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Immunologic and Biologic Study of Human Chorionic Gonadotropin *

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Immunological methods have been used to measure human chorionic gonadotropin (HCG) in normal and pathological pregnancies (1–3). Immunohistological methods with the fluorescent antibody technique have also been used to localize HCG in placenta and tumors (4, 5). The antisera used in the above studies were produced with HCG preparations that contained contaminants and, therefore, contained antibodies to a multiplicity of extraneous antigens in addition to HCG. Antibodies to some contaminants can be removed from HCG antisera by adsorption with urine, serum, or their extracts (1, 3, 6). Previous studies, however, have demonstrated that absorption methods probably do not make such antisera HCG specific (7). This investigation is concerned with the immunological characteristics of several commercial HCG preparations in an attempt to evaluate the reliability and validity of the HCG antisera previously employed (1–5).

Seven antigens are demonstrable in commercial HCG preparations by immunoelectrophoresis. Five of these contaminating antigens are common to HCG and nonpregnant urine extract and can be demonstrated by immunoelectrophoresis and hemagglutination. HCG antisera after adsorption with normal male urine powder contain antibodies to two antigens unique to pregnancy urine extracts and urinary HCG preparations. These two antigens are dissimilar but share partial immunological identity by immunoelectrophoresis. Chromatographic separation of HCG preparations into biologically active and inactive fractions has made it possible to evaluate the relationship between immunological and biological activities. The two antigenic moieties unique to pregnancy are different and distinct from the material possessing the biological activity of HCG. It appears, therefore, that the biological activity of HCG cannot be attributed to the two antigenic moieties unique to pregnancy urine that can be demonstrated by hemagglutination and immunoelectrophoresis. These pregnancy antigens, distinct from the biologically active HCG, are probably the causative agents in preparing antisera highly effective in testing for pregnancy. They probably do not possess biological activity, however, and antisera directed to one or both of them are probably not specific for the localization of HCG (biologically active) histologically. Demonstration of precipitating or hemagglutinating antibodies against biologically active HCG was not possible in four different antisera tested. All four of these antisera did, however, contain antibodies capable of neutralizing the biological activity of HCG.

Methods

Human chorionic gonadotropin (HCG). HCG, lot no. 90231, 90259, and 90272, containing 3,400, 2,410, and 2,710 U of HCG per mg, respectively, was obtained commercially.[1]

Cellulose acetate electrophoresis (7). Electrophoresis using cellulose acetate strips [2] (8) was performed on the Shandon Universal electrophoresis apparatus 2548 [2] with barbital buffer at pH 8.6, ionic strength 0.08, for 1.5 hours at 0.4 ma per cm of strip width. A Heathkit model PS-3 [3] was the current power supply. The electrophoretic strips were stained with light green S.F. or Ponceau S and nigrosin.

Hemagglutination and hemagglutination inhibition methods. The hemagglutination and hemagglutination inhibition methods employed have been described by Arquilla and Stavitsky (9). Modifications in the method used for HCG have been previously described (7).

Immunoelectrophoresis (7). Immunoelectrophoretic studies by the Scheidegger modification (10) were per-

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[1] Vitamerican Corp., Little Falls, N. J.

[2] Consolidated Laboratories, Chicago, Ill.

[3] Heath Co., Benton Harbor, Mich.

formed on the LKB electrophoresis apparatus 6800A[4] with 1% Ionager no. 2[2] in barbital buffer at pH 8.6, ionic strength 0.08. Electrophoresis was performed at 4.5 ma per slide for 1 to 3 hours. Incubation for development of precipitin lines was done at room temperature for 16 to 40 hours in a moist chamber. The staining of precipitin lines was accomplished with 0.5% phloxine B[5] and 0.5% eosin Y[5] in 95% ethanol.

Bioassay in the intact immature male rat. The rat prostate and accessory organ method of Diczfalusy (11) was employed for HCG bioassay with 21-day old Long-Evans rats. Formalin (10%) was used for tissue fixation, and results were expressed in grams per 100 g body weight (7).

Preparation, processing, and use of antisera. The immunization of rabbits and processing and storage of HCG antiserum have been previously described (7). Identical procedures were used to obtain antisera to normal male urine extract and to an HCG fraction.

All lots of antisera used were individually pretested immunoelectrophoretically to determine the optimal antigen concentration required for maximal precipitin line formation. Further tests were performed to define the zones of antibody and antigen excess and to detect optimal proportions of other antigen-antibody systems.

Several lots of commercially available rabbit antihuman serum[6] were used for immunoelectrophoretic studies. A pool of horse antiserum to Vitamerican HCG[7] was used exclusively for immunoelectrophoresis.

Preparation of human urine powders. Crude human urine powders from normal males, normal females, and pregnant females were obtained by acetone precipitation (12). The crude acetone precipitate was suspended in saline and dialyzed against water at 4° C. The residue after dialysis was separated by centrifugation and discarded. The dialyzed supernatant fluid was lyophilized, and about 1 mg of extract was obtained per 4 to 5 ml of freshly voided urine. Extracts used were freshly prepared by dissolving weighed amounts of powder in barbital buffered saline,[8] and the insoluble residue was removed by centrifugation.

Various concentrations of the water insoluble residue of normal male urine acetone precipitate were dissolved in barbital buffered saline and tested immunoelectrophoretically with HCG antiserum. Adsorption with the water insoluble residue failed to alter the characteristics of the antisera with respect to their ability to react with HCG immunoelectrophoretically.

Bioassay studies performed with intact, immature male rats showed no detectable gonadotropic activity in 5 mg (25 ml urine) of normal male or normal female urine powders (p 0.0001). Similar amounts of identically prepared pregnant female urine powder contained HCG (p 0.92).

