

Evidence for a gonadotropin from nonpregnant subjects that has physical, immunological, and biological similarities to human chorionic gonadotropin

(COOH-terminal peptide of human chorionic gonadotropin β subunit/radioimmunoassay/human luteinizing hormone/gel filtration)

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ABSTRACT Substances from urinary extracts of normal, nonpregnant subjects and human pituitary gonadotropin preparations were found to react similarly to human chorionic gonadotropin (hCG) in a radioimmunoassay system that is highly specific for hCG and without crossreactivity to human luteinizing hormone (hLH). The antiserum was produced in a rabbit immunized with a bovine albumin conjugate of the unique carboxyl-terminal peptide (residues 123-145) isolated from a tryptic digest of the reduced, S-carboxymethylated hCG β subunit. The antibody recognition site on the peptide was found to reside on the last 15 amino acid residues of the carboxyl-terminal peptide, as evidenced by the competitive binding activities against ¹²⁵I-labeled hCG of a series of peptides chemically synthesized according to the carboxyl-terminal sequence of hCG β . In order to elucidate the nature of the crossreacting substance in urinary extracts, a human postmenopausal urinary preparation (Pergonal) and a kaolin-acetone extract of urine from a patient with Klinefelter's syndrome were subjected to gel chromatography on Sephadex G-100. The results indicate that fractions showing immunocrossreactivity with the antiserum to hCG β -carboxyl-terminal peptide coeluted with ¹²⁵I-labeled hCG which was separated distinctly from hLH. The same fractions from this postmenopausal urinary gonadotropin preparation exhibited *in vitro* biological activity proportional to the immunocrossreactivity of the hCG-specific antiserum. Concentration of postmenopausal women's urine by acetone precipitation retained approximately five times more immunoreactivity per unit volume than kaolin-acetone extraction, when assayed with the antiserum to hCG β -carboxyl-terminal peptide.

The development of practical, sensitive, and specific assays for distinguishing human chorionic gonadotropin (hCG, choriogonadotropin) from human luteinizing hormone (hLH, lutropin) in serum and urine specimens, collected for diagnostic purposes from pregnant women and persons with neoplasms secreting hCG, has been a major objective of the Reproduction Research Branch, National Institute of Child Health and Human Development, and its collaborators. Radioimmunoassays, specific for hCG in serum, have been developed and one of these has been applied extensively in clinical practice (1) and in clinical investigation (2-4). With these assays it was observed that extracts of urine from persons who were not pregnant or did not have a neoplasm as well as extracts of human pituitary tissue, thought not to contain hCG, showed significant cross-reactivity with ¹²⁵I-hCG. These data led to the assumption that, while this assay appeared to be specific for hCG in serum, it

could not be used to distinguish hCG from hLH in pituitary and urinary extracts.

Efforts to develop assays to make this distinction have continued by producing antisera, in rabbits, directed at antigenic sites that are known to be present in hCG but not in hL (5). The immunogen employed was a peptide isolated after tryptic digestion of an S-carboxymethylated, desialylated preparation of purified hCG β subunit. The amino acid composition and NH₂-terminal sequence analysis indicate that the peptide was composed of 23 amino acids, unique to the COOH-terminus of the hCG β subunit (6, 7). Unexpectedly, in radioimmunoassays using this antiserum and ¹²⁵I-hCG, both the urinary extracts previously thought not to contain hCG and pituitary extracts were found to give rise to dose-response curves indistinguishable from those of highly purified hCG. These results were similar to those of the earlier assays (2). In order to elucidate the basis for these unexpected observations, radioimmunoassays were carried out prior to and following gel filtration of these extracts on Sephadex G-100. The results indicate that substances with immunological, physical, and biological properties of hCG are present in both crude and partially purified urinary gonadotropin preparations, as well as in pituitary extracts, and these are distinguished from hLH.

