μm) was purchased from Schleicher & Schuell. Nonidet P-40 was obtained from Shell Chemical Company.

All chemicals are either analytical or reagent grade.

Methods

1. Preparation of samples

The frozen urine was thawed and centrifuged at 10,000 rpm for 1h to remove sediments. The supernatant (about 20 ml) was dialyzed (27 DM, Union Carbide) at $4^{o}C$ against DD-H_2O overnight to remove salts and low molecular weight substances (<10,000 daltons). The urine was subsequently concentrated by lyophilization and dissolving in 0.6 ml of DD-H_2O. An aliquot was removed for determination of hCG. The remaining solution was kept at $-20^{o}C$. Otherwise indicated, it was used throughout the experiments.

1.1 Determination of hCG

The urinary hCG was determined by Ouchterlony double immuno-diffusion as described by Williams and Chase (Williams & Chase, 1971).

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Purified hCG of known concentration was used for calibration. In my experiment, 3.5 ml of 1% melting agar (1 g of special nobel agar in 100 ml of 0.85% NaCl containing 0.05% sodium azide) was applied on a

clean microscopic slide (1"x3") and wells (0.3 cm in diameter) were subsequently formed. Purified urinary hCG (kindly provided by Miss Rachanee Kongsayreepong), 0.1, 0.3, 0.5, 1.0, 2.0 and 3.0 μg in 7 μl, were filled in each peripheral well (as shown in Fig. 4A). The central well contained 7 μl of anti-hCG_D. The gel was placed in a moisture chamber and incubated for 18-24 h at room temperature (28-30^oC). The sensitivity of this method was determined by the lowest concentration of hCG which could be detected from the gel and this value was used for determination of hCG in urine samples.

The concentration of hCG in urine samples was estimated as described above. Two-fold serial dilution of each sample was prepared from the concentrated urine and 7 μl of each dilution was filled in the corresponding peripheral wells. The central well contained 7 μl of anti-hCG_D. Crude hCG (0.3 mg/ml of hCG, Sigma) was used simultaneously as the positive control. The gel was placed in a moisture chamber and incubated for 18-24 h at room temperature.

To make a permanent slide, the gel was press-dried with a weight (10 g/cm^2) for 10 min then immersed in 0.85% NaCl for 1 h to remove non-precipitated proteins and subsequently transferred to distilled water for 15 min to remove NaCl from the gel. The gel was press-dried again and dried at 50^oC before staining with 0.5% Coomassie brilliant blue R-250, 50% ethanol and 10% glacial acetic acid for 5 min, destained in 50% ethanol and 10% glacial acetic acid until the background was clear, then rinsed in distilled water and air-dried.

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1.2 Determination of proteins

Proteins were determined by the method modified from Lowry (Lowry *et al.*, 1951). Bovine serum albumin (Sigma fraction V) was used as standard. The assayed mixture contained 0.1 ml of 1:10 dilution of urine sample (unconcentrated), 1 ml of freshly prepared potassium-sodium tartrate-$CuSO_4$ reagent and 0.1 ml of phenol reagent.

2. Preparation of anti-αhCG

Anti-αhCG was prepared by the method of Vaitukaitis (Vaitukaitis *et al.*, 1971). Each rabbit (3-month-old New Zealand white rabbit) was immunized by intradermal injection 30-50 sites on shaved back and proximal limbs with the total of 2 ml of the emulsion prepared by dissolving 25 μg of purified αhCG (kindly provided by Dr. Kavi Ratanabanangkoon) in 1 ml of 0.85% sterile saline solution and this mixture was homogenized at $4^{o}C$ with 1 ml of Freund's complete adjuvant (Difco Laboratories). Then 0.5 ml of mixed vaccine (Diphtheria, Tetanus toxoids and Pertussis, Connaught Laboratories) was injected subcutaneously at 2 separate sites. After the immunization, 1 ml of blood was drawn every 7 day intervals by central ear artery puncture. The blood was allowed to clot at room temperature for ½ h followed by centrifugation at 3,000 rpm for 10 min to remove red blood cells. The fresh serum was tested for antiserum titer and antiserum specificity. One month later, the booster injection was given, using incomplete adjuvant instead of the complete one. Seven days after the second injection, 30-40 ml of blood was collected. The serum was separated and stored in 0.5 ml aliquots at $-70^{o}C$.

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2.1 Determination of antiserum titer

The method for determination of antiserum titer was Ouchterlony double immunodiffusion as previously described in section 1.1. 1 μg/ 7μl of purified αhCG was filled in the central well. The peripheral wells contained 7 μl of each two-fold dilution of rabbit immunized serum. Rabbit preimmunized serum was used as the negative control. The free diffusion was allowed to occur in a moisture chamber for 18-24 h at room temperature. The gel was stained, destained and dried as described in section 1.1.

2.2 Determination of antiserum specificity

The specificity of anti-αhCG was determined by immunoelectrophoresis (Grabar & Williams, 1955). 3.5 ml of 1% melting agarose (1 g of agarose gel in 100 ml of Veronal buffer pH 8.6 containing 0.9 g% sodium barbital, 0.05% sodium azide) was applied on a clean microscopic slide (1"x3"). One trough between 2 wells was made as shown in Fig. 10C. 1 μg each of purified αhCG or βhCG (provided by Dr. Watcharee Hiranyavasit) was filled in each well. The gel was placed in an electrophoretic apparatus (Gelman instrument company) containing Veronal buffer. Whatman paper no. 1 was used to connect the gel to the electrode chamber. The electrophoresis was performed at a constant voltage (15 V/cm) until the tracking dye (bromophenol blue) reached the anode edge of the gel (about 3 h). The power was disconnected and a trough was cut (6.5 cm) and filled with 70 μl of anti-αhCG. The gel was placed in a moisture chamber for 18-24 h at room

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temperature. The precipitin band was observed and the gel was stained, destained and dried as described in section 1.1.

3. Determination of molecular charge of hCG

In order to compare the electrophoretic mobilities of hCG from normal pregnant women and from patients, Tandem-CIE was performed according to Krøll (1973) and Rajatasriprasert (1982).

One gram of agarose gel (Kallestad Co.) in 100 ml of Tris-glycine buffer pH 8.6 (0.038 M Tris-HCl, 0.1 M glycine and 0.01% sodium azide) was boiled until solubilized. 8 ml of the gel solution was applied on a glass plate (6x10x0.1 cm). Two wells (0.3 cm in diameter) with 1 cm distance were punched with the pattern shown in Fig.5. The concentrated urines to be compared were adjusted with Ouchterlony double immunodiffusion to obtain 0.5 μg/7 μl of hCG and alternately filled in those wells. The glass plate was placed in an electrophoretic apparatus (Gelman instrument company) containing Tris-glycine buffer pH 8.6. Electrophoresis was carried out at 10V/cm for 1 h at room temperature (25°C). After the first dimensional electrophoresis, the power was disconnected and the gel was cut into strips (1 cm wide). Each strip which contained separated proteins was transferred by a razor blade to another glass plate (5x5x0.1 cm). The remaining part of the plate was covered with 2.5 ml of the 1% gel solution (56°C) containing anti-hCG_D (50 μl/plate). The second dimensional electrophoresis was performed at perpendicular direction to the first one at 5V/cm for 18-20 h at room temperature (25°C). The gel was stained, destained and dried as described in section 1.1. The interdistances

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between two peaks of hCG in both runs were measured and compared with each other.

4. Determination of molecular size of hCG and its subunits

To study on molecular size of intact hCG and its subunits, SDS-PAGE and electrophoretic transfer were performed.

4.1 Separation of normal hCG and subunits on SDS-PAGE

A mixture of known molecular weight proteins : 10 μg of BSA (68K), 10 μg of IgG containing H-chain (53K) and L-chain (23K), 20 μg of ovalbumin (43K) and 10 μg of myoglobin (17K) were dissolved in sample buffer (0.0625 M Tris-HCl, pH 6.8, 1% (W/V) SDS, 10% (V/V) glycerol and 0.001% (W/V) bromophenol blue with 5% 2-mercaptoethanol. 6 μg/20 μl of crude hCG (Sigma) and 12 μg/40 μl of crude hCG were dissolved in sample buffer and subjected to 12% SDS-polyacrylamide gel (9x16x0.1 cm) with 3% stacking gel as described by Laemmli (Laemmli, 1970). In order to separate the hCG subunits, 12 μg/40 μl of the crude hCG was dissolved in sample buffer containing 5% 2-mercaptoethanol and subjected to the similar gel. Electrophoresis was conducted in a vertical slab gel containing electrode buffer (0.025M Tris, 0.192 M glycine and 0.1% (W/V) SDS, pH 8.3) and carried out with a constant current of 20 mA per gel for 3h. The proteins were visualized by staining for 2h with 0.2% (W/V) Coomassie brilliant blue R, 50% methanol, 7% acetic acid and destained in 5% methanol and 7% acetic acid. The molecular weights of marker proteins were plotted versus the relative mobilities. The molecular weights of normal intact hCG and subunits were determined from the standard curve.

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Besides, protein patterns from nonpregnant urine and from human serum were demonstrated. Nonpregnant urine was performed the same as described in section 1 and human serum was diluted 1:10 with DD-H_2O. 40 μg/5 μl of serum proteins and 125 μg/5 μl of nonpregnant urinary proteins were dissolved in sample buffer with 5% 2-mercaptoethanol and electrophoresed simultaneously with the marker proteins.

4.2 Separation of molar hCG and subunits on SDS-PAGE

Concentrated urine sample containing approximately 3 μg/10 μl of hCG was dissolved in sample buffer without mercaptoethanol. In order to separate the hCG subunits, 6 μg/20 μl of hCG was dissolved in sample buffer containing 5% 2-mercaptoethanol. The proteins were subjected to electrophoresis as described in section 4.1. Crude hCG (Sigma) using as the positive control was performed the same as urine sample and electrophoresed simultaneously.

4.3 Electrophoretic transfer of hCG and subunits from polyacrylamide gel to the nitrocellulose paper

After the electrophoresis, proteins from SDS-polyacrylamide gel were electrophoretically transferred to a nitrocellulose sheet (S&S, pore size 0.45 μm) as described by Burnette (Burnette, 1981). A sandwich was performed with the following successive layers (see Fig. 3):-

1. a porous polyethylene supporter, 14.5x14.5x0.6 cm;
2. three sheets of Whatman no. 3 soaked in the transfer buffer;
3. the SDS-polyacrylamide gel (9x9x0.1 cm) with the stacking gel removed;
4. a sheet of nitrocellulose cut to the size of the gel, prewet in DD-H_2O before equilibrating in the transfer buffer;

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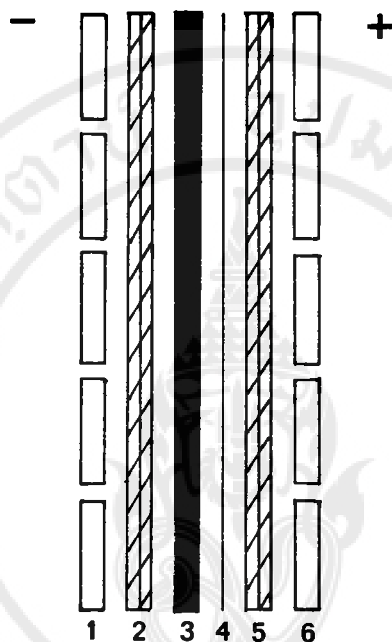

Figure 3 Diagram showing the assembly of electrophoretic transfer.

(1) a porous polyethylene supporter

(2) three sheets of Whatman no. 3

(3) the SDS-polyacrylamide gel

(4) a sheet of nitrocellulose

(5) another three sheets of Whatman no. 3

(6) another porous polyethylene supporter

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5. another three sheets of Whatman no. 3 soaked in the transfer buffer, and
6. another porous polyethylene supporter, 14.5x14.5x0.6 cm.

This sandwich was assembled quickly to avoid dehydration of the gel and great care was taken to remove all air bubbles which would lead to the uneven transfer. Then it was placed between the electrodes with the nitrocellulose sheet toward the anode. The transfer buffer (20 mM Tris-base, 150 mM glycine and 20% methanol, pH 8.3) was added to fill the chamber (laboratory-made electroblotter, based on the design of Bittner). Electrophoretic transfer was usually performed at the constant voltage of 10V/cm (distance between electrodes) for 2 and 12 h.

After the transfer, proteins on the nitrocellulose sheet were visualized by staining for 5 min in solution containing 0.1% amido black-45% methanol-10% acetic acid and rapidly destained in 45% methanol-10% acetic acid. The destained sheet was rinsed in distilled water and blotted between filter paper. The gel after transfer was stained with amido black for 2 h and destained until the background was clear.