Adsorption of antiserum. Weighed amounts of extracts were added to antiserum, and the mixture was allowed to stand at room temperature for a minimum of 3 hours. The adsorbed antiserum (supernatant fluid) was then obtained by centrifugation at 2,000 rpm for 10 minutes. Twice the minimal amount of extract required to delete the maximal number of precipitin lines or cause maximal inhibition of hemagglutination was used. When an antiserum was adsorbed with more than one material, each extract was added sequentially.

Preliminary studies indicated that 50 to 125 mg of normal male urine powder (250 to 650 ml of urine) was necessary to fulfill the criteria outlined for adsorptions. Adsorption with urine was not practical, since the amount required represented quantities greater than previously used (7) and antiserum would be diluted to a low order of sensitivity.

Column chromatography with diethylaminoethyl cellulose. HCG, 250 mg, dissolved in 10 ml of buffer was added to a 2.5- × 30.0-cm column packed with diethylaminoethyl cellulose (DEAE). The DEAE was equilibrated with 0.03 M, pH 8.3, tris(hydroxymethyl)aminomethane (Tris) buffer, before packing the column. A gradient of sodium chloride (0.0 to 0.2 M) was obtained by allowing the eluant (0.4 M sodium chloride in 0.03 M Tris buffer) to be diluted with an equal volume of 0.03 M Tris buffer in a magnetically stirred mixing chamber before flowing into the column. The flow rate used in these chromatographic studies was 2 to 5 ml of effluent per minute. Five-ml fractions were collected with a GME volumetric fractionator, model V15,[2,9] continuously scanned for proteins at 280 mμ with a GME ultraviolet adsorption meter, model UV-280IF,[9] and recorded (Rectiriter).[10]

The cores of the protein peaks were confirmed by adsorption at 280 mμ with Beckman model DB spectrophotometer,[11] dialyzed against water at 4° C, and lyophilized. The lyophilized powders were weighed and dissolved in barbital-buffered saline just before use.

Results

Characterization studies of HCG preparations. A maximum of seven distinct precipitin lines was observed by immunoelectrophoresis of HCG preparations with concentrations ranging from 1 to 500 mg per ml. An example of the immunoelectrophoretic pattern with horse HCG antiserum is shown in Figure 1. The precipitin lines are

[4] LKB Instruments, Inc., Washington, D. C.

[5] Allied Chemical Corp., National Aniline Division, New York, N. Y.

[6] Hyland Laboratories, Los Angeles, Calif.

[7] Generously supplied by Dr. T. Asher of Hyland Laboratories.

[8] Diluent for hemagglutination, immunoelectrophoresis, and bioassay was barbital buffered saline (13), pH 7.4, containing 5×10^{-4} M $MgCl_2$ and 1.5×10^{-3} M $CaCl_2$ per L.

[9] Gilson Medical Electronics, Middleton, Wis.

[10] Texas Instruments, Inc., Houston, Texas.

[11] Beckman Instruments, Inc., Fullerton, Calif.

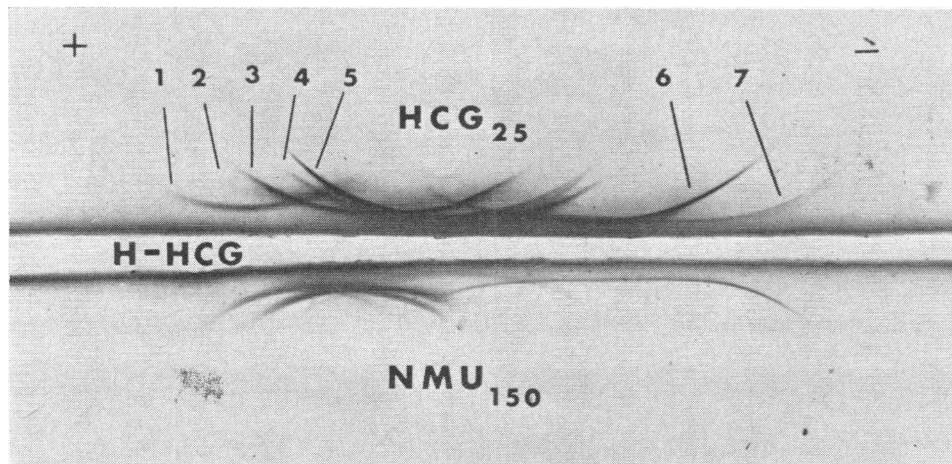

FIG. 1. IMMUNOELECTROPHORETIC PATTERNS OF HCG AND NORMAL MALE URINE EXTRACT AGAINST HORSE HCG ANTISERUM. HCG_{25} = human chorionic gonadotropin preparation (25 mg per ml); H-HCG = horse HCG antiserum; and NMU_{150} = normal male urine extract (150 mg per ml).

numbered 1 to 7 from anode to cathode. These numbers will be used in referring to the homologous antigen or antibody responsible for each precipitin line. Precipitin line 2 was faint and did not reproduce well in the photograph. The HCG antisera from three rabbits used in these studies showed similar immunoelectrophoretic patterns and contained 10,000 to 50,000 hemagglutination units (Table I, line 1).

Antigens common to HCG and normal male urine extracts (50 to 800 mg) were demonstrated by immunoelectrophoresis with HCG antiserum (Figure 1). Five precipitin lines (no. 1, 2, 3, 4, and 7) were obtained with extracts of normal male urine. The precipitin line similar to line 2 was faint and is not reproduced in the figure shown. Identical immunoelectrophoretic results have also been obtained with nonpregnant female urine extracts.