MATERIAL AND METHODS

Hormone Preparations. (A) Urinary Extracts. The following human urinary extracts, prepared and variably purified by the methods of Frank *et al.* (8), Albert (9), Donini *et al.* (10), and Canfield *et al.* (11) were tested in radioimmunoassays: (1) second international reference preparation of human postmenopausal gonadotropin (2IRPHMG) with biological activity of 40 international units (IU) per ampoule or 1 IU = 0.2295 mg for both hLH and human follicle-stimulating hormone (hFSH, follitropin); (2) first international standard for human urinary FSH/LH (interstitial cell-stimulating hormone, ICSH) for bioassay containing 46 IU of hLH and 54 IU hFSH per ampoule or 1 IU hFSH = 0.11388 mg and 1 IU hLH = 0.13369 mg of protein (as measured by bioassay, with 2IRPHMG standard); these two preparations were generously supplied by Dr. Derek Bangham, acting for the International Laboratory for Biological Standards of the World Health Organization; (3) Pergonal (Cutter Laboratories, lot PR2168), a commercial preparation of postmenopausal women's urine, manufacturer's potency estimate of 75 IU FSH and 75 IU LH per ampoule (bioassay, 2IRPHMG); (4) Albert fraction A kaolin-acetone extract, from a 24 hr urine specimen containing 500 mouse uterus units (bioassay) and 36.75 IU (radioimmunoassay, 2IRPHMG) hLH

Abbreviations: h, human; CG, chorionic gonadotropin (choriogonadotropin); LH, luteinizing hormone (lutropin); FSH, follicle-stimulating hormone (follitropin); 2IRPHMG, second international reference preparation of human postmenopausal gonadotropin.

collected from a male with Klinefelter's syndrome; (5) a purified preparation of hCG-CR119, as well as a preparation of α and β subunits derived from hCG-CR119; hCG-CR119 contained 11,600 IU, CR119 α 14.5 IU, and CR119 β 5.2 IU per mg of protein (bioassay, second international standard hCG); the biological activity of both subunit preparations is attributable to the presence of 0.05–0.2% undissociated and/or reassociated hCG (12, 13); (6) an extract, prepared by acetone precipitation of an aliquot of a 75 liter pool of postmenopausal urine, containing 0.26 IU LH and 0.36 IU FSH per mg of nondialyzable solid (radioimmunoassay, 2IRPHMG); (7) an analogous kaolin-acetone extract made from the same 75 liter pool of postmenopausal urine was used to compare the efficiency of the acetone precipitation versus kaolin-acetone extraction for recovering immunoreactivity.

(B) Pituitary Extracts. The following pituitary extracts were tested in radioimmunoassays: (1) first international reference preparation of human pituitary FSH and LH/ICSH for bioassay (1IRP-pituitary) containing 25 IU LH and 10 IU FSH per ampoule or 1 IU LH = 0.0668 mg and 1 IU FSH = 0.1670 mg of protein (bioassay, 2IRPHMG); (2) a purified preparation of hLH (LER 960VI) distributed by the National Institutes of Health for use as tracer in radioimmunoassay for hLH, which contains 4620 IU LH per mg of protein (bioassay, 2IRPHMG); (3) a partially purified pituitary fraction, LER 1966, containing 415 IU FSH and 355 IU LH per mg protein (bioassay, 2IRPHMG).

(C) Modified HCG β Subunits. To prepare *S*-carboxymethyl (SCM-hCG β) and *S*-carboxamidomethyl (SAM-hCG β) hCG β derivatives, 10 mg of hCG β (CR119) were dissolved in 1 ml of 0.5 M Tris-HCl, pH 8.5, containing 8 M urea and 0.1 M EDTA. Under N₂, 1.3 ml of 0.3 M dithiothreitol were added and incubation was continued for 30 min at 37°. Alkylating agent (2 ml of 2 M iodoacetic acid or iodoacetamide) was added and the reaction was continued for an additional 30 min in the dark at 37°. The protein solutions were dialyzed against deionized water and lyophilized.

(D) Synthetic Peptides. Tetrapeptide (residues 142–145 of hCG β subunit) and eicosapeptide (residues 126–145) were a gift from Dr. V. C. Stevens of Ohio State University.

Dodecapeptide (residues 134–145) and octaeicosapeptide (residues 118–145) were synthesized by the standard Merrifield solid phase method (14). Cleavage from resin and removal of protecting groups were accomplished by treatment with liquid hydrogen fluoride.