4.4 Immunochemical detection of hCG and subunits on the nitrocellulose sheet

After electrophoretic transfer, the nitrocellulose sheet (usually not stained with amido black) was immersed in buffer 1 (5% BSA in 10 mM Tris, 150 mM NaCl and 0.01% sodium azide) for 1 h at room temperature with continuous shaking. In this step the unbound sites of the sheet would be saturated by nonreactive molecule (BSA) in order to minimize the background. The sheet was washed in buffer 2 (10 mM Tris,

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150 mM NaCl and 0.01% sodium azide, pH 7.4) and blotted to remove excess solution before incubation with shaking for 2 h at room temperature in specific antiserum diluted 1:10 with buffer 3 (2% BSA in buffer 2) and 3 ml of this solution was used for a sheet of 100 cm^2. After incubation, the sheet was washed for 10 min in 100 ml of buffer 2, for 20 min in 200 ml twice of buffer 4 (0.05% Nonidet P-40 in buffer 2), and again for 10 min in 100 ml of buffer 2 in order to remove the excess unbound antiserum. The sheet was blotted and further incubated for 2 h in either FITC-conjugated swine anti-rabbit IgG diluted 1:20 or peroxidase-conjugated goat anti-rabbit IgG diluted 1:100 with buffer 3 (3 ml/100 cm^2 of the nitrocellulose sheet was used). The excess unbound anti-IgG was washed out as described above and the proteins were visualized under short wavelength UV-light for fluorescein-conjugated anti-rabbit IgG. In case of peroxidase-conjugated anti-rabbit IgG, the proteins were visualized by color reaction according to Pelliniemi (Pelliniemi *et al.*, 1980). The sheet was incubated at room temperature in 100 ml of 50 mM Tris-HCl buffer, pH 7.6 containing 0.2 mg/ml diaminobenzidine and 0.005% H_2O_2 until the brown product was visible (about ½ h). The reaction was terminated by washing in distilled water. The sheet was dried between filter paper and stored in darkness to prevent fading of the patterns.

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RESULTS

1. Levels of hCG and protein in urine of pregnant women

Concentrations of both hCG (section 1.1) and total protein (section 1.2) were separately determined in urine samples from 4 normal and 10 molar pregnancies.

HCG concentration was estimated by Ouchterlony double immunodiffusion using either diluted or concentrated samples. Although the method does not provide accurate measurement, it is simple and practically convenient. Fig. 4A showed standard calibration using purified hCG. The amounts of the immunoprecipitates correlated with the hCG concentration. The lowest detectable concentration of hCG by this method was about 0.5 μg. On the other hand, Fig. 4B and Fig. 4C demonstrated the immunoprecipitates of hCG in the urine samples at various concentrations from normal and molar pregnancies, respectively. The lowest concentration giving detectable immunoprecipitate (for example, x8 in Fig. 4B) was therefore assumed to contain 0.5 μg of hCG. The original concentration of hCG in the urine was estimated and summarized in Table 1. Seven molar patients (no. 2-4, 7-10) exhibited urinary hCG concentrations higher than that from the normal. One molar patient (no. 5) was about the same, and two molar patients (no. 1,6) were lower than the normal. The precipitin bands of molar hCG and standard hCG (Sigma) against anti-hCG_D fused completely with each other. This indicated the similarity in antigenic determinant between

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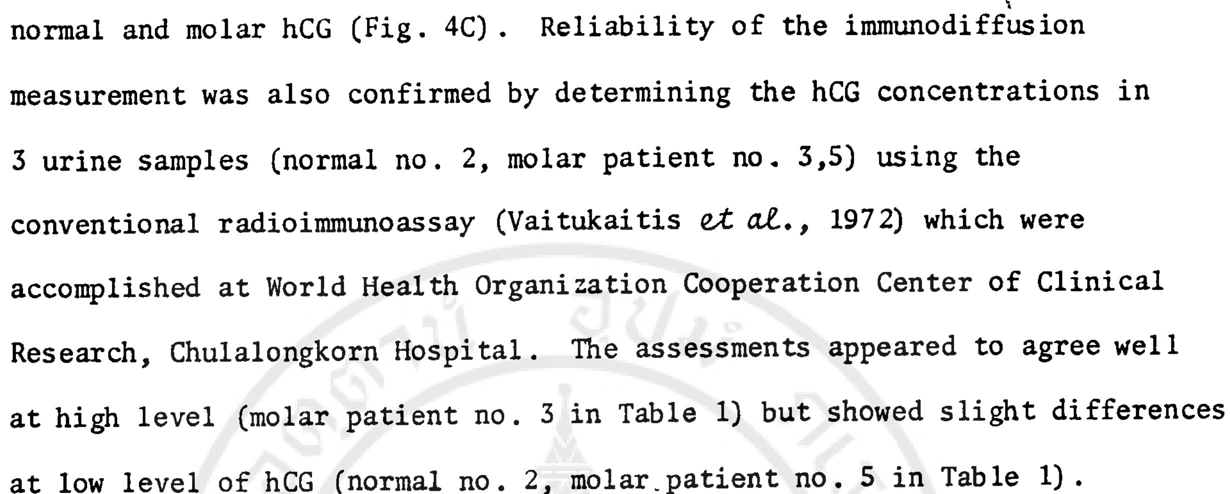

normal and molar hCG (Fig. 4C). Reliability of the immunodiffusion measurement was also confirmed by determining the hCG concentrations in 3 urine samples (normal no. 2, molar patient no. 3,5) using the conventional radioimmunoassay (Vaitukaitis *et al.*, 1972) which were accomplished at World Health Organization Cooperation Center of Clinical Research, Chulalongkorn Hospital. The assessments appeared to agree well at high level (molar patient no. 3 in Table 1) but showed slight differences at low level of hCG (normal no. 2, molar patient no. 5 in Table 1).

In comparison to the wide range of hCG level, total protein concentration in the urine of normal pregnant women was in the range of 2.5-3.5 mg/ml but its level varied from 1.4-5.7 mg/ml in molar patients (Table 1). It should be noticed that the hCG and total protein concentrations in molar urines were not parallel. Therefore, the ratio of hCG/protein in the molar patients varied considerably (Table 1). However, the ratio in molar patients was higher than in normal.

2. Comparison of molecular charge between normal and molar hCG

In addition to other distinct properties of molar hCG, the hormone has been found lacking of some terminal sugar residues in their carbohydrate chains (Choy *et al.*, 1979). The loss of the carbohydrate represents about 10% reduction in molecular weight of the intact hCG extracted from normal pregnant urine. Since sialic acid residues are located terminally at the hCG carbohydrate chains (Bahl, 1977), lacking of sialic acid in molar hCG as reported by Choy is expected to reduce both its negative charge and electrophoretic mobility. The present experiment was therefore undertaken to determine electrophoretic

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Figure 4 Measurement of urinary hCG concentration by Ouchterlony double immunodiffusion.

A. Sensitivity for detection of hCG by Ouchterlony double immunodiffusion
Purified hCG, 0.1, 0.3, 0.5, 1.0, 2.0 and 3.0 μg in 7 μl, were applied in each peripheral well. The central well contained 7 μl of anti-hCG_D.

B,C. The typical pattern of hCG from normal and molar pregnant urines, respectively.
The central well contained 7 μl of anti-hCG_D. Each peripheral well contained 7 μl of 2-fold concentration of urine samples (normal no. 1-4, molar patient no. 1-10). 7 μl of standard hCG (std, Sigma, 0.3 mg/ml) was used as the positive control.

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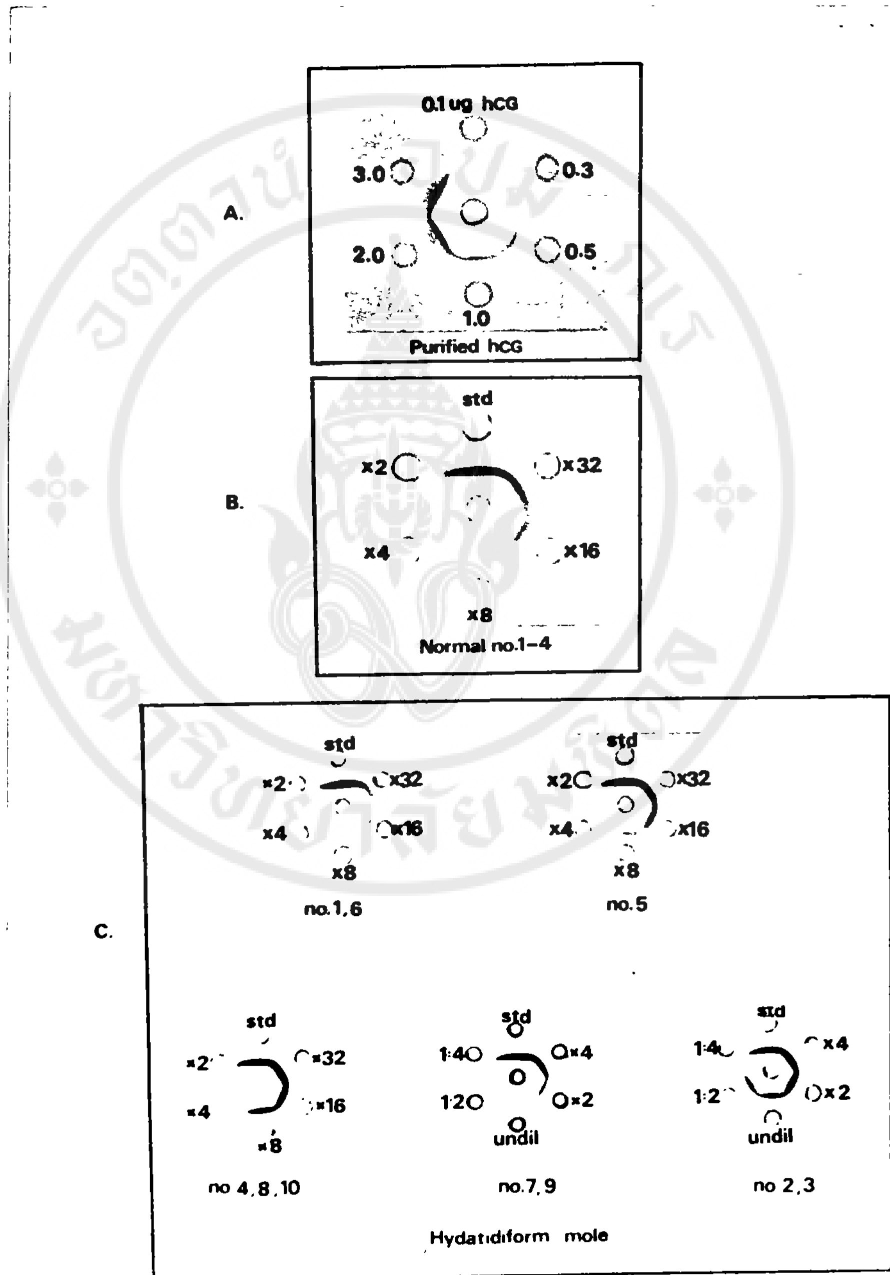

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Table 1 Levels of hCG and protein in urine from women with normal and molar pregnancies.

Patient number	hCG (approx concn μg/ml)	Protein (mg/ml)	hCG/protein %
Normal pregnancy: 1	8.93	2.45	0.36
2	8.93 (6.7)[a]	2.70	0.33
3	8.93	3.45	0.26
4	8.93	2.75	0.32
Molar pregnancy: 1	4.47	3.25	0.14
2	142.86	3.10	4.60
3	142.86 (144.5)[a]	2.28	6.27
4	17.86	2.50	0.71
5	8.93 (4.5)[a]	2.20	0.41
6	4.47	1.40	0.32
7	71.43	1.75	4.08
8	17.86	3.65	0.49
9	71.43	5.65	1.26
10	17.86	3.65	0.49

[a]values in parentheses represent the level of hCG determined by RIA

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The original concentration of urinary hCG was calculated from

$$\frac{0.5}{7} \times \frac{1000}{c} \quad \mu g/ml$$

whereas $\frac{0.5}{7}$ = detectable amount of hCG (0.5 μg) in 7 μl

c = folds of concentration

normal pregnancy:	no. 1-4 were concentrated		8	folds
molar pregnancy:	no. 1,6	"	16	"
	no. 5	"	8	"
	no. 4,8,10	"	4	"
	no. 7,9	"	1	"
	no. 2,3	"	½	"

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mobilities of normal and molar hCG using Tandem-CIE as described in section 3. The interdistances between two precipitin peaks of hCG were measured following the electrophoresis.

When standard hCG was compared together, the interdistances were not different as shown in Fig. 5A indicating their equal mobilities. Similar comparison of hCG from normal pregnant urine (no. 1) also showed no difference in their electrophoretic mobilities (Fig. 5B). The continuous precipitin lines of the two peaks indicated the immunochemical identity of hCG.

Subsequently, the electrophoretic mobilities between normal (no. 1) and individual molar hCG were compared (Fig. 6, no. 1-10). Their interdistances were measured from the glass plate as indicated in the picture (Fig. 6) and summarized in Table 2. Each sample was done in duplicate to assure that the results were reproducible. From the results, the mobility of molar hCG was slower than that of the normal, as shown by the longer interdistances between the two peaks on the right plate than those on the left. It indicated the less negative charge of hCG from molar pregnancy. The variation of the interdistances among the patients was probably due to variation of negative charge of hCG from each patient. In addition, the peaks of molar hCG frequently showed broad and asymmetric shape (Fig. 6 , no. 3-10). These characteristics could be due to diffusion of molar hCG during the first dimensional electrophoresis. The continuous precipitin lines of the two peaks indicated the immunochemical identity of hCG from both sources. One interesting point was that the presence of an additional peak of hCG found in molar urines no. 3,5.

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Figure 5 Reproducibility of Tandem-CIE.

A. Crude hCG (Sigma) containing 0.5 μg/7 μl of hCG was applied to each well (S) of the two glass plates which were electrophoresed simultaneously in horizontal direction at 10V/cm for 75 min followed by the second dimensional electrophoresis in vertical direction at 5V/cm for 18-20 h in the presence of anti-hCG_D (50 μl/plate). The immunoprecipitate was shown in dark line after staining and photography.

B. Tandem-CIE of urine from normal pregnancy (N) containing 0.5 μg/7 μl of hCG. The experimental conditions were identical to Fig. 5A except the first dimension was electrophoresed for 60 min instead of 75 min.