Immunoelectrophoresis of HCG preparations with rabbit antiserum against normal male urine extracts failed to demonstrate the five (no. 1, 2, 3, 4, and 7) precipitin lines. Hemagglutination studies with the same antiserum tested against HCG conjugated sheep erythrocytes had a low titer (Table I, line 2). The poor hemagglutinating titer and paucity of precipitating antibodies in these antisera were probably due to the low concentration of antigens in the extract used for immunization.

TABLE I

*Summary of hemagglutination and hemagglutination inhibition studies**

Line	Rabbit antiserum† to:	Inhibiting antigen‡				
		Saline	HCG_{10}	NMU_{10}	NFU_{10}	PFU_{10}
1	HCG	25,000	0	6,250 to 12,500	6,250 to 12,500	200
2	NMU	1,280	0	40	40	20
				$F\ I_{10}$	$F\ II_{10}$	
3	HCG (NMU)	10,000	0	400	0	
4	F II (NMU)	10,000	0	50	0	
5	HCG (NMU, F I)	0				

* Numbers indicate hemagglutination units that are reciprocals of titers. HCG = human chorionic gonadotropin preparation, NMU = normal male urine extract, NFU = normal female urine extract, PFU = pregnant female urine extract, F I = Fraction I, and F II = Fraction II.

† Adsorbed antiserum followed by letters in parentheses denoting adsorbent used.

‡ Subscripts indicate amount of antigen in micrograms used for inhibition. Reaction with rabbit antiserum is indicated by inhibition of hemagglutination of sheep erythrocytes to which HCG was conjugated.

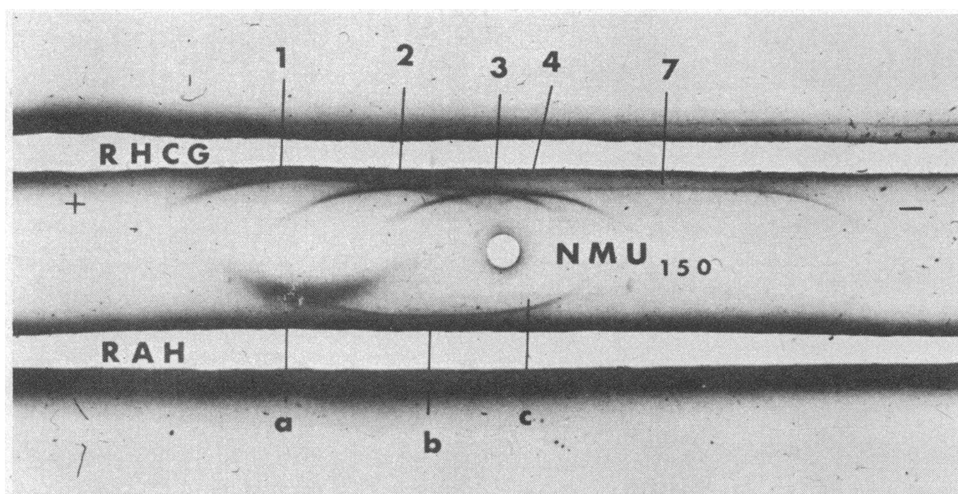

FIG. 2. SERUM PROTEIN CONTAMINANTS DEMONSTRABLE IN NORMAL MALE URINE EXTRACT. R–HCG = rabbit HCG antiserum; NMU_{150} = normal male urine extract (150 mg per ml); and RAH = rabbit antihuman serum.

The presence of serum protein contaminants in HCG, tested immunoelectrophoretically with rabbit antihuman serum demonstrated two precipitin lines. Two similar precipitin lines were also observed when various dilutions (1/1 to 1/100) of human serum were tested with rabbit HCG antisera. These serum protein contaminants (precipitin lines b and c) were also identified in normal male urine extracts (Figure 2). Precipitin line c, not visible in the photograph, was a short arc extending from just below the cathodic portion of the antigen well and appeared to merge with the anodic tip of precipitin line b. Precipitin line c (Figure 2) has been observed only under these conditions and indicates the presence of an eighth antigen in HCG preparations. Of the seven precipitin lines (no. 1 to 7) previously demonstrated with HCG and its homologous antiserum, only precipitin line 2 appears to be due to a serum protein antigen. Precipitin line a has not been observed in each of three lots of HCG. Further studies show that antigen a possessed the electrophoretic mobility of serum albumin and b and c migrated as alpha globulins.

Each of three lots of a commercial HCG preparation contains a minimum of seven antigenic moieties capable of eliciting precipitating antibodies in rabbits. An eighth antigen (precipitin line c, Figure 2), not ordinarily detected, also appears to be present in HCG preparations. A minimum of five of these antigenic moieties is common to HCG preparations and nonpregnant human urine extracts. There appear to be two antigens in each of three HCG preparations tested that demonstrate immunological cross reaction with antigens in serum.

Studies of similarities and dissimilarities between HCG and normal male, normal female, and pregnant urines by adsorption. Five (no. 1, 2, 3, 4, and 7) of the seven antigens demonstrated were common to commercial HCG preparations and nonpregnant human urine extracts, whereas two antigens (no. 5 and 6) appeared unique to HCG preparations. To further study similarities and dissimilarities between HCG preparations and antigens in both pregnant and nonpregnant urine, adsorptions of HCG antisera with urine powders were performed.