Peptides other than the four peptides mentioned above were synthesized also by the solid phase method, except *S*-methylcysteinyldehydroalanine was coupled to the resin prior to the elongation of peptide. Peptides were cleaved from the resin by 1 M HCl in glacial acetic acid. *O*-Benzyl groups were removed by HBr gas in trifluoroacetic acid containing anisole. Thus, the peptides produced from this series contain COOH-terminal *S*-methylcysteinyl amide. Arginine is in the form of *N*-nitroguanidinoarginine. *S*-Methylcysteinyl amide residue was not included in the counting of chain length as shown in Fig. 1. Amino acid analyses of the synthetic peptides yielded compositions expected from the respective sequences.

Antisera. (A) Anti-hCG Serum (H80). Antiserum H80 was generated in a rabbit immunized with hCG-CR119 by the method of Vaitukaitis *et al.* (15). At a tube dilution of 1:75,000, it bound 25–30% of trace quantities of ¹²⁵I-hLH or ¹²⁵I-hCG and less than 2% of ¹²⁵I-hFSH. When this serum was reacted with trace quantities of ¹²⁵I-hLH, 50% inhibition of binding required 200 ng of hLH (1IRP-pituitary) and 10.6 ng of hFSH/hLH (LER 1966). Up to 25 ng of human thyrotropin

(Pierce Fraction IV) had no detectable crossreactivity.

(B) Anti-hCG β Serum (Sb6). Production and characteristics of this antiserum have been described previously by Vaitukaitis *et al.* (2). A radioimmunoassay using this antiserum employing either ¹²⁵I-hCG or ¹²⁵I-hCG β as tracer distinguished hCG from hLH in human serum, but inhibition of binding was observed with both pituitary and urinary extracts containing hLH. Its crossreactivity with hLH was significantly lower than that of H80 antiserum.

(C) Anti-hCG β -COOH-Terminal Peptide Serum (H93). Immunogen preparation (trienicosaglycopeptide from *S*-carboxymethylated, desialylated hCG β conjugated to bovine serum albumin) and immunization procedure were similar to those described by Louvet *et al.* (5). When antiserum H93 was diluted 1:8000, it bound 20–25% of trace quantities of ¹²⁵I-hCG, and did not bind ¹²⁵I-hLH. The following highly purified glycoprotein hormones, at the dose level in parenthesis per tube, did not inhibit the binding of ¹²⁵I-hCG with this antiserum: hLH-LER-960VI (23 IU by bioassay, 2IRPHMG standard), hFSH-LER-1575 (40 IU by bioassay, 2IRPHMG), NIH pregnant mare serum gonadotropin (100 IU by bioassay, 2IRPHMG) and human thyrotropin, Pierce Fraction IV (4 μ g).

Radioimmunoassay. All radioimmunoassays were carried out by the double-antibody technique as described previously (16), except that tubes were incubated initially at 37° for 2 hr, then at 4° for an additional 12–16 hr. Second antibody (sheep antiserum to rabbit gamma globulin) was added at the end of that time, and incubation was continued for another 4–6 hr at 4° before bound and free hormone were separated by centrifugation and aspiration of the supernatant. Computations of potencies and calculations and comparisons of slopes of dose-response lines were done by a modified computer program described by Rodbard and Lewald (17).

Sephadex G-100 Column Chromatography. A 1.48 \times 190 cm column of Sephadex G-100 was equilibrated with 0.05 M Tris-HCl-0.1 M NaCl, pH 7.8. The flow rate of the column was 15 ml/hr. All samples were dissolved in 2 ml of the same buffer used to equilibrate the column, but containing a trace amount of ¹²⁵I-hCG in 1% bovine serum albumin solution. Two milliliter fractions were collected.

RESULTS

To identify the antigenic determinants for the anti-hCG β -COOH-terminus serum (H93), a series of peptides, synthesized according to the amino acid sequence of the COOH-terminal 28 residues of hCG β subunit determined by Morgan *et al.* (6, 7), was tested. The assay consisted of a 1:8000 final dilution of the serum reacted with trace quantities of ¹²⁵I-hCG. Results are shown in Fig. 1. Inhibition of binding increased with increasing chain length. The shortest COOH-terminal peptide tested, a tetrapeptide (not shown in Fig. 1), yielded immunoreactivity equivalent to 5.9 ng of hCG-CR119 per μ g of the peptide. The immunoreactivity reached a plateau at the pentadecapeptide. These data were interpreted to indicate that any peptide in which as few as four and as many as 15 of the COOH-terminal amino acid residues of hCG β subunit were available for antigenic recognition would be reactive in this radioimmunoassay system. Furthermore, peptide chains elongated beyond these initial 15 amino acid residues would be indistinguishable by this assay. In contrast, these synthetic peptides were not immunoreactive with the Sb6 anti-hCB β serum or the H80 anti-hCG serum assay systems.