- cathode

+ anode

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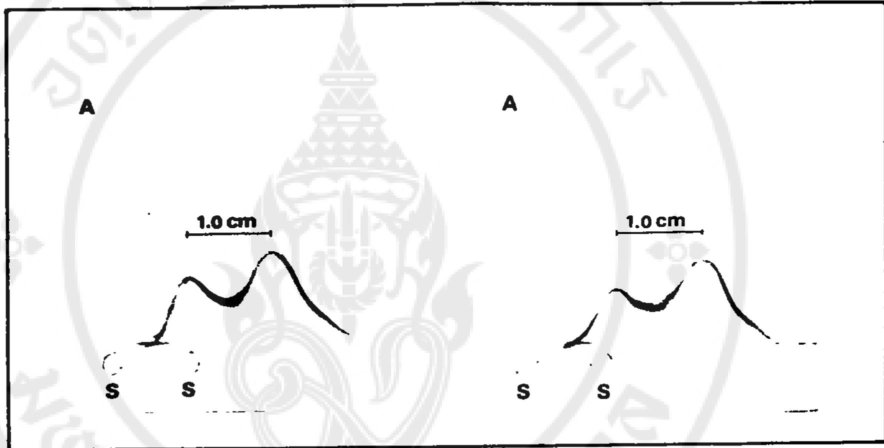

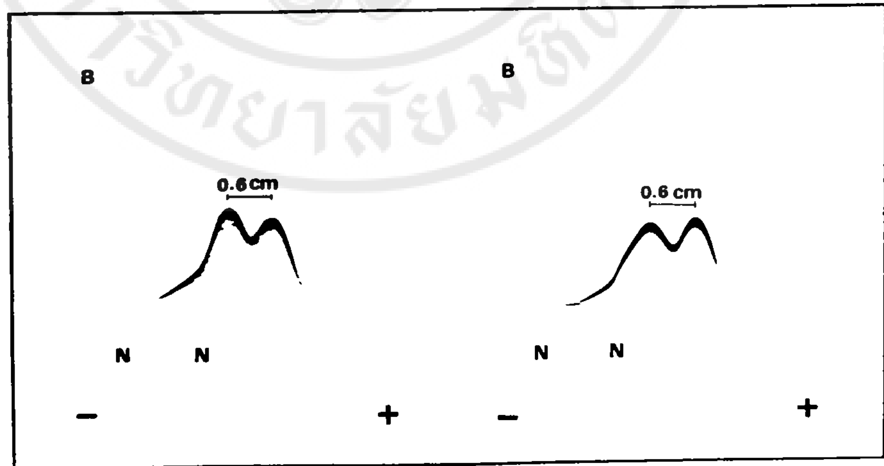

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Figure 6 Comparison of Tandem-crossimmunoelectrophoretic mobility between normal and molar urinary hCG.

Concentrated urines (0.5 μg/7 μl of hCG) from normal pregnant woman (N) and from each molar patient (P) were applied to the wells as indicated. Other conditions were identical to Fig. 5B.

1-10. indicated patient number.

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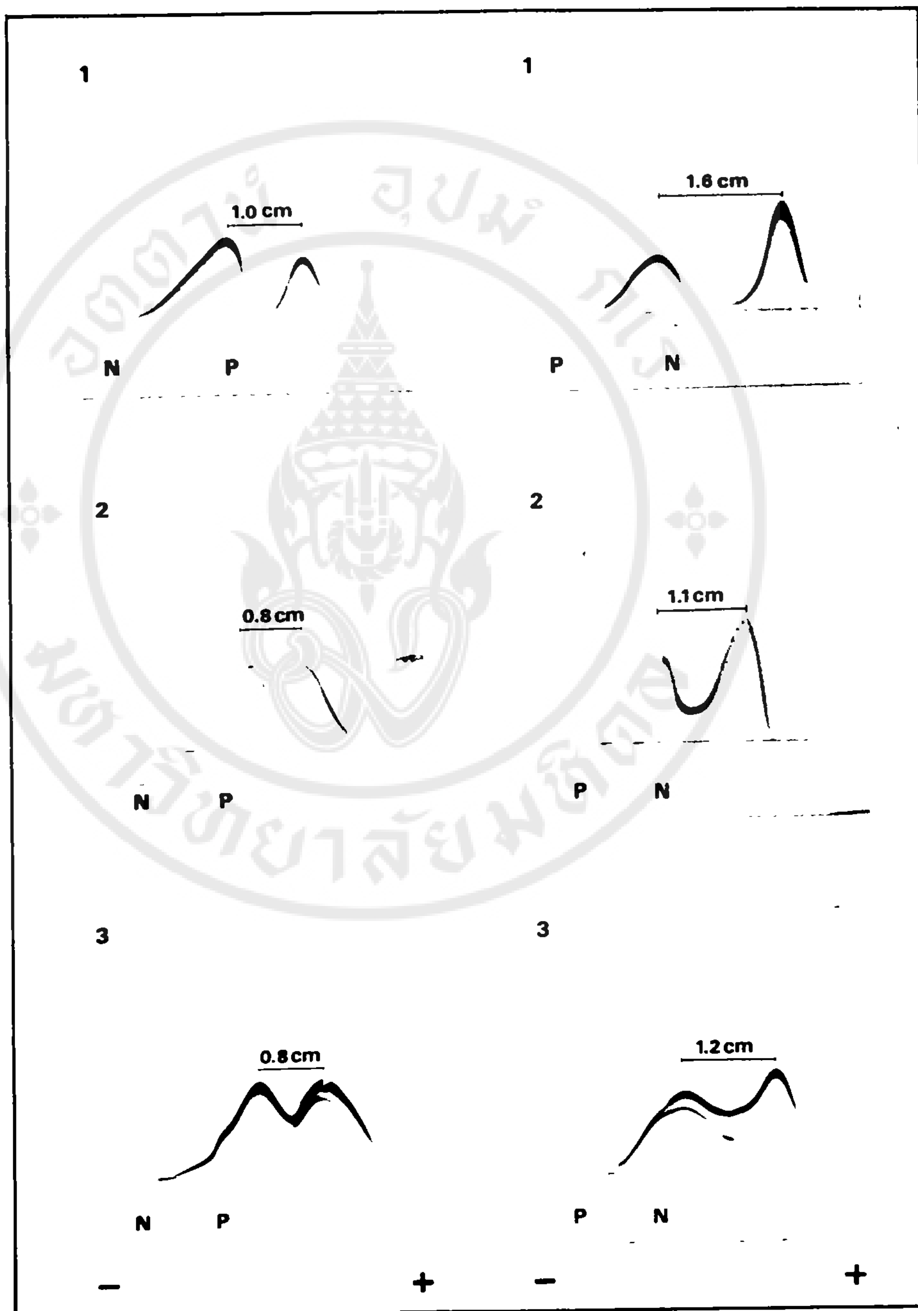

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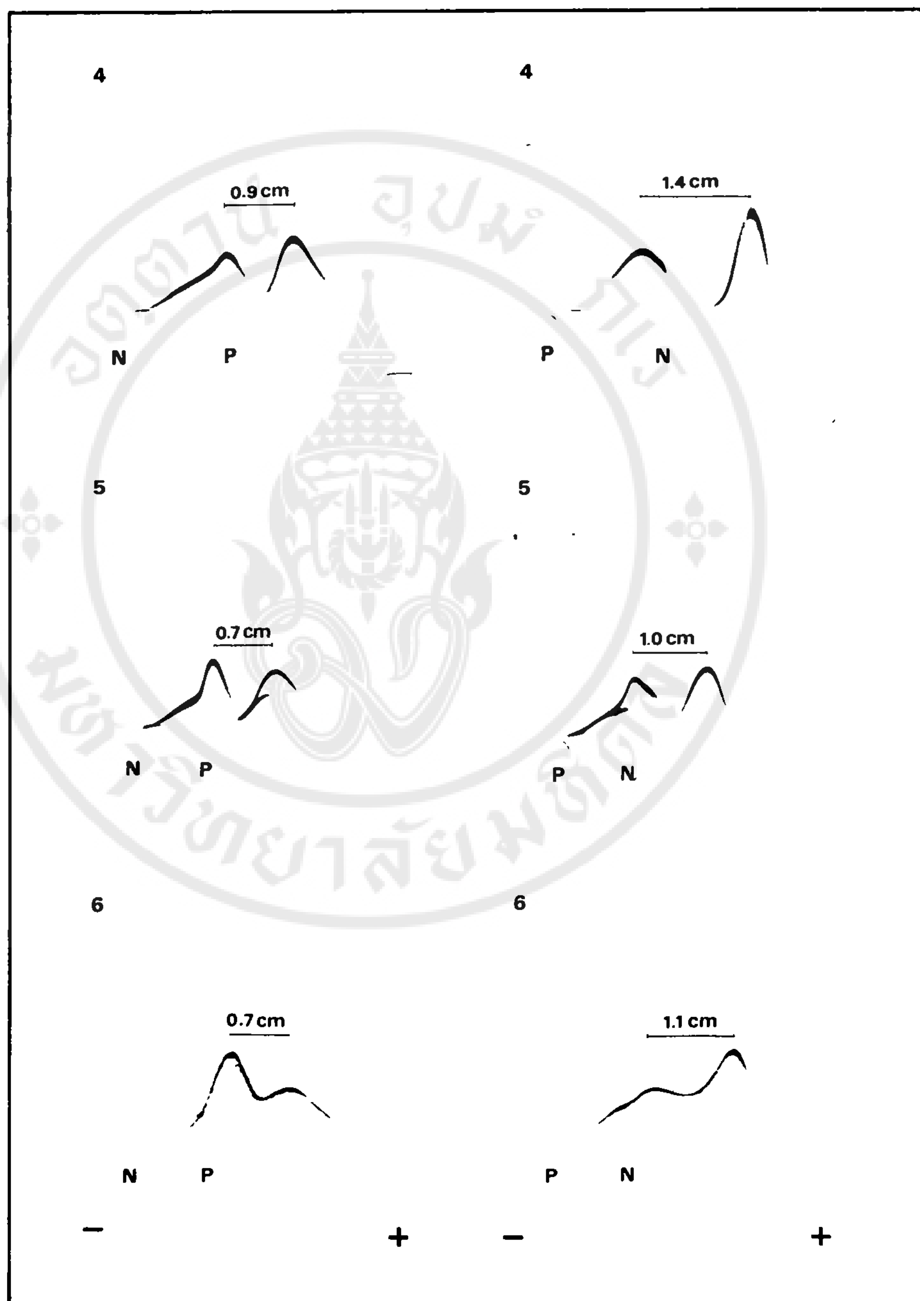

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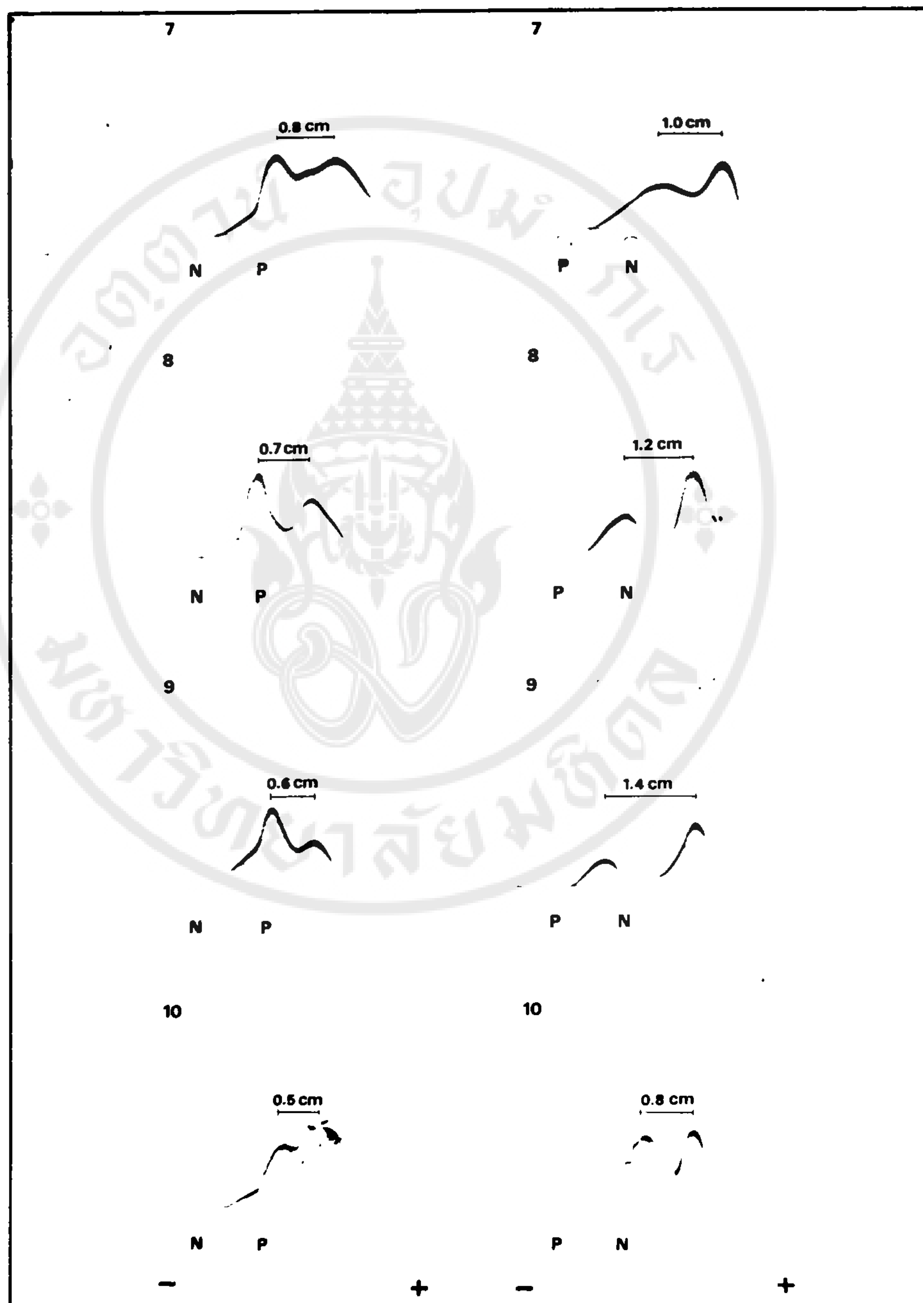

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Table 2 Interdistances from Tandem-CIE between normal and molar hCG obtained from Figs. 5 and 6.