Two merging precipitin arcs with a single spur, which correspond to precipitin lines 5 and 6, were consistently observed by immunoelectrophoresis of HCG preparations with each of three rabbit HCG antisera adsorbed with normal male urine extract. Horse HCG antiserum was used in the example shown (Figure 3, top half of montage) and is identical to results obtained with rabbit HCG antisera. These antisera showed a decrease from 25,000 to a minimum of 6,250 hemagglutination units. As much as 800 mg of normal male urine powder per ml of HCG antisera was used in adsorptions with no detectable change in the immunoelectrophoretic pattern or further decrease

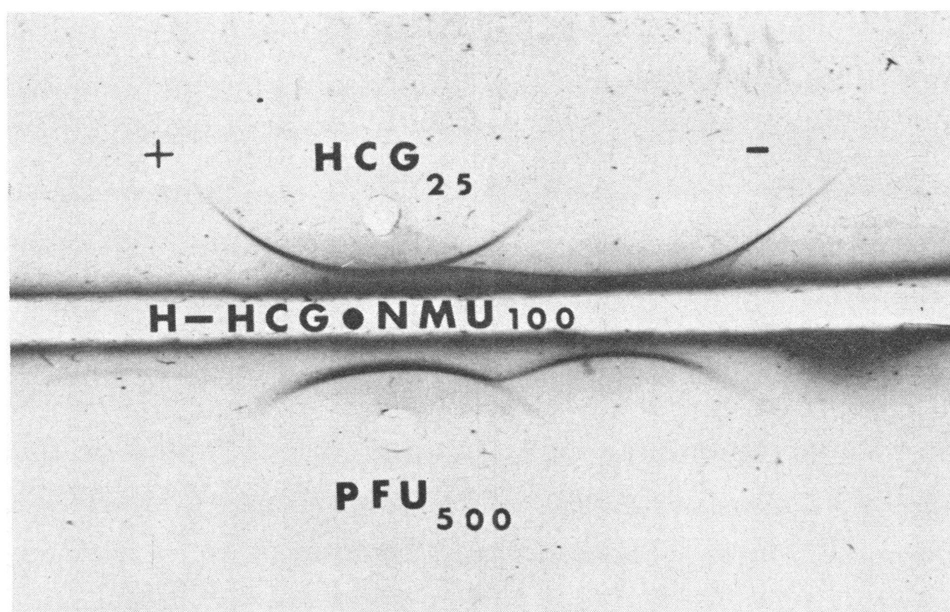

FIG. 3. ILLUSTRATION OF ANTIGENS UNIQUE TO PREGNANCY BY ADSORPTION OF HCG ANTISERUM WITH NORMAL MALE URINE EXTRACT. HCG_{25} = human chorionic gonadotropin preparation (25 mg per ml); H-HCG·NMU_{100} = horse HCG antiserum (two different lots) adsorbed with normal male urine extract (100 mg per ml of antiserum); and PFU_{500} = pregnant female urine extract (500 mg per ml).

in hemagglutination titer. Nor was the HCG neutralizing ability of these three antisera adsorbed in this fashion altered when tested biologically (Table II, line 3). Similar immunoelectrophoretic, hemagglutination, and bioassay results were also obtained when these HCG antisera were adsorbed with urine extracts from nonpregnant females.

It has been possible to demonstrate that pregnant female urine extracts contain all of the antigenic moieties demonstrable in HCG preparations with the methods employed. A total of seven antigens common to HCG and pregnant female urine can be demonstrated by immunoelectrophoresis with HCG antisera. By varying the concentration of the pregnant female urine extract, the five antigenic moieties common to HCG preparations and pregnant and nonpregnant urine extracts were

TABLE II
*Summary of bioassay studies**

Line	Antigen (biological units)	Rabbit antiserum† to:		Mean wt, prostate and accessory sex organs‡	p
	mg		*ml*	*g/100 g body wt*	
1	HCG, 0.004 (10 U)		0	0.25	
2	HCG, 0.004 (10 U)	HCG	0.02	0.15	0.0001
3	HCG, 0.004 (10 U)	HCG (NMU)	0.02	0.17	0.0001
4	HCG, 0.004 (10 U)	HCG (NMU, F I)	0.02	0.15	0.0001
5	HCG, 0.004 (10 U)	F II	0.05	0.17	0.0001
6	F II, 0.002 (10 U)	HCG	0.05	0.16	0.0001

* HCG = human chorionic gonadotropin preparation, NMU = normal male urine extract, F I = Fraction I, and F II = Fraction II.

† Adsorbed antiserum followed by letters in parentheses denoting adsorbent used.

‡ Average SD per group = 0.02 g per 100 g body weight. Range of SD per group = 0.01 to 0.03 g per 100 g body weight.

demonstrable. Precipitin lines 5 and 6 were observed with higher concentrations (500 mg per ml) of pregnancy urine extracts (Figure 3, lower half of montage). The horse HCG antiserum used in this illustration was incompletely adsorbed, as evidenced by a faint third precipitin line near the anode. Further studies indicated that this third line was precipitin line 1 and could be removed by adsorption without affecting the two merging precipitin lines (no. 5 and 6). The antigenic similarity existing between HCG preparations and pregnant female urine extracts was further demonstrated by complete adsorption of demonstrable antibodies (precipitating and hemaglutinating) in rabbit HCG antisera.

These studies further confirm the presence of five contaminant antigens (biologically inactive) common to HCG preparations and all human urines. Furthermore, two antibody species present in HCG antisera have been shown to be directed to antigens unique to HCG preparations and pregnancy urine. These antigens share an immunological identity but are distinct and separate as indicated by the single spur (Figure 3).

The hemagglutination of HCG conjugated cells (Table I, line 2) by antisera against extracts of normal male urine was significantly less (1,280) than seen with HCG antisera (25,000). Inhibition of hemagglutination of HCG conjugated cells by antisera against extracts of normal male urine was very similar with extracts of pregnant and nonpregnant urines (Table I, line 2). The inhibition of hemagglutination of HCG antisera with normal urine extracts was relatively minor (6,250) compared with the marked inhibition (200) obtained with pregnancy urine extracts (Table I, line 1). Therefore, the components that are responsible for most of the immunological activity of HCG antisera as measured by hemagglutination and hemagglutination inhibition are probably contributed by the two "pregnancy antigens."