To test this interpretation further, the antigenic potency of *S*-carboxymethyl-hCG β subunit, *S*-carboxamidomethyl-hCG β subunit, hCG β subunit, and hCG were compared. Results are

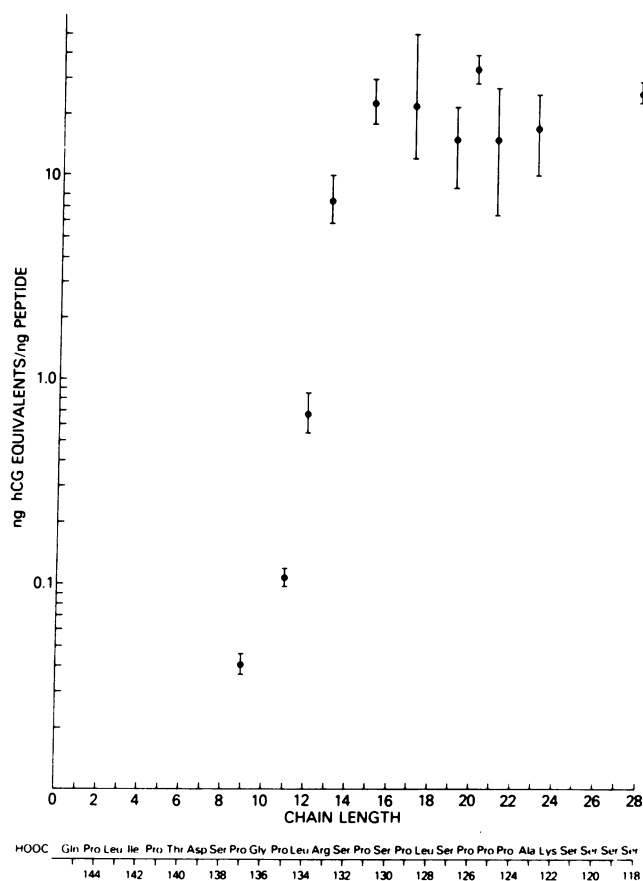


FIG. 1. Competitive binding activities against ¹²⁵I-hCG of synthetic peptides analogous to the COOH-terminal sequence of hCG β . Ordinate indicates ng of unlabeled hCG-CR119 required to exhibit inhibition of ¹²⁵I-hCG binding to antiserum per ng of peptides. Amino acid sequence of the COOH-terminal peptide is shown on the lower abscissa. Methods used were similar to radioimmunoassay procedure, which is described in the text.

shown in Fig. 2, where it can be seen that slopes of dose-response curves for these materials were indistinguishable and masses required for equivalent inhibition of binding were virtually the same. On the basis of these data, it seemed reasonable to consider that this assay would be specific for any peptide in which these antigenic determinants shared with

hCG β were accessible for recognition under these assay conditions. It was therefore surprising to observe that four preparations of postmenopausal urine extracts: (1) Pergonal (lot PR 2168), (2) first international standard for urinary FSH/LH (ICSH), (3) second international reference preparation of human menopausal gonadotropins, and (4) an acetone precipitate of pooled postmenopausal nuns' urine, none of which extracts were expected to contain hCG or hCG β , all completely inhibited binding of tracer hCG with dose-response curves parallel to that for purified hCG (Fig. 2). In addition, two pituitary extracts, the first international reference preparation for bioassays of pituitary FSH/LH (1 IRP) and LER 1966, gave rise to similar dose-response curves. Furthermore, potency estimates based upon these dose-response curves were not consistently related to the LH biological activity of these preparations, suggesting that the hLH and apparent 4-15 COOH-terminal amino acid hCG β immunoreactivities varied independently.