Patient number	Interdistances between two peaks (cm)[a]	
	Left plate	Right plate
Standard	1.0	1.0
Normal pregnancy: 1	0.6	0.6
Molar pregnancy: 1	1.0	1.6
2	0.8	1.1
3	0.8	1.2
4	0.9	1.4
5	0.7	1.0
6	0.7	1.1
7	0.8	1.0
8	0.7	1.2
9	0.6	1.4
10	0.5	0.8

[a]The distances indicated were measured directly from the electrophoretic glass plates.

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From this study, the mobilities of molar hCG from all patients were obviously slower than those of the normal indicating less negative charge of the former.

3. Comparison of molecular size between normal and molar hCG

The results in previous section indicated that molar hCG used in the present studies might lack some terminal sugar residues. As a consequence, the molar hCG could be smaller in molecular size than the normal. Therefore, the following experiments were designed to distinguish the molecular size of molar hCG using SDS-PAGE followed by Western blotting.

3.1 SDS-PAGE of normal intact hCG and subunits

A mixture of known molecular weight proteins containing BSA (68K), H-chain of IgG (53K), ovalbumin (43K), L-chain of IgG (23K), myoglobin (17K) were subjected to 12% SDS-PAGE as described in section 4.1. Their molecular weights were plotted versus the relative mobilities (Fig. 8). The positions of crude hCG (Sigma, Fig. 7, lane 1) and its dissociated β and α subunits (Fig. 7, lane 2) electrophoresed simulataneously with the marker proteins were 2, 3.3 and 4.5 cm, respectively from the origin. The molecular weights of hCG, βhCG and αhCG estimated from standard curve (Fig. 8) were 52,000, 33,000 and 22,000, respectively.

Besides, serum collected from normal healthy men and urine from normal healthy nonpregnant women were also compared. Serum proteins and nonpregnant urinary proteins contain essentially albumin (mol wt 68K, Fig. 7, lanes3,4) and free from hCG (Berggard, 1970). Therefore, both of them were used as negative controls for further studies.

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Figure 7 Coomassie blue stain of hCG and subunits after SDS-PAGE.

The 1st lane was crude hCG (Sigma, 6 μg/20 μl of hCG).
The 2nd lane was 12 μg/40 μl of crude hCG dissociated into β and α subunits by 2-mercaptoethanol.
The 3rd lane was human serum (40 μg/5 μl of proteins).
The 4th lane was concentrated urine from nonpregnant women (125 μg/5 μl of proteins).
The 5th lane was marker protein: 10 μg each of BSA (68K), IgG (53K and 23K), myoglobin (17K) and 20 μg of ovalbumin (43K).

Figure 8 Relationship between molecular weights of proteins and their mobilities in SDS-PAGE.

Molecular weights of marker proteins in Fig. 7 were plotted versus their relative mobilities. The molecular weights of hCG and subunits were estimated from this curve.

$$\text{The relative mobility} = \frac{\text{The distance of protein migration}}{\text{The distance of tracking dye}}$$

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FIGURE 7

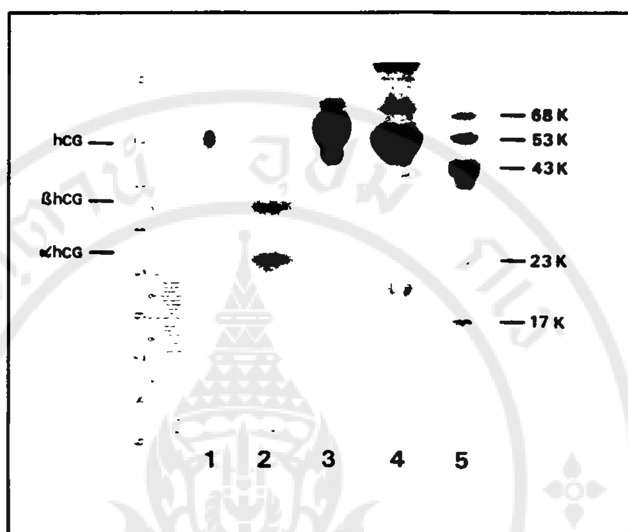
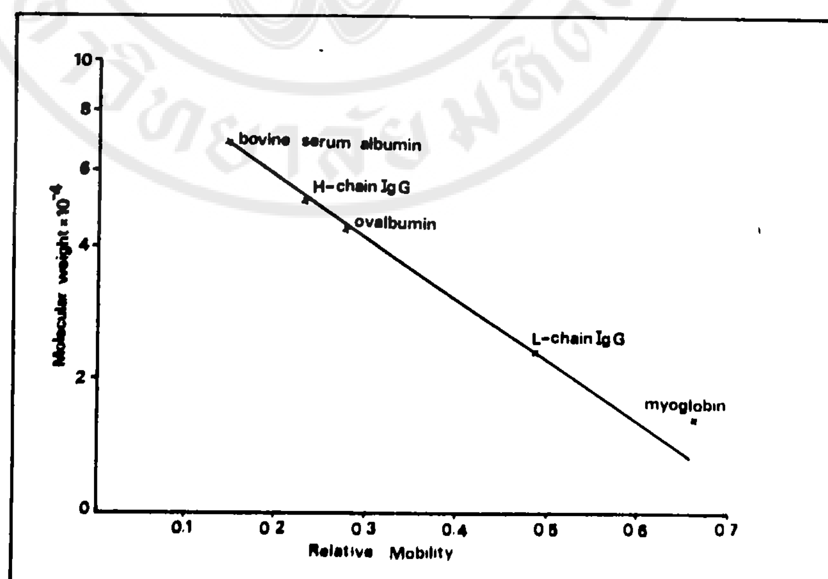


FIGURE 8 STANDARD CURVE FOR DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE



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3.2 <u>Transfer efficiency of hCG subunits from polyacrylamide gel to the nitrocellulose paper</u>

SDS-PAGE provides high resolution for separating proteins which their molecular weights are different but the proteins are not accessible to further investigation due to the limit pore size of the gel (Towbin *et al.*, 1979). To solve this problem, the proteins were transferred from the gel onto a nitrocellulose paper and analyzed by immunochemical method.

HCG is composed of 2 dissimilar subunits. The abnormality of intact hCG might be derived from either or both subunits. Therefore, it is interested to investigate the molecular changes of the β and α subunits from molar hCG. Since the blotting of hCG has not been previously reported, the optimum condition for transferring β and α subunits from SDS-polyacrylamide gel must be established.

Crude hCG (Sigma, 12 μg/40 μl of hCG) was dissociated into β and α subunits and separated on SDS-PAGE. The details of the method were described in section 4.1. The marker proteins (BSA, H-chain of IgG, ovalbumin, L-chain of IgG, myoglobin) and serum proteins were used as positive controls of this process. Proteins in the gel were transferred to nitrocellulose sheets (pore size 0.45 μm) for 0,2,4,15 and 24 h. After the transfer, the remaining proteins on the gels and the proteins which had been transferred onto the nitrocellulose sheets were visualized by staining with amido black (as described in section 4.3). The results showed that under the indicated conditions, most of the serum proteins were partially transferred within 2 h of blotting (Fig. 9B). Increasing amount of the proteins were removed from the gel

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onto the nitrocellulose sheet at 4 h (Fig. 9C) and 15 h (Fig. 9D). Complete transfer was observed at 24 h as shown by no protein could be detected in the original gel after blotting (Fig. 9E). Besides, the rate of the proteins transferred depended on their molecular weights. Low-molecular-weight proteins (myoglobin, ovalbumin, β and α subunits of hCG) were completely transferred at 15 h but high-molecular-weight proteins (BSA, serum proteins) still remained in the gel (Fig. 9D). The results also showed that electrophoretic transfer was able to elute all the proteins from the SDS-gel to a nitrocellulose sheet without significant loss of resolution.

In comparison, the results indicated that subunits of hCG could be transferred from SDS-gel to a nitrocellulose sheet extensively within 2 h (Fig. 9B). Longer blotting time could lead to diffuse bands (Fig. 9E). The transfer was performed in all subsequent studies for either 2 h or 12 h.

3.3 Immunochemical detection of hCG and subunits on nitrocellulose sheets

Since hCG in urine sample would be used without purification in section 3.4, the immobilized hormone and subunits on the nitrocellulose paper must be detected by immunochemical staining. For this purpose, the specificity and sensitivity of the system were very important.

3.3.1 Specificity for detection of hCG and subunits

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The specificities of rabbit anti-hCG and anti-βhCG have been previously demonstrated (Hiranyavasit, 1983). On the other hand, anti-αhCG prepared during this study showed 1:4 of antiserum titer after

Figure 9 Blotting efficiency of hCG subunits from SDS-polyacrylamide gel to nitrocellulose papers.

The upper parts were proteins left in the gels stained with amido black after the blotting transfer for 0,2,4,15 and 24 h, respectively.

Corresponding nitrocellulose blots (A-E) shown in the lower parts were stained with amido black.

The 1st lane of each section was marker proteins: 10 μg each of BSA (68K), IgG (53K and 23K), myoglobin (17K) and 20 μg of ovalbumin (43K).

The 2nd lane was 12 μg/40 μl of crude hCG dissociated into β and α subunits by 2-mercaptoethanol.

The 3rd lane was human serum (40 μg/5 μl of proteins).

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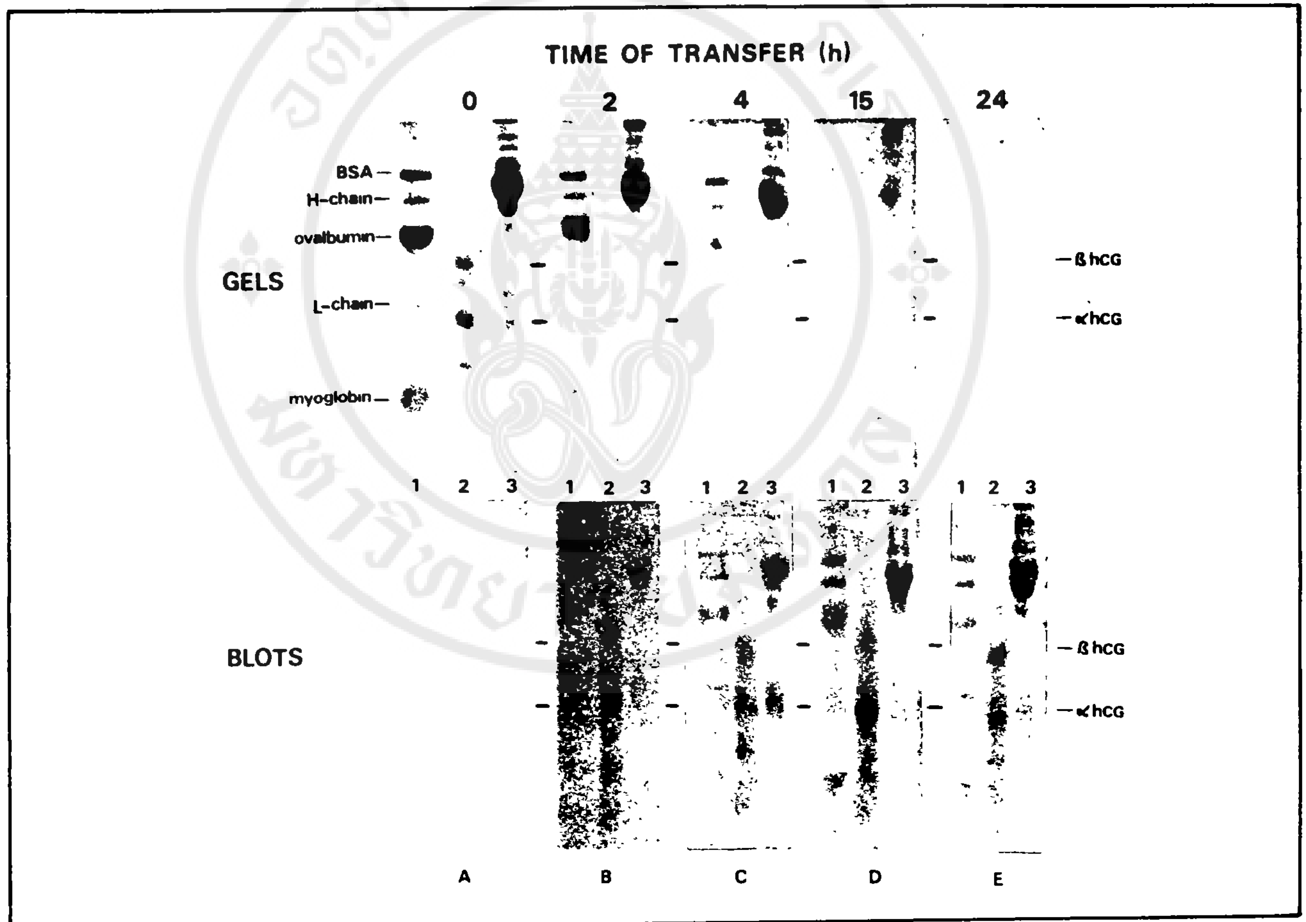

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5 weeks of immunization (Fig. 10B) compared to the negative control using rabbit preimmuned serum (Fig. 10A). There was no cross-reaction of anti-αhCG to βhCG as illustrated by the absence of the immunoprecipitate (Fig. 10C).