These studies also demonstrate that HCG antisera can be made reliable for determining pregnancy by proper adsorption methods. The persistence of two different antibody species, however, implies that adsorbed antisera against HCG preparations are not HCG specific.

Chromatographic fractionation of HCG preparations. Figure 4 is a chromatogram of HCG lot no. 90272 in which two major peaks were obtained and labeled Fractions I and II, respectively. Fraction I was consistently obtained as a sharp spike with a sodium chloride concentration (14) greater than 0.01 M and less than 0.05 M. Rechromatography of both fractions, under identical conditions, showed no significant changes in the patterns. Similar chromatographic results were obtained with HCG lot no. 90231.

HCG preparation: Vitamerican Corporation Lot No. 90272
Column: DEAE Cellulose, 2.5 x 45 cm
Buffer: Tris, 0.03 m, pH 8.3
Eluant: NaCl, 0.4 m, in Tris buffer

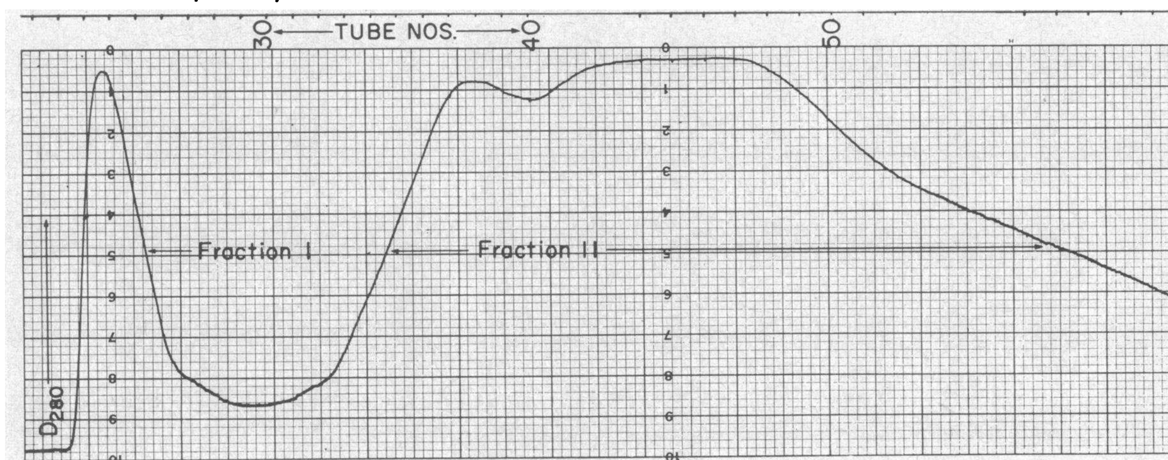

FIG. 4. CHROMATOGRAPHY OF HCG.

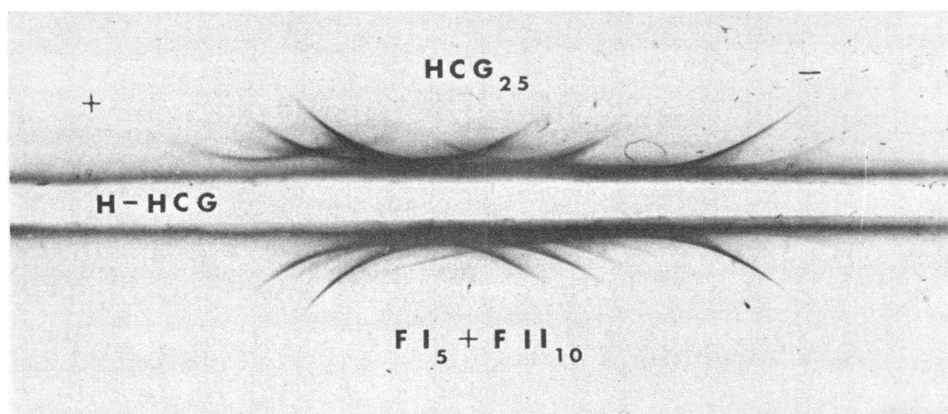

FIG. 5. MIXTURES OF FRACTIONS I AND II EXAMINED IMMUNOELECTROPHORETICALLY WITH HCG ANTISERUM. HCG_{25} = human chorionic gonadotropin preparation (25 mg per ml); H-HCG = horse HCG antiserum; and F I_5 + F II_{10} = mixture of Fraction I (5 mg per ml) and Fraction II (10 mg per ml).

Chromatograms of HCG lot no. 90259 were similar to the other two lots of HCG except for a bifid first peak (chromatogram not shown). The bifid nature of Fraction I, HCG lot no. 90272, was demonstrated by further chromatographic studies. Cellulose acetate electrophoresis, hemagglutination, hemagglutination inhibition, immunoelectrophoresis, and bioassay studies repeatedly failed to demonstrate qualitative differences of components in the three lots of HCG used. The bifid first peak in HCG lot no. 90259 apparently represents a significant quantitative variation peculiar to this lot. The chromatographic results were repeated several times with each HCG preparation.

The specific biological activity of the effluent fractions was not determined. Random qualitative determinations were made and detectable biological activity was present with the ascending portion and descending portion of Fraction II and in the long trailing tail that follows Fraction II.

The amounts recovered comprised about 14% and 36% of the starting HCG preparation for Fraction I and Fraction II, respectively. When each of these fractions was rechromatographed, 70 to 75% of the sample added was recovered. About 45% of the HCG (biological activity) was recovered after Fraction II was chromatographed.