It should also be emphasized that neither Sb6 anti-hCG β serum nor H80 anti-hCG serum showed immunocrossreactivity toward S-carboxymethyl- on S-carboxamidomethyl-hCG β , indicating that antigen binding sites are dependent on conformation. Although to a lesser degree than was observed in the H93 assay, immunopotency estimates by Sb6 assay of those same preparations shown on Fig. 2 were not consistently related to hLH biological activity. Estimates by H80 assay showed much more consistency with hLH biopotency. These findings indicate that specificity of the Sb6 antiserum lies in the structural characteristics of hCG β which are distinguishable from hLH, and not in the region of the unique COOH-terminal sequence.

To further characterize the substances in pituitary and postmenopausal urinary extracts having antigenic determinants common to those of COOH-terminal region of hCG β , Pergonal was subjected to gel filtration on a Sephadex G-100 column. The 140 fractions collected were assayed not only with the H93 anti-hCG β -COOH-terminal serum, but also with the Sb6 anti-hCG β serum (which distinguishes hCG from hLH in serum), the H80 anti-hCG serum (which distinguishes hCG and hLH from hFSH and human thyrotropin in serum and pituitary and urinary extracts, but does not distinguish hCG from hLH in a mixture of these two hormones) and the H31 anti-ovine FSH serum (18) (which is specific for hFSH in serum and in urinary and pituitary extracts). Results are shown in Fig. 3A-E.

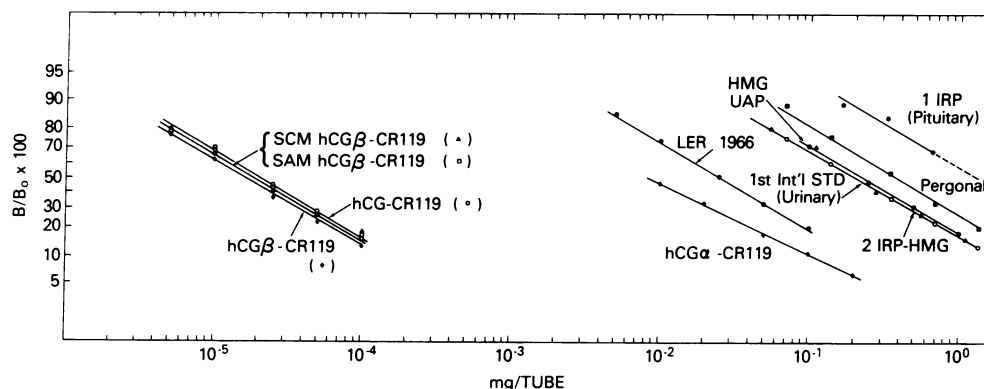


FIG. 2. Dose-response curves of gonadotropin preparations in a radioimmunoassay system based on an antiserum to hCG β -COOH-terminal peptide (H93) diluted 1:8000. B = cpm bound in the presence of ¹²⁵I-hCG-CR119 and unlabeled ligand; B₀ = cpm bound in the presence of ¹²⁵I-hCG alone. The line for Pergonal was placed according to the biological activity cited by the manufacturer, assuming identical specific bioactivity to 2IRPHMG. SCM is S-carboxymethyl; SAM, S-carboxamidomethyl. 2IRP-HMG is second international reference preparation of human postmenopausal gonadotropins; HMG UAP is human postmenopausal gonadotropins, urinary acetone precipitate.