Using the above mentioned antisera, detection of intact hCG and subunits by immunofluorescence technique was illustrated in Fig. 11. Crude hCG (Sigma) containing 0.25, 0.75, 1.25 and 2.5 μg of hCG in 10 μl were dissolved in sample buffer without or with 2-mercaptoethanol and subjected separately to SDS-PAGE for 3 h. The proteins were subsequently transferred to nitrocellulose sheets followed by detection with specific antiserum and FITC-conjugated anti-rabbit IgG.

When the intact hCG was incubated with anti-hCG_D, it showed 1 band at 2 cm from the origin (Fig. 11A). The fluorescent intensity increased from 0.25 μg to 2.5 μg, respectively. No band was observed when human serum was used (Lane 1) which indicated the specificity of the system. The position of the band on the nitrocellulose sheet correlated well with that of intact hCG on the previous gel (Fig. 7, lane 1). When the intact hCG was dissociated into subunits and incubated with anti-βhCG, there was 1 band at 3.3 cm from the origin (Fig. 11B) whereas incubation with anti-αhCG resulted to another single band at 4.5 cm from the origin (Fig. 11C). The bands observed were presumably β and α hCG respectively whose mobilities were correlated with those presented on the SDS-PAGE (Fig. 7, lane 2).

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These results indicated that hCG, β and α subunits could be transferred from SDS-polyacrylamide gel to the nitrocellulose paper without complete loss of their immunochemical properties.

3.3.2 Sensitivity for detection of hCG and subunits

As little as 0.25 μg of hCG was required for detection of intact hCG (Fig. 11A, lane 2) and its β subunit (Fig. 11B, lane 2) but at least 0.75 μg of hCG was required for detection of α subunit (Fig. 11C, lane 3). However, the most intense band was observed at 2.5 μg of hCG (lane 5). Therefore, urine samples were concentrated to obtain approximately 3 μg of hCG for further studies.

3.4 Detection of molecular size of molar hCG and subunits

In order to compare the molecular size between normal and molar hCG, urines from both sources were analyzed on SDS-polyacrylamide gel followed by blotting using the same condition as described in section 3.3.

Concentrated urines (3 μg/10 μl of hCG) from 4 normal pregnant women and 10 molar patients (prepared as described in section 1) were subjected to SDS-PAGE for 3 h. The results were illustrated in Fig. 12. The amounts of total proteins in each lane were different in order to obtain 3 μg/10 μl of hCG which previously shown to be suitable (Fig. 11, lane 5). Although 3 μg of standard hCG could be seen clearly, the amount of urinary hCG was hardly identified and overwhelmed by other proteins in the urine (Fig. 12).

When the first gel which contained 2 normal pregnant women (no. 1-2) and 5 patients with molar pregnancy (no. 1-5) were blotted for 2 h, incubated with rabbit anti-hCG_D and detected by FITC-conjugated anti-rabbit IgG (Fig. 13A) or peroxidase-conjugated anti-rabbit IgG (Fig. 13B), molar hCG from each sample showed 1 major band at the same

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Figure 10 Titer and specificity of rabbit anti-αhCG.

Ouchterlony double immunodiffusion of rabbit preimmuned serum (A) or rabbit αhCG antiserum (B) against purified αhCG.

The central well contained 1 μg of purified αhCG. The peripheral wells contained 7 μl of 2-fold-dilution of either rabbit preimmuned serum (A) or rabbit αhCG antiserum after 5 weeks of immunization (B).

C. Specificity of rabbit anti-αhCG by immunoelectrophoresis. The upper and lower wells contained 1 μg of either purified α or β hCG, respectively. The electrophoresis was performed at 15V/cm for 3 h. The trough was cut and filled with 70 μl of rabbit anti-αhCG. The gel was incubated for 18-24 h and staining.

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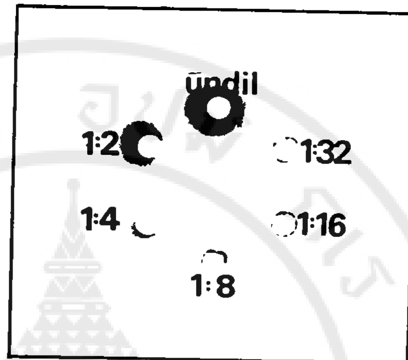
A.



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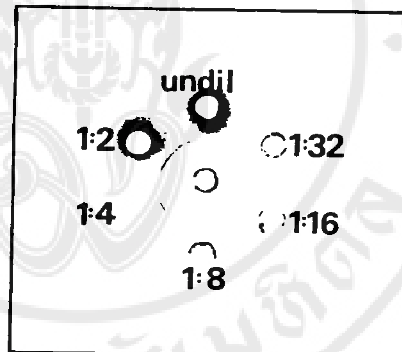

C.

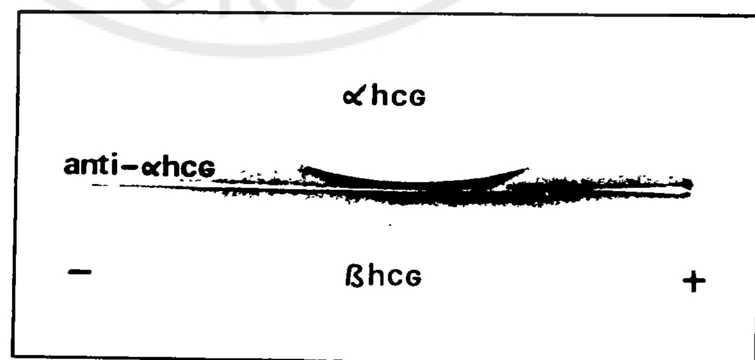

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Figure 11 Immunofluorescence detection of hCG and subunits on nitrocellulose papers.

The 1st lane of each section were negative controls using human serum (40 μg/5 μl of proteins).

The other lanes (2-5) were crude hCG (Sigma) containing 0.25, 0.75, 1.25 and 2.5 μg of hCG in 10 μl. The proteins were dissolved in sample buffer without 2-mercaptoethanol (A) or with 5% 2-mercaptoethanol (B,C) and subjected to 12% SDS-PAGE for 3 h. The proteins were blotted for 2 h on nitrocellulose papers which subsequently incubated with anti-hCG_D (A), anti-βhCG (B) and anti-αhCG (C) at dilution 1:10. The immune complex was detected by fluorescein-conjugated anti-rabbit IgG (dilution 1:20).

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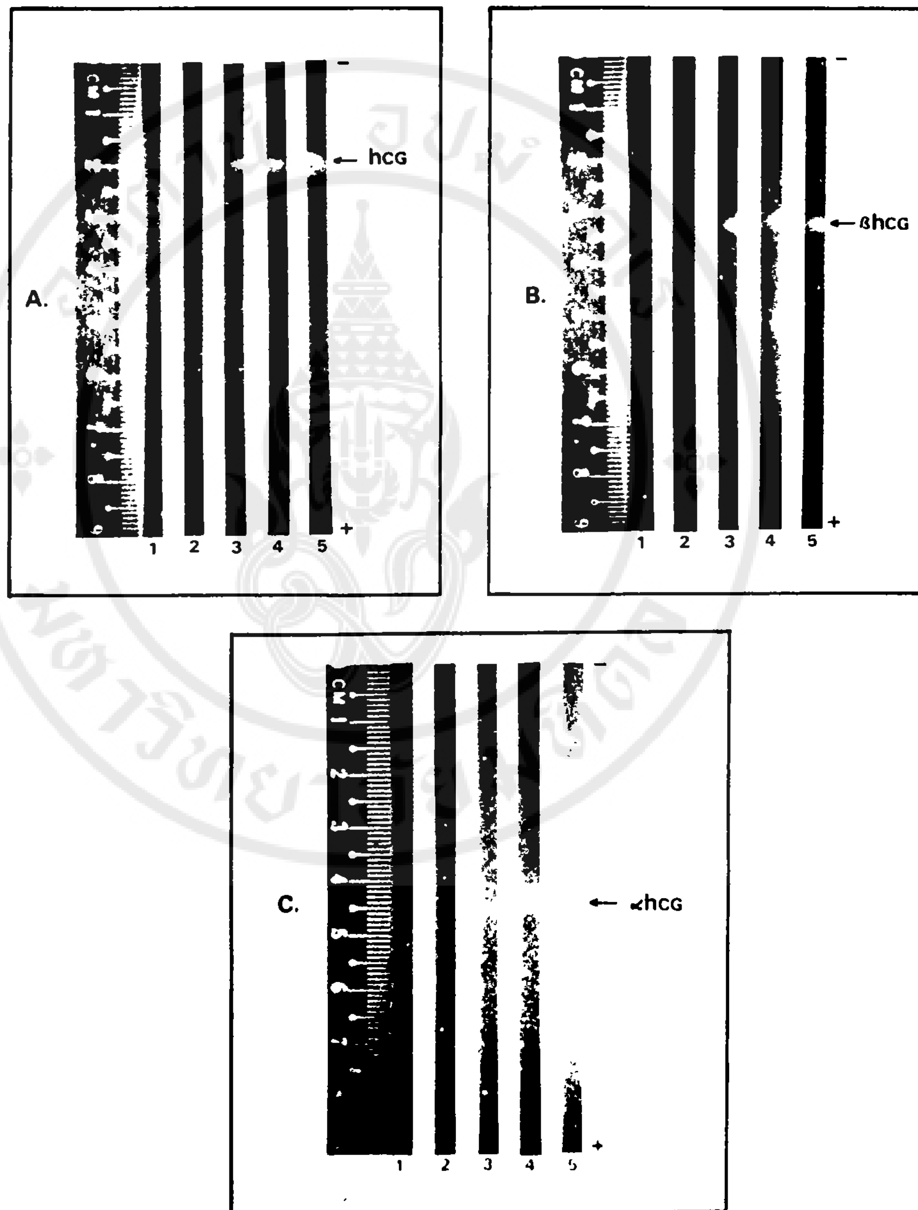

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position as that from the normal (2 cm from the origin). The apparent molecular weight of molar hCG calculated from the standard curve in Fig. 8 was about 52,000 which was undistinguishable from the normal hCG (Fig. 13). In some patients, an additional band of molecular weight 43,000 was detected (Fig. 13, molar patient no. 2,3,5). One interesting point was the appearance of a low-molecular-weight immunoreactive hCG-like substance (apparent mol wt 17,000) in both normal and molar urines. The same results were obtained whether using anti-hCG_D (Fig. 13) or different batch of the antiserum (anti-hCG_W, data not shown). None of the bands was observed when urine from nonpregnant women was used (Fig. 13, lane 1) which indicated the specificity of the system.

When molar hCG was dissociated into subunits, incubated with anti-βhCG and detected by either immunofluorescence (Fig. 14A) or immunoperoxidase (Fig. 14B) method. The results showed that molar hCG from each patient (no. 1-5) contained not only a single band of molecular weight 33,000 at 3.3 cm from the origin, but also a second band moving faster near the position of αhCG, 4.4 cm from the origin (apparent mol wt 23,000). The additional band was observed in 3 from 5 patients (no.2,3,5).

Detection by immunofluorescence has the disadvantage of giving the unstable product and requiring ultraviolet accessories for observation. Alternatively, peroxidase-conjugated antibody is more sensitive and give more stable product than the fluorescein-conjugated one (Bergquist *et al.*, 1976). Therefore, immunoperoxidase method was chosen in the following studies.

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When molar hCG was dissociated into subunits and detected with anti-αhCG followed by peroxidase-conjugated antibodies. Molar hCG from

all patients showed 1 band at the same position as that from the normal (4.5 cm from the origin, apparent mol wt 22,000) indicating no difference in molecular size of αhCG from both sources (Fig. 15).

As mentioned earlier, the amounts of proteins electrophoretically transferred increased with time (see results in section 3.2). In addition, low-molecular-weight proteins left the gel easier than high-molecular-weight proteins did. From previous experiments, blotting time of 2 h might cause the incomplete recovery of high-molecular-weight proteins. To confirm the previous results, two modifications were made. Firstly, the running time of SDS-PAGE was increased to 3½ h in order to obtain better separation of the proteins. Secondly, blotting transfer was performed longer than 2 h to obtain complete recovery of the proteins.

Immunoperoxidase detection of urinary hCG and subunits from normal pregnant women (no. 1-2) and molar patients (no. 1-5) after increasing the running time of SDS-PAGE to 3½ h and blotting time to 12 h were shown in Fig. 16. The results were similar to those from Figs.13-15 except that increasing amount of proteins were obtained from 12 h of blotting.

