Purification and characterization of guinea-pig chorionic gonadotrophin*

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Summary. A human chorionic gonadotrophin-like protein (GF-1, 1.0 g) from the placentae of 50 guinea-pigs killed at Day 26 of gestation was purified by pH and ammonium salt fractionation followed by column chromatography on DEAE-Sephadex and filtration on Sephadex G-100. Relative to the Second International hCG standard (MRC 61/6) GF-1 had an immunological potency of 21 000 i.u./mg as measured in a specific hCG- β radioimmunoassay and, using the ovarian ascorbic acid depletion assay, an apparent biological potency of 24 064 i.u./mg. Isoelectric focussing yielded 6 bands between pH 4.4 and 5.7 and the material comprised two non-covalently linked subunits. The Stokes' radii were 3.40 nm for the native preparation, and 2.38 nm and 3.15 nm for GF-1- α and GF-1- β subunits respectively. The guinea-pig placenta therefore produces a chorionic gonadotrophin which on purification has physico-chemical, biological and immunological properties similar to those of hCG.

Introduction

The presence of placental gonadotrophins is well documented for primates (Aschheim & Zondek, 1927; Hodgen, Tullner, Vaitukaitis, Ward & Ross, 1974; Hobson & Wide, 1976) and equids (Cole & Hart, 1930; Allen, 1979) but chorionic gonadotrophin (CG)-like proteins have been reported for few other animals. Biologically active gonadotrophins with gonadotrophin profiles similar to hCG and its subunits are present in the placentae of the rat, mouse and hamster (Wide & Hobson, 1978), and implanting mouse blastocysts synthesize and release a glycoprotein allied to chorionic gonadotrophin (Fishel & Surani, 1980). These CG-like proteins have also been reported in the rock hyrax and spring-hare (Gombe, Oduor-Okelo & Else, 1980). Bambra (1981), using an immuno-histochemical technique involving a specific antiserum against the hCG