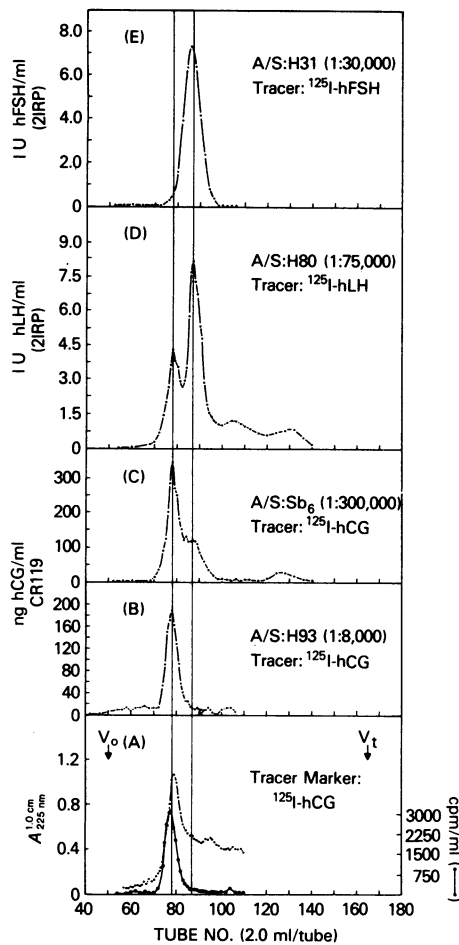


FIG. 3. Sephadex G-100 gel chromatographic patterns of human postmenopausal gonadotropin preparation (Pergonal) as determined by radioimmunoassay systems from a single column run using antisera of different specificities. Two ml containing 300 IU (bioassay, 2IRPHMG) were applied to the column. A/S is antiserum, with dilution given in parentheses. V_o is void volume; V_t is total column volume.

In Fig. 3A, the elution volume of ^{125}I -hCG is shown. A symmetrical peak of radioactivity was seen, beginning with tube 70, reaching a fastigium (peak) at tube 78, and declining to baseline at tube 87. For this column the partition coefficient K_{av} is 0.24 and the V_e/V_o ratio (elution volume/void volume) is 1.56 for ^{125}I -hCG.

Fractions collected when the Pergonal preparation was applied to this column were assayed with the H93 antiserum to hCG β -COOH-terminal peptide, and the results are shown in Fig. 3B. A symmetrical peak of immunoreactivity was found to be coincident with that for the ^{125}I -hCG radioactivity in Fig. 3A. When the same fractions were assayed with the Sb6 anti-hCG β serum, a coincident immunoreactivity peak was seen (Fig. 3C), but this peak was asymmetrical, with a shoulder on the descending limb, extending from tubes 83 to 90. As can be seen in the data presented below with the H80 assay, we believe this shoulder represents some crossreactivity of Sb6 antiserum with hLH.

When these fractions were assayed with the H80 anti-hCG serum, two overlapping peaks of immunoreactivity were seen (Fig. 3D). The first and smaller of these was coincident with those seen in the other two assays, whereas the second and larger peak was coincident with the shoulder seen by the Sb6 assay (Fig. 3C). This second peak reached its fastigium in tube 87 and

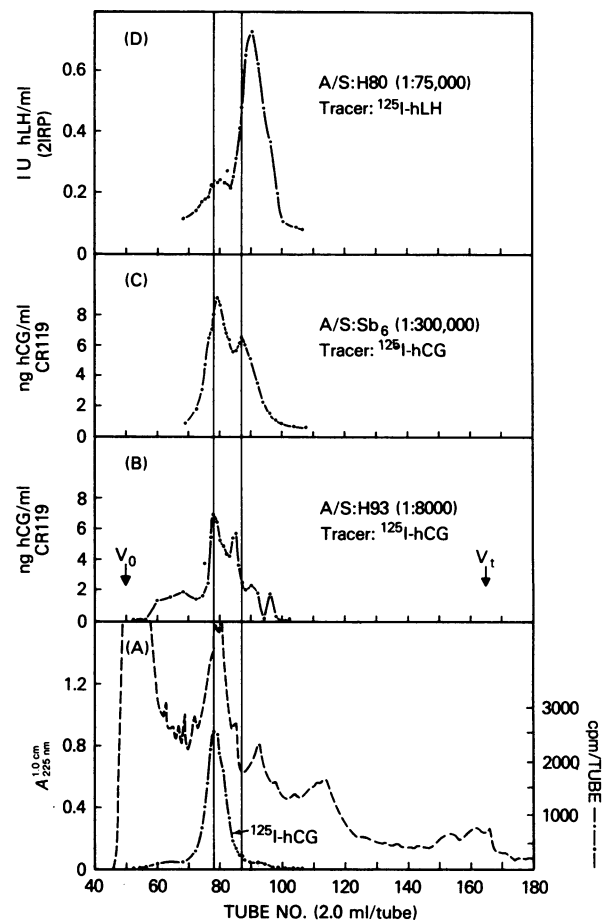


FIG. 4. Sephadex G-100 gel chromatographic patterns of kaolin-acetone extract of urine (330 ml) obtained from a patient with Klinefelter's syndrome. The kaolin-acetone extract applied to the column represents $\frac{1}{3}$ of a 24 hr collection. Abbreviations, chromatographic conditions, and radioimmunoassay methods are identical to those of Fig. 3.

was coincident with the peak of hFSH immunoreactivity found when these fractions were tested with the H31 radioimmunoassay for hFSH (Fig. 3E).