Immunoperoxidase detection of urinary hCG and subunits from normal pregnant women (no. 3-4) and molar patients (no. 6-10) were shown in Fig. 17. Molar urine from each patient showed 1 major band of hCG at the same position as that from the normal (Fig. 17A). However, urine from the other three patients (no. 7,9,10) showed an additional band moving slightly faster than the normal. A low-molecular-weight immunoreactive hCG-like substance (apparent mol wt 17,000) was still found in both normal and molar urines.

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Immunoperoxidase detection of the subunits from both sources were compared to each other. The results demonstrated that the β subunit of molar hCG from the three patients (no. 7,9,10) was composed of another band moving faster near the position of α subunit (Fig. 17B). The α subunit from all patients showed only single band at the position identical to the normal which indicated that urinary α subunit from both sources was equal in molecular size (Fig. 17C).

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Figure 12 SDS-PAGE of urinary proteins from women with normal and molar pregnancies.

The 1st lane was marker proteins: 10 μg each of BSA (68K), IgG (53K and 23K), myoglobin (17K) and 20 μg of ovalbumin (43K).

The 2nd lane was crude hCG (Sigma, 3 μg/10 μl of hCG).

The other lanes were concentrated urine samples (3 μg/10 μl of hCG) from 4 normal pregnant women and 10 patients with molar pregnancy.

The proteins were subjected to 12% SDS-PAGE with a constant current of 20 mA per gel for 3 h and visualized by staining with coomassie brilliant blue R.

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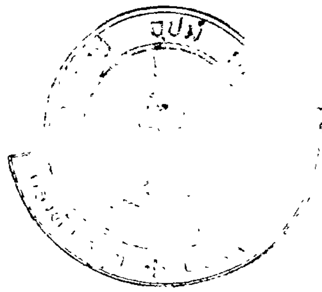

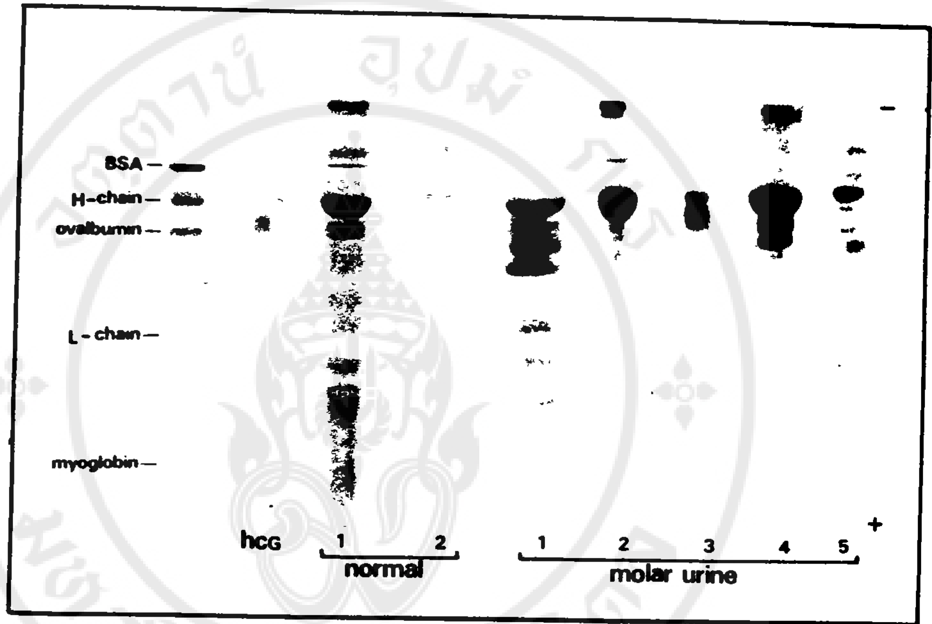

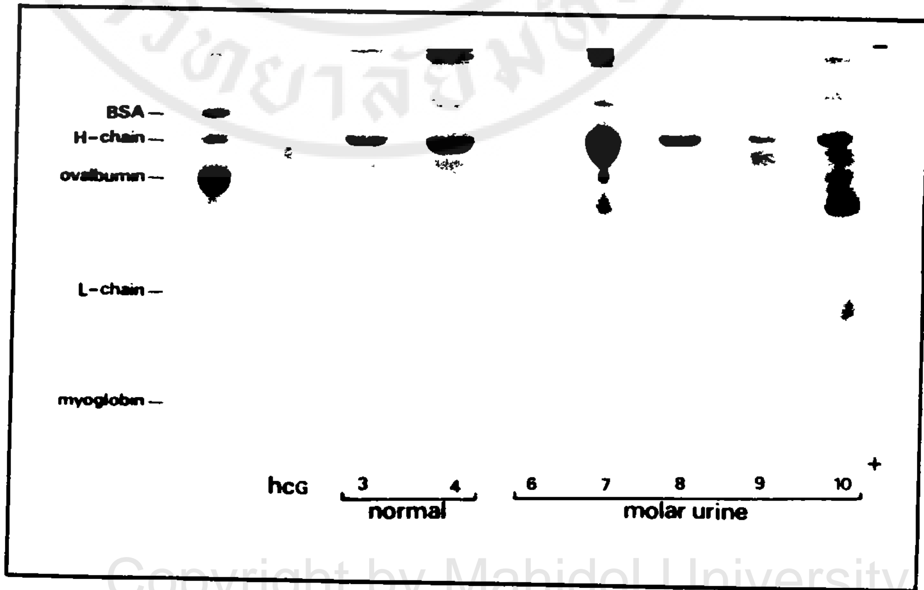

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Figure 13 Comparison of molecular size between normal and molar urinary intact hCG by SDS-PAGE.

The hormones were detected by anti-hCG_D followed by either FITC-conjugated anti-rabbit IgG (A) or peroxidase-conjugated anti-rabbit IgG (B).

The 1st lane (C) was concentrated urine from nonpregnant women (125 μg/5 μl of proteins).

The 2nd lane (S) was the positive control using crude hCG (3 μg/10 μl of hCG).

The other lanes were concentrated urine samples (normal no. 1-2, molar no. 1-5) containing 3 μg/10 μl of hCG.

The proteins were subjected to SDS-PAGE for 3 h and blotted for 2 h on nitrocellulose papers which subsequently incubated with rabbit anti-hCG (dilution 1:10). The immune complex was detected by either fluorescein-conjugated anti-rabbit IgG (dilution 1:20) or peroxidase-conjugated anti-rabbit IgG (dilution 1:100) for 2 h.

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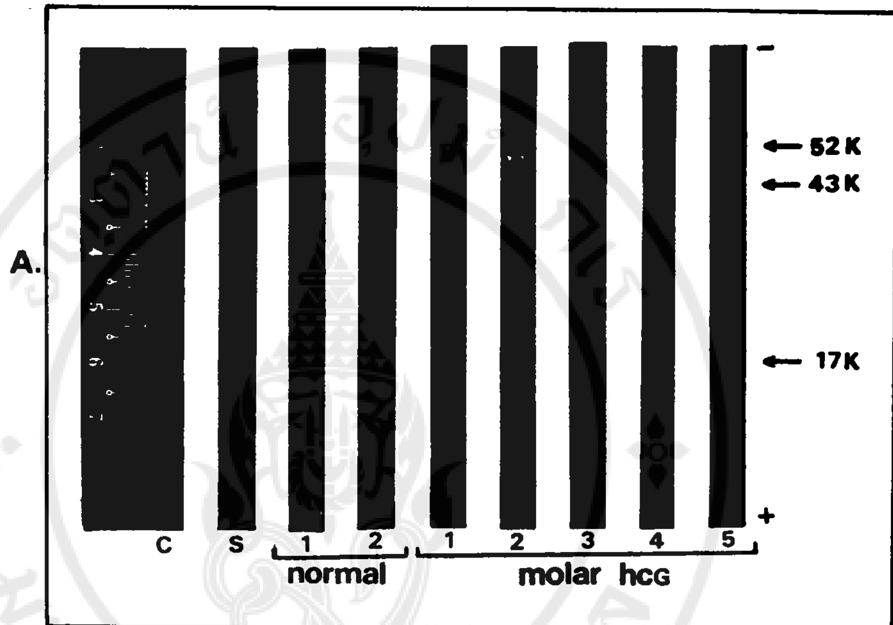

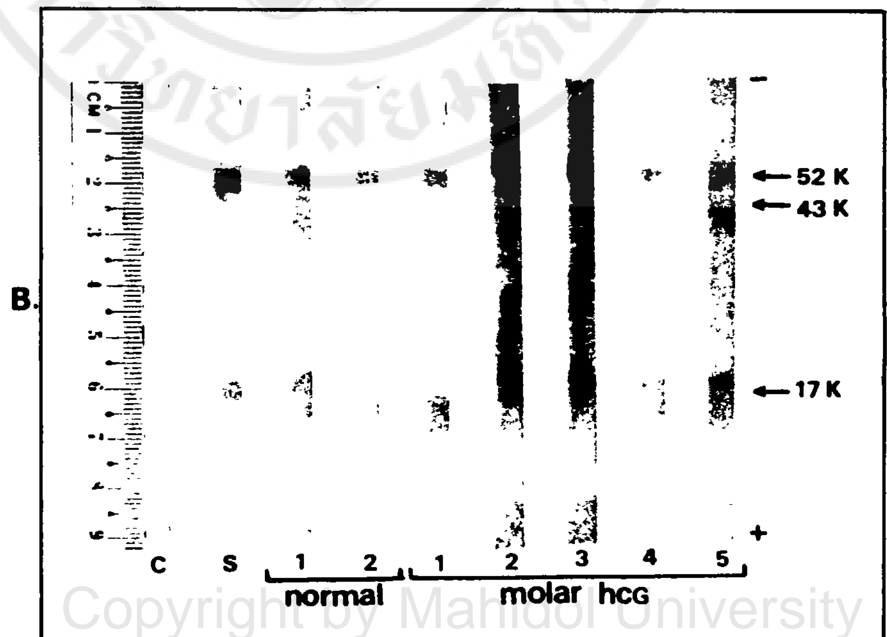

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<u>Figure 14</u> Comparison of molecular size between normal and molar urinary βhCG by SDS-PAGE

The hormones were detected by anti-βhCG followed by either FITC-conjugated anti-rabbit IgG (A) or peroxidase-conjugated anti-rabbit IgG (B).

The 1st lane (C) was concentrated urine from nonpregnant women (125 μg/5 μl of proteins).

The 2nd lane (S) was the positive control using crude hCG (6 μg/20 μl of hCG).

The other lanes were concentrated urine samples (normal no. 1-2, molar no. 1-5) containing 6 μg/20 μl of hCG.

The proteins were dissociated with 5% 2-mercaptoethanol and subjected to SDS-PAGE for 3 h. The proteins were blotted for 2 h on nitrocellulose papers which subsequently incubated with rabbit anti-βhCG (dilution 1:10). The immune complex was detected by either fluorescein-conjugated anti-rabbit IgG (dilution 1:20) or peroxidase-conjugated anti-rabbit IgG (dilution 1:100) for 2 h.

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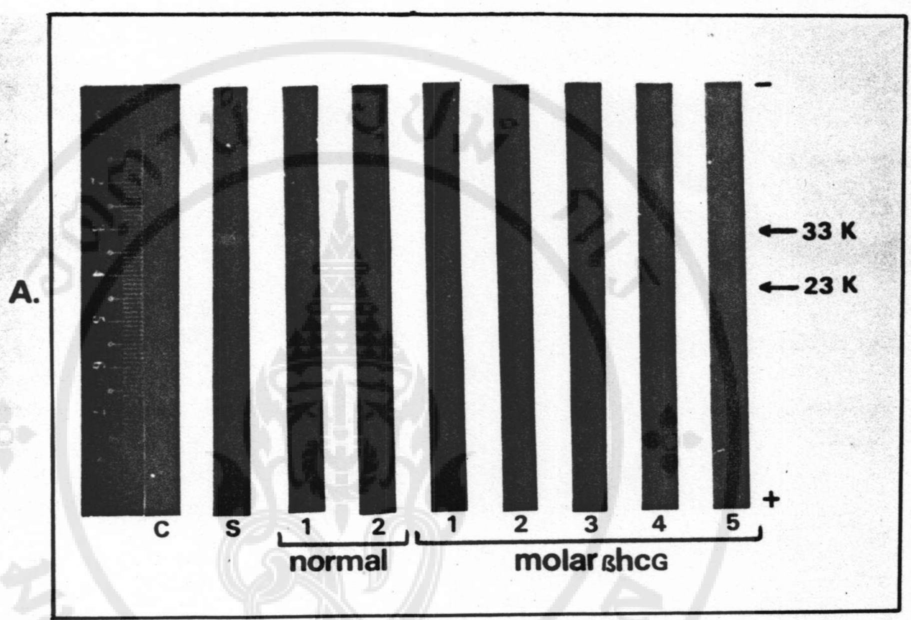