Cellulose acetate electrophoresis of HCG preparations and a mixture of Fraction I and Fraction II resulted in identical patterns. Fraction I revealed two protein zones migrating toward the cathode that corresponded to two similar zones with HCG. Fraction II presented a dumbbell-shaped pattern that corresponded to an oblong and more diffuse electrophoretic pattern observed with HCG preparations.

Significant antigenic alteration was not demonstrated when mixtures of Fraction I and Fraction II were examined immunoelectrophoretically with HCG antisera (Figure 5). A single precipitin line (no. 1) nearest the anode is not seen in the recombined mixture. The missing antigen represents the only element eliminated by the chromatographic procedure and probably adhered to the column. Immunoelectrophoresis of recombined mixtures of Fractions I and II tested against HCG antisera adsorbed with normal male urine powders reproduced the two merging precipitin arcs (no. 5 and 6) with a single spur previously demonstrated (Figure 3) and shown to be unique to pregnancy.

Chromatography apparently did not alter the immunological or biological activities of any of the three HCG preparations. Hemagglutination and hemagglutination inhibition studies demonstrated Fraction II to contain specific activity (immunological) comparable to HCG preparations (Table I, lines 3 and 4). Rabbit antisera to Fraction II demonstrated hemagglutination activity similar to HCG antisera (Table I, line 4). Biological neutralization of gonadotropic activity in HCG or Fraction II was readily accomplished by antisera against either HCG or Fraction II (Table II, lines 5 and 6).

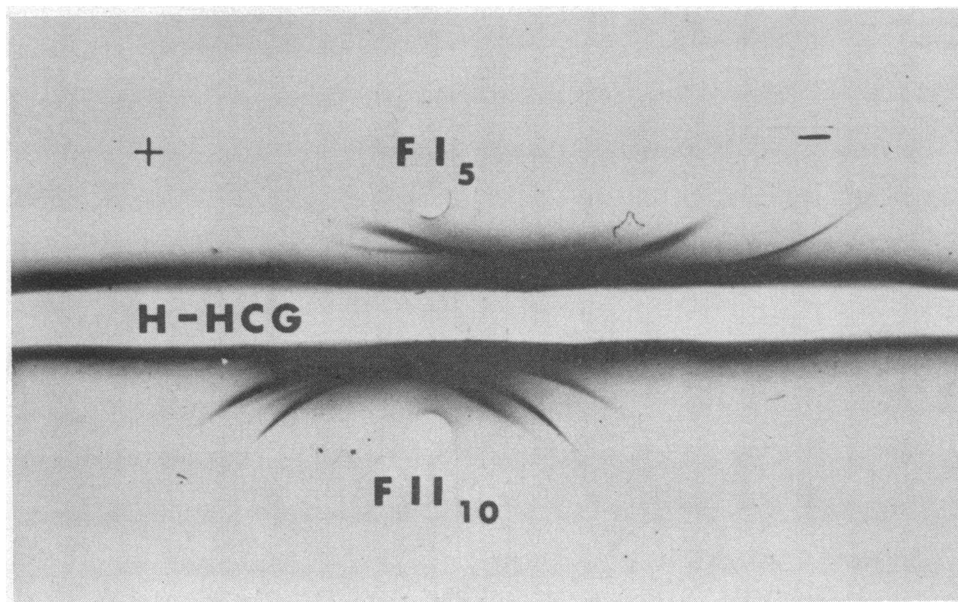

FIG. 6. IMMUNOELECTROPHORETIC STUDY OF FRACTIONS I AND II WITH HCG ANTISERUM. F I_5 = Fraction I (5 mg per ml); H–HCG = horse HCG antiserum; and F II_{10} = Fraction II (10 mg per ml).

The immunoelectrophoretic study of Fractions I and II with HCG antiserum is shown in Figure 6. Each fraction was characterized by four precipitin lines. Only three of the precipitin lines in Fraction I photographed well. Further immunoelectrophoretic studies show that Fraction I contained antigens 4, 5, 6, and 7 and Fraction II contained antigens 2, 3, 4, and 5. Antigens 4 and 5 appear to be common to both fractions. Fraction I contains the pregnancy antigen 6 (Figure 7). Pregnancy antigen 5, however, appears in both Fraction I (Figures 6 and 8) and Fraction II (Figure 7).

Bioassay of the chromatographic fractions by

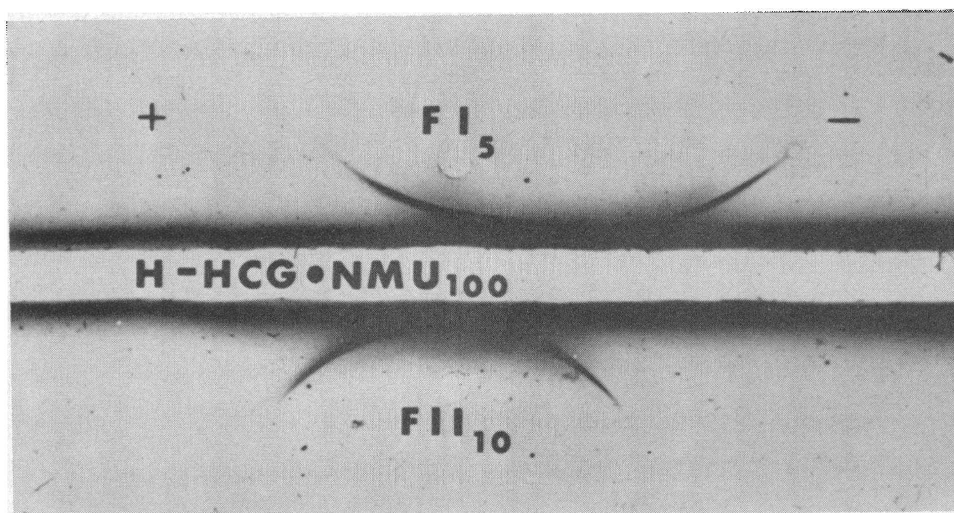

FIG. 7. IMMUNOELECTROPHORETIC STUDY OF FRACTIONS I AND II WITH HCG ANTISERUM ADSORBED WITH NORMAL MALE URINE EXTRACT. F I_5 = Fraction I (5 mg per ml); H–HCG·NMU_{100} = horse HCG antiserum adsorbed with normal male urine extract (100 mg per ml); and F II_{10} = Fraction II (10 mg per ml).