Collectively, these data were interpreted to indicate that the substances in the postmenopausal urine extract that shared antigenic determinants with the COOH-terminal region of hCG β also exhibited the physical characteristics of hCG as manifested by elution pattern in Sephadex G-100 column chromatography.

Despite the fact that immunoreactivity similar to that for hCG was demonstrated in four separate postmenopausal urine extracts, the remote possibility of inadvertent contamination of all four preparations with pregnancy urine or urine from persons with neoplasms cannot be eliminated. Accordingly, a 24 hr urine specimen was collected from a young man with Klinefelter's syndrome and was processed by the method of Albert (9). An aliquot of this kaolin-acetone extract containing biopotency of 500 mouse uterus units and 36.75 IU LH (immunoassay, H80 antiserum, 2IRPHMG) was applied to the Sephadex G-100 column. Fractions were collected and assayed in the same manner as had been done for Pergonal. Results are shown in Fig. 4A-D. Despite the fact that this urine extract is crude as compared to Pergonal, an immunoreactive peak coincident with that for ^{125}I -hCG was seen in all three assays (Fig. 4B-D). In addition, the shoulder and overlapping second

peak recognized by Sb6 anti-hCG β serum assays of Pergonal fractions were seen in the corresponding fractions of this crude urine extract.

Although the V_e/V_0 ratio of this substance in postmenopausal urine extracts is more consistent with that reported for hCG than for hCG β (13), hCG β is antigenically potent in all the assays used in these studies. To eliminate the possibility that the immunoreactivity was due exclusively to a substance or substances similar to hCG β , tubes 75, 78, and 81 from the Sephadex G-100 fractions of Pergonal were bioassayed by the method of Dufau *et al.* (19), which requires intact hCG to induce steroidogenesis. The greatest biological activity was in tube 78, commensurate with the maximal immunoreactivity in the H93 assay for the COOH-terminal peptide of hCG β .

DISCUSSION

The observation that postmenopausal urine extracts contained a substance with the biological properties similar to hCG was reported in 1960 by Albert and Derner (20). This conclusion was reached on the basis of the "biologic fingerprint" of 2IRPHMG. It was not possible to distinguish incidental contamination with pregnancy urine from an endogenous source of an hCG-like gonadotropin in postmenopausal women.

Although we cannot eliminate the possibility of inadvertent contamination of pooled specimens by pregnancy urine or urine from persons bearing neoplasms, the possibility of such contamination seems extremely remote in the case of the acetone precipitates we obtained from postmenopausal nuns' urine and a urine extract from a patient with Klinefelter's syndrome. Acetone precipitation was found to be a more effective means of concentrating the immunoreactive substance than the kaolin-acetone extraction. Kaolin-acetone extracts of specimens containing up to 50 mouse uterus units of total urinary gonadotropin did not contain measurable immunoreactivity in the H93 assay (21).

Since urinary preparations contained substances that shared antigenic determinants with those of the COOH-terminal peptide of hCG β , the autogenous source seems more plausible. In addition, the observation that similar antigenic activity was present in pituitary extracts suggests that the pituitary may be a source of this substance. To our knowledge, neither the observation that pituitary extracts contain a substance with such antigenic properties nor the possibility that the pituitary may be a source of similar substances in human urinary extracts has been reported hitherto. However, it has been reported that extracts of human testis contain a substance with physical and antigenic properties of hCG (22). These observations raise questions as to whether the mere presence of hCG as a "marker" (23) can be useful for diagnosing or monitoring treatment of some human neoplasms. Thus, the validity of hCG as a "marker" must depend on considerations such as its origin, levels, and patterns of secretion. Equally important implications for the design of highly sensitive qualitative tests for hCG in

serum and urine for pregnancy tests are obvious. Moreover, irrespective of the source of these substances that can be detected antigenically in pituitary and urinary extracts, these observations have implication for human reproductive physiology and pathophysiology, and ultimately for clinical practice and investigation.

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