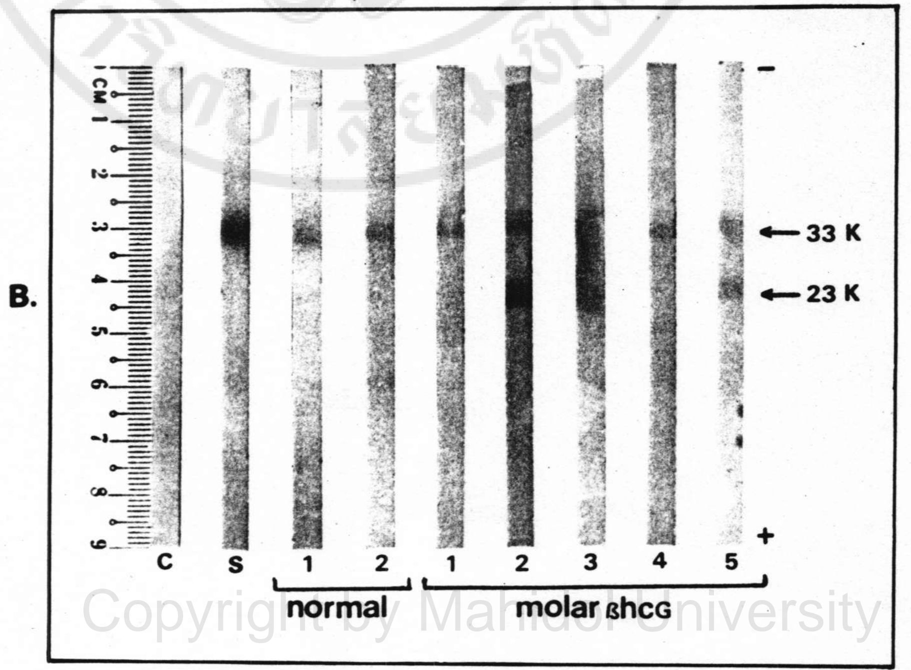

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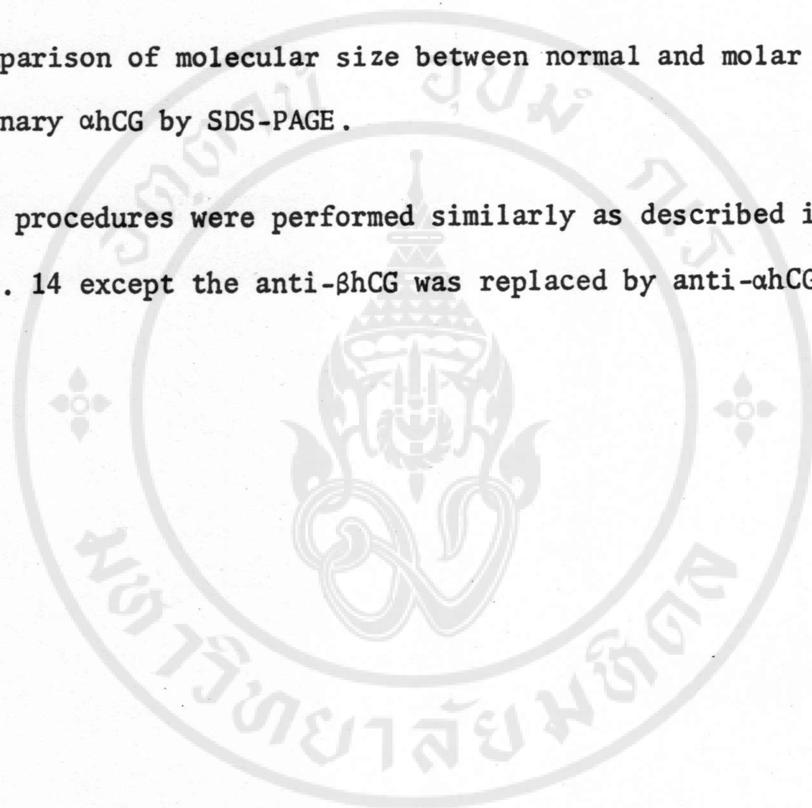
<u>Figure 15</u> Comparison of molecular size between normal and molar urinary αhCG by SDS-PAGE.

The procedures were performed similarly as described in Fig. 14 except the anti-βhCG was replaced by anti-αhCG.



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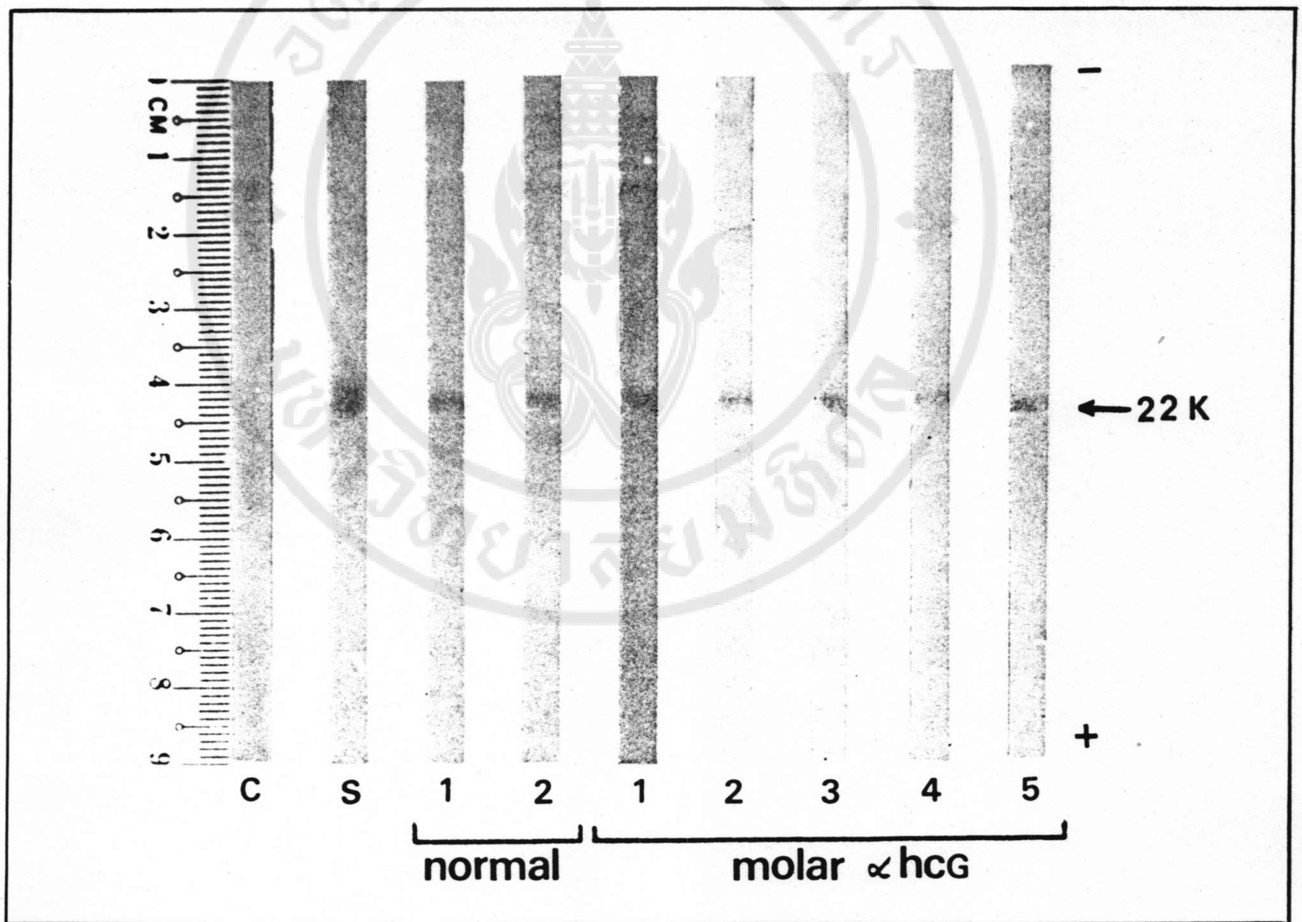

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Figure 16 Effects of increasing electrophoretic time during SDS-PAGE and blotting on the comparison of molecular size of urinary hCG and subunits.

The 1st lane of each section (C) were negative controls using concentrated urine from nonpregnant women (125 μg/5 μl of proteins).

The 2nd lane of each section (S) were positive controls using crude hCG (Sigma, 3 μg/10 μl of hCG).

The other lanes were concentrated urines from normal pregnant women (no. 1-2) and patients with molar pregnancy (no. 1-5).

Concentrated urines (3 μg/10 μl of hCG) were dissolved in sample buffer without 2-mercaptoethanol (A) and concentrated urines (6 μg/20 μl of hCG were dissolved in sample buffer with 5% 2-mercaptoethanol (B,C). The proteins were subjected to 12% SDS-PAGE for 3½ h and blotted for 12 h on nitrocellulose papers which subsequently incubated with anti-hCG_D (A), anti-βhCG (B) and anti-αhCG (C) at dilution 1:10. The immune complex was detected by peroxidase-conjugated anti-rabbit IgG (dilution 1:100).

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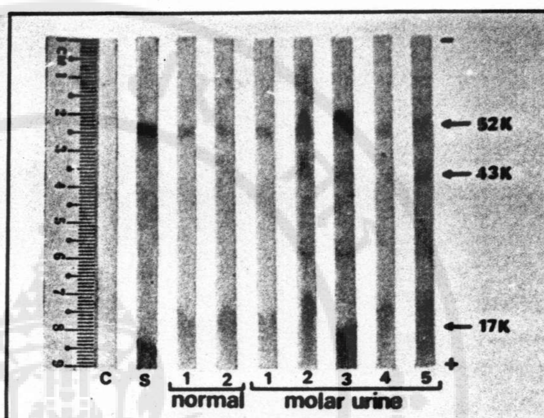
A. INTACT HCG



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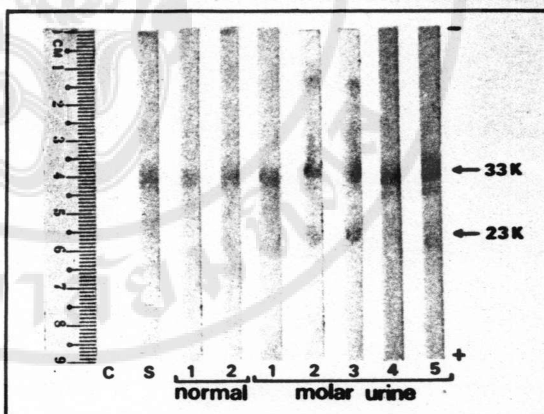

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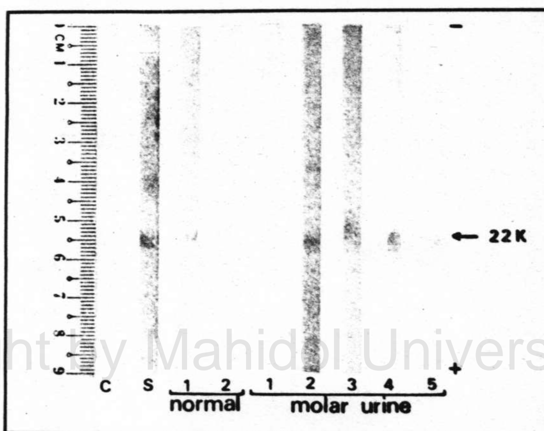

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Figure 17 Effects of increasing electrophoretic time during SDS-PAGE and blotting on the comparison of molecular size of urinary hCG and subunits (continued).

The 1 st lane of each section (C) were negative controls using concentrated urine from nonpregnant women (125 μg/ 5 μl of proteins).

The 2nd lane of each section (S) were positive controls using crude hCG (3 μg/10 μl of hCG).

The other lanes were concentrated urine samples from normal pregnant women (no. 3-4) and patients with molar pregnancy (no. 6-10). The urine samples were performed the same as described in Fig. 16.

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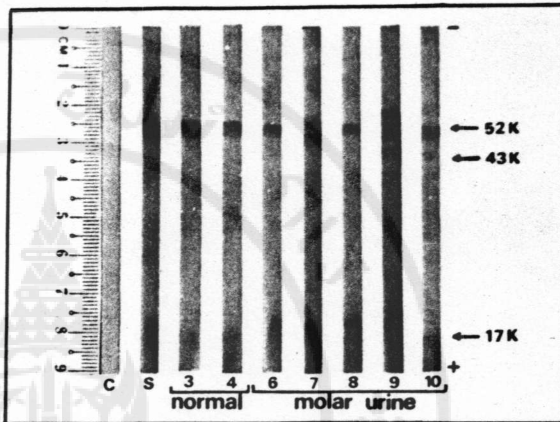
A. INTACT HCG



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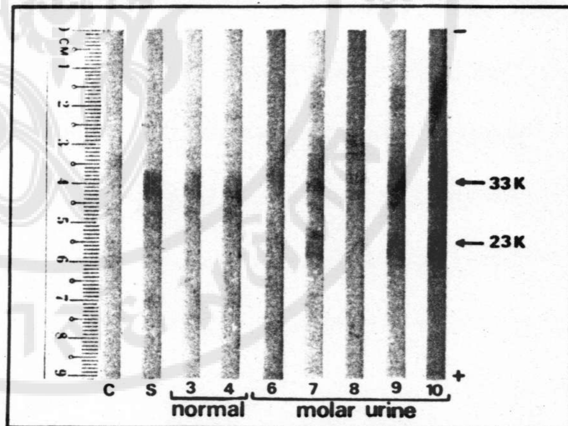
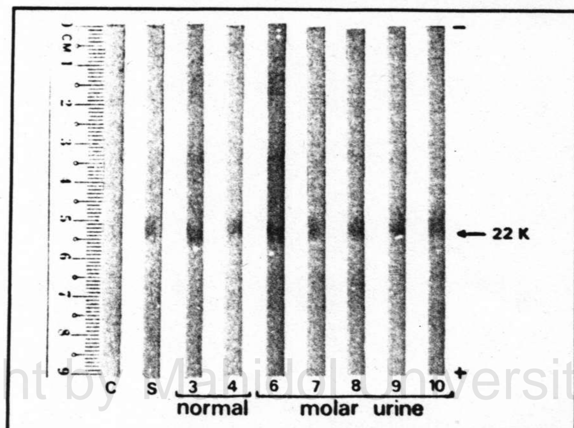


C. ∝ SUBUNIT



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DISCUSSION

In this study, molecular charge and molecular size of urinary hCG from normal pregnant women and from patients with hydatidiform mole were investigated. The information obtained may lead to better development of early diagnosis and rapid treatment of the hydatidiform mole disease.