the rat prostate and accessory organ method showed Fraction I (0.1 mg) to contain no detectable gonadotropic activity (p 0.0001). Fraction II, obtained from each of three lots of HCG and tested separately, contained 4,000 to 6,000 U per mg.[12] The chromatographic procedure has doubled the specific biological activity of Fraction II as compared to the activity in the HCG preparations.

In these series of experiments it has been possible to detect variations in commercial HCG preparations that appear to be quantitative rather than qualitative differences in the antigens present. DEAE column chromatography resulted in partial separation of antigens and complete exclusion of biologically active HCG from Fraction I. The studies also indicate that Fraction II contains the major portion of the hemagglutinating activity (Table I, lines 3 and 4) and all of the biological activity recovered.

Immunologic and biologic study of HCG. The separation of antigens into two fractions and concentration of the immunological and biological activity into a single fraction was achieved by column chromatography. However, there was significant contamination of Fraction I with moieties from Fraction II. Immunoelectrophoretic studies testing Fraction I with rabbit antiserum against Fraction II demonstrated four precipitin lines identical to those observed with the homologous antigen (Fraction II). Only two of the major precipitin arcs are easily visible in the photograph shown (Figure 8). Consequently, it is probable that Fraction I contains antigens (2 and 3) in addition to antigens 4, 5, 6, and 7. When Fraction II antisera, adsorbed with normal male urine powder, were tested against Fractions I and II, a single precipitin arc possessing the characteristics of pregnancy antigen 5 was obtained with each fraction. The two rabbit antisera against Fraction II contain demonstrable precipitating antibodies to only one of the two pregnancy antigens previously described.

Fraction I contains the pregnancy antigens 5 and 6. It is not surprising, therefore, that HCG or Fraction II antisera adsorbed with both normal male urine powder (50 to 125 mg per ml of antiserum) and Fraction I powder (2 to 5 mg per ml of antiserum) fail to give precipitin lines when tested against either HCG or Fraction II. Furthermore, HCG or Fraction II antisera so adsorbed contained no demonstrable hemagglutinating antibodies (Table I, line 5). Similar results were obtained with antisera from three

[12] Fraction II (HCG lot no. 90259) was bioassayed at 4,773 U per mg by South Mountain Laboratories, Inc., Maplewood, N. J., using the immature rat uterine weight method employing a 3-dose assay with 15 rats per dose level.

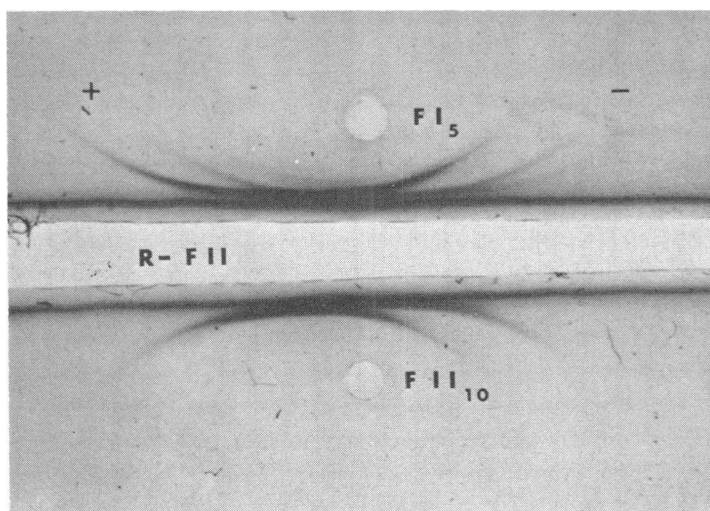

FIG. 8. IMMUNOELECTROPHORETIC STUDY OF FRACTIONS I AND II WITH RABBIT ANTISERUM TO FRACTION II. F I_5 = Fraction I (5 mg per ml); R–F II = rabbit antiserum to Fraction II; and F II_{10} = Fraction II (10 mg per ml).

rabbits immunized with HCG preparations and also antisera from two rabbits immunized with Fraction II.

Four HCG antisera from which all detectable precipitating and hemagglutinating antibodies were removed by adsorption were capable of completely neutralizing the biological activity of 10 U of HCG tested in the male rat (Table II, line 4). Consequently, we feel that HCG, biologically active or inactive, failed to elicit the production of precipitating antibodies in the five rabbits immunized. It is possible that biologically inactive HCG elicits precipitating antibodies that do not cross-react with biologically active HCG and may be responsible for one or both of the pregnancy antigens described.

The antibodies in HCG antisera that neutralize the biological activity of HCG were not associated with antigens responsible for precipitating or hemagglutinating antibodies present in the same antisera. The precipitating and hemagglutinating antibodies in antisera directed against HCG preparations and specific for pregnancy are elicited by the two pregnancy antigens previously described and not by biologically active HCG.