1. Level of hCG in urine of pregnant women

It was found from qualitative measurement of hCG from 4 normal pregnant women and from 10 patients with hydatidiform mole that most of the patients excreted hCG in higher level than the normal (Table 1). The results agreed with the previous reports of Goldstein *et al.* (1974) and Hiranyavasit (1978) by using radioimmunoassay (RIA). In order to avoid using of radioactive compound which is perceived hazardous and required an expensive nuclear counting apparatus, an alternative non-isotopic method (Ouchterlony double immunodiffusion) was used instead in the present study. The immunodiffusion method is disadvantageous of lower sensitivity and accuracy than the RIA (Williams & Chase, 1971; Spellacy *et al.*, 1974). From this experiment, the errors mostly occurred in the steps of making dilution and approximate calculation. Comparison between the urinary hCG level as measured by Ouchterlony double immunodiffusion and by RIA showed some differences (Table 1). However, the normal level of hCG at the first trimester of gestation obtained in this study was still in the normal range of

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0.8-12 μg/ml (McCarthy *et al.*, 1964).

Despite clearly exhibiting clinical symtoms, hCG levels varied among the molar patients in this study and were not essentially always higher than normal (Table 1). The results agreed with previous observation by other investigators (Bagshawe & Wilde, 1965). On the other hand, variations of hCG and protein concentrations in the urines might also depend on other factors such as water content in the urine, complication and development of the disease. The unusually low level of hCG found in 3 molar patients (no. 1,5,6) might be resulted from the chemotherapy which has been previously reported to effect the hCG level (Than *et al.*, 1981; Lee *et al.*, 1982). Nevertheless, all urine samples were collected from the patients whose clinical diagnosis was confirmed to be hydatidiform mole.

2. Difference in molecular charge between normal and molar hCG

The carbohydrate portions have been proved to be important in the action of many glycoproteins. Removal of the carbohydrate results to decrease in biological activity of glycoprotein hormones (Dufau, 1971; Kalyan & Bahl, 1983). Prasad *et al.* (1979) have demonstrated three forms of rat α-lactalbumin which their amino acids are similar but different in the number of sialic acid residues. Removal of sialic acid reduces the negative charge and the electrophoretic mobility of this glycoprotein. Van Hell *et al.* (1966) have also found that electrophoretic mobilities of hCG from different preparations are variable but parallel to sialic acid content in the hormone. Since molar hCG has been reported to lack some terminal sugar residues especially sialic acid

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(Choy *et al.*, 1979; Imamura, 1980), the molecular charge of molar hCG was, therefore, investigated in the present study by using Tandem-CIE.

Since the isoelectric point of intact hCG is 4.3-6.6 (Nwokoro *et al.*, 1981), therefore, hCG moves from cathode to anode during electrophoresis in Fig. 5. Comparison of electrophoretic mobilities between normal and molar hCG indicated that the molar hCG from all patients moved slower than the normal (Fig. 6 and Table 2) which implied lower negative charge of the former. The reproducibility of the system was shown in Fig. 5 (A,B) suggesting that the difference in mobilities of hCG from both sources should not be an artefact. The variation of the interdistances obtained among the patients was probably due to the difference in negative charge of molar hCG from each patient. The continuous precipitin lines between normal and molar hCG indicated their immunochemical identity. The presence of another precipitin peak which occurred consistently in some particular molar hCG samples (no.3,5) was unexplainable. Clausen (1969) has suggested that a second line of precipitation or artefacts can occur in slowly precipitating system or inconstant temperature system.

Bahl and Marz (1974) have suggested that the protein moiety is solely responsible for the immunological properties of hCG. Choy *et al.* (1979) report that molar hCG has the same amino acid composition as that in the normal but lower in sugar content especially sialic acid. Therefore, the lack of sialic acid of molar hCG might result to the loss of the hormone negative charge.

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3. Western blotting of hCG and subunits

The loss of negative charge in molar hCG (in section 2) might occur from lacking of terminal sugar moieties, especially the sialic acid, on the molar hCG molecule. If it was correct, the molecular size of molar hCG was expected to be smaller than that of the normal. Since SDS-PAGE provides high resolution for separating proteins differing in molecular weight, it was therefore selected in the present study.

The molecular weights of intact hCG, βhCG and αhCG on SDS-PAGE were 52,000, 33,000 and 22,000, respectively (Figs. 7,8). This result agreed with the observation of Choy *et al.* (1979). The actual molecular weights of hCG, βhCG and αhCG calculated from their chemical composition are 38,000, 23,000 and 14,700, respectively (Bahl, 1977). Determination of molecular weight by SDS-PAGE is overestimated because hCG is a glycoprotein. As a consequence of the reduced amounts of SDS-bound for glycoprotein, the mobility in the gel is reduced and apparent molecular weight is increased (Gordon, 1975).

The elution of many proteins, such as SV40 viral polypeptides, murine erythrocytic ghosts and some marker proteins were affected by various conditions (Lin & Kasamatsu, 1983; Gershoni & Palade, 1983). They have found that the electroelution of protein from SDS-gel to the nitrocellulose paper depends on many factors (e.g. pore sizes of both the gel and the nitrocellulose paper, molecular weights and net charges of the proteins, electrical current, time of transfer, etc.).

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Therefore, the optimum condition for transferring hCG to the nitro-

cellulose paper was examined in the present study.

It was first discovered in the present study that hCG and its subunits could be electrophoretically transferred from SDS-polyacrylamide gel to the nitrocellulose paper (Fig. 9). Moreover, the proteins could resume their native conformations and exhibit their immunochemical properties. The amounts of β and α hCG transferred increased at longer blotting time and were completely transferred within 15 h (Fig. 9D). This result agrees with the report of Burnette (1981) that proteins (mol wt 12,000-100,000) require about 20 h at 8V/cm in order to obtain complete transfer. It was not possible to estimate the transfer efficiency of β and α subunits of hCG since amido black staining on a nitrocellulose paper could not be acheived quantitatively (Fig. 9, A,E). The staining must be done in short time otherwise the nitrocellulose sheet would be disintegrated if it is left in the acidic methanol longer than 5 min (Burnette, 1981). Another possibility was that proteins might be lost during transfer especially in longer blotting time. Little amount of the proteins transferred could move through the first sheet of nitrocellulose and appear on the second one in 24 h of blotting (Data not shown). Lin and Kasamatsu (1983) have also demonstrated that some proteins (mol wt 14,000-30,000) are observed on the second sheet of nitrocellulose after 4 h and 13 h of blotting.

The systems for detection of hCG and individual subunits by immunofluorescence were proved to have specificity as shown by the presence of one band of intact hCG, βhCG and αhCG (Fig. 11). Human serum proteins showed no cross-reactivity to any of the antisera (Fig. 11, lane 1). Besides, the positions of hCG and its subunits

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observed on the nitrocellulose paper correlated well with those present on the original gel.

As little as 0.25 μg of hCG could be detected for intact form and β subunit of hCG but 3 times higher concentration of hCG were required for detection of α subunit (Fig. 11). This is due to the variation of each antiserum. Burnette (1981) has suggested that the sensitivity in blotting is a function of the specific antibody titer of the immune serum being utilized.

4. Molecular size of normal and molar hCG

Comparison of molecular size following SDS-PAGE between normal and molar urinary intact hCG indicated that all molar hCG contained mainly the hCG of molecular size similar to normal. However, small amount of additional low molecular size of hCG (apparent mol wt 43,000) was found in 6 from 10 patients (no. 2,3,5,7,9,10, Fig. 16A and Fig. 17A). The same results were obtained by using anti-hCG_D (Fig. 16A and Fig. 17A) or different preparation of the antiserum (anti-hCG_W, data not shown). These results were consistent with the observation of Hiranyavasit *et al.* (1979) that purified hCG from molar patients is composed of a low molecular size of hCG in addition to the normal form.

Moreover, certain quantities of a low-molecular-weight immunoreactive hCG-like substance (apparent mol wt 17,000) were detected in both normal and molar urines (Fig. 16A and Fig. 17A). This substance could also react with anti-βhCG but not anti-αhCG (data not shown). Masure *et al.* (1981) have recently reported the presence of a small molecular size immunoreactive hCG-like substance when urines from normal pregnant

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women and from a patient with gestational trophoblastic disease are separated on Sephadex G-100. They have shown that the substance is a glycoprotein displaying immunological cross-reactivity with hCG, βhCG but not αhCG or the C-terminal portion of βhCG. Besides, the concentration of this substance is approximately equal to that of normal hCG. This observation raised the possibility that this substance might be a secretory or a degradative product of the hCG molecule. The nature of the "small" hCG is presently unclear.

The β subunit of normal and molar hCG was characterized as shown in Fig. 16B and Fig. 17B. All molar patients contained mainly normal βhCG. However, low molecular size of βhCG which its concentration was approximately equal to that of the normal was detected near the α position in urines of 6 from 10 patients (no. 2,3,5,7,9,10). No difference in molecular size of αhCG between normal and molar patients was found (Fig. 16C and Fig. 17C). The heterogeneities of both intact hCG and β subunit were detected from the same patients (no. 2,3,5,7,9, 10). Therefore, the abnormality of molar hCG might reside on the β subunit.

The appearance of low molecular forms of βhCG in urine of molar patients might represent degradative product of hCG or βhCG. Wehmann and Nisula (1980) have shown that purified βhCG injected to a circulation can be degraded to a carbohydrate-containing fragment and excreted in the urine. Besides, low molecular form of βhCG is reported to be present in the human placenta (Good *et al.*, 1977) suggesting that it might be an incomplete synthesis of βhCG.

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Hironori (1983) has examined the molecular forms of hCG and subunits from normal pregnant women and patients with trophoblastic disease using gel filtration chromatography. No change in molecular size of αhCG is found, the large and small molecular forms of hCG and small molecular forms of βhCG in addition to the normal form are detected in placental extracts of the first trimester in normal pregnancy, vesicular fluid and urine samples, suggesting that the heterogeneities in molecular size of hCG and βhCG are present at the sites of their production and secretion.

In the present studies, urinary molar hCG from all patients showed significantly slower electrophoretic mobilities than the normal indicating the less negative charge of the former. Molar hCG has been reported to lack some terminal negative-charged sialic acid residues (Choy *et al.*, 1979). Therefore, loss of sialic acid of molar hCG decreased both negative charge and electrophoretic mobility. The total number of sialic acid residues is 16 molecules per molecule of intact hCG (Kessler *et al.*, 1979). The variation of electrophoretic mobility among the patients was probably due to different degree of the remaining sialic acid content. Difference in molecular size between normal and molar hCG could not be clearly observed in some patients. It was possible that the differences might be too small to be detected by SDS-PAGE. Weber and Osborn (1969) have demonstrated that proteins could not be resolved in SDS-PAGE unless their molecular weights are at least 10% difference. Since the molecular weight of sialic acid is 309, a minor variation in carbohydrate content in some molar patients would not be discernible by SDS-PAGE.

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The reason for the observed heterogeneity in the carbohydrate side chains of hCG is still unclear. It might be due to the defective biosynthesis, especially in glycosylation. According to the recent works, hCG is synthesized by sequential addition of monosaccharides to protein portion and the initial glycosylation occurs during translation of polypeptides in endoplasmic reticulum followed by the terminal glycosylation during the passage of the protein through Golgi apparatus. The fucose and sialic acid are added in the terminal (Hussa, 1980; Henner *et al.*, 1983). Therefore, the abnormality of molar hCG might arise from the deficiency of a specific enzyme needed for the synthesis of the complex carbohydrate side chain especially sialic acid.

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SUMMARY

Urinary human chorionic gonadotropin (hCG) from normal pregnant women and from patients with hydatidiform mole was analyzed directely without purification. The difference in molecular charge was investigated by Tandem-CIE and difference in molecular size was characterized by SDS-PAGE followed by electrophoretic transfer and immunochemical detection. The results could be summarized in the followings:-

1. Molar hCG was immunochemically identical to normal hCG but its electrophoretic mobility in non-denaturing condition was slower than that of the normal. This implied the less negative charge of molar hCG.

2. Intact hCG, β and α subunits from both normal and molar patients could be electrophoretically transferred from SDS-polyacrylamide gel to the nitrocellulose paper with significant recovery of their immunochemical properties.

3. Both normal and molar urines contained normal hCG (apparent molecular weight 52,000) and large quantities of a low-molecular-weight immunoreactive hCG-like substance (apparent molecular weight 17,000).

4. Molar hCG (6 from 10 patients) appeared to be heterogeneous in its molecular form. It was composed of a low molecular size of hCG (apparent molecular weight 43,000) in addition to normal hCG.

5. The β subunit (6 from 10 patients) showed heterogeneity. It contained 2 forms, one of them had the molecular size equal to that

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of the normal (apparent molecular weight 33,000) and the other had lower molecular size (apparent molecular weight 23,000).

6. The α subunit showed no difference in molecular size between normal and molar hCG.

7. The heterogeneity of intact hCG of individual patient agreed with that of the β subunit indicating that the abnormality of molar hCG might reside on the β subunit.

8. Correlation could not be established in the present studies between the loss of negative charge of molar hCG and reduction of their molecular size.

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