Discussion

The majority of immunological studies of HCG have been performed with antiserum obtained by immunizing rabbits with commercial preparations (1–3, 6, 7). The presence of contaminants in these preparations has been demonstrated by electrophoresis, immunodiffusion, or immunoelectrophoresis (6, 7, 15, 16). The number of contaminants shown to be present has been variable, depending on the preparations and characterization techniques used. Adsorption methods using serum or urine have been employed to increase the HCG specificity of the antiserum (1, 3, 6). A previous study from this laboratory questioned the validity of adsorption and the specificity of the antiserum after adsorption (7).

Immunoelectrophoresis of commercial HCG preparations with homologous antisera (Figure 1) consistently demonstrates seven precipitin lines. This number and the pattern of precipitin lines have been reproduced with antisera from three different rabbits. An eighth antigen probably is present in the HCG preparations employed. Antigens 1, 2, 3, 4, and 7 are contaminants, lack gonadotropic activity, and are common to all human urines. Only two of these contaminant antigens demonstrate precipitin lines (Figure 2, b and c) with rabbit antihuman serum and appear to be of serum protein origin. These two contaminants may represent the "long $alpha_2$" and the second $alpha_2$ globulins described in urine by Berggård (17). Berggård was unable to demonstrate these antigens in human serum. Antisera against the commercial HCG preparations employed in this study can cause two precipitin lines (Figure 2, precipitin lines b and c) similar to those observed by Berggård when tested against whole human serum. The remaining contaminating antigens in HCG preparations probably represent urinary macromolecules, not originating in whole serum. This is not surprising, since at least two-thirds of urinary macromolecules have no serum counterpart (18).

The adsorption of HCG antiserum with urine is not practical, since the amount required represents quantities greater than commonly used and would dilute the antiserum to a very low order of sensitivity. After adsorption with normal, nonpregnant human urine powders, two precipitating antibody species remain in HCG antisera demonstrable by immunoelectrophoresis (Figure 3). These precipitin lines (no. 5 and 6) showed no change in character or intensity, despite adsorption with increasing increments of extracts up to 800 mg (4 L of urine) per ml of antisera. These antigens unique to pregnancy urine are distinct but share partial immunological identity. It is possible, therefore, to prepare antisera against HCG preparations, by adsorptions with extracts of nonpregnant urine, which can be used as reliable indexes of pregnancy. Such antisera, however, contain at least two antibody species and, therefore, cannot be considered specific for any single moiety.

It was decided that these materials had to be purified and characterized before reliable and valid antisera for measuring and localizing HCG could be produced. Two major fractions were repeatedly obtained by chromatography. One antigen (no. 1) was no longer discernible after DEAE chromatography. All of the remaining antigens (no. 2, 3, 4, 5, 6, and 7) were detectable in Fraction I. Fraction II, however, contained antigens 2, 3, 4, and 5.

The possibility exists that antigens 4 and 5 may be macromolecules that dissociate to give antigenic moieties responsible for precipitin lines 2 and 3. In this way the presence of all of the antigens in Fraction I that come off the column first may be explained. Both pregnancy antigens 5 and 6 were present in Fraction I, whereas Fraction II contained only pregnancy antigen 5. No detectable gonadotropic activity (p 0.0001) was present in Fraction I (0.1 mg) when repeatedly tested in male rats. Therefore, the possibility that pregnancy antigens 5 and 6 are not associated with biologically active HCG was considered.

The contamination of Fraction I suggests the possibility of adsorbing all precipitating antibodies from HCG antisera. Several antigens, however, were present only in trace amounts; consequently, large quantities of Fraction I would be required. Therefore, a combination of normal male urine and Fraction I powders was used to remove all precipitating and hemagglutinating antibodies from HCG antisera. Neither Fraction I nor normal male urine extracts contained any measurable amounts of biologically active HCG. Consequently, the possibility of preparing antisera devoid of demonstrable precipitating and hemagglutinating antibodies but capable of neutralizing the biological activity of HCG was tested. This test was performed with antisera from four rabbits (Table II, line 4). Complete neutralization of biologic activity (10 U of HCG) was achieved. A previous study (*7*) in which the biologic neutralizing ability of HCG antisera was titrated showed that neutralizing activity is negated before loss of hemagglutinating activity. Hemagglutination is markedly more sensitive for measuring antibodies than is the biological neutralization of HCG. Therefore, it was considered unlikely that the antibodies responsible for neutralization were the same as those causing hemagglutination. The presence of biological neutralizing antibodies in adsorbed HCG antisera devoid of precipitating and hemagglutinating antibodies is, therefore, best explained by assuming that the antibodies which neutralize HCG are directed to antigen (or antigens) different and distinct from the antigens eliciting the production of precipitating and hemagglutinating antibodies. Immunological methods probably measure moieties not responsible for the activity of HCG preparations measured with biological methods. This thesis was previously proposed, and supporting evidence was presented by Wide (1).

Summary

1) Seven antigens are demonstrable in commercial human chorionic gonadotropin (HCG) preparations by immunoelectrophoresis. Five of the seven antigens present are contaminants, lack gonadotrophic activity, and are common to HCG preparations and acetone precipitated urine extracts.

2) HCG antisera after adsorption with nonpregnant urine extracts contain precipitating antibodies to two antigens unique to pregnancy urines and HCG preparations. Such antisera appear highly specific for pregnancy.

3) The two antigens unique to pregnancy are dissimilar but share partial immunological identity by immunoelectrophoresis. The precipitating and hemagglutinating antibodies in HCG antisera are directed against these antigens.

4) The antibodies that neutralize the gonadotrophic activity of HCG as tested in the rat are independent of precipitating and hemagglutinating activity in antisera prepared against three different commercial HCG preparations.

5) Antibodies directed against biologically active HCG are detected with certainty only by neutralization experiments. Current immunological methods appear to be of questionable value in the detection, assay, and localization of biologically active HCG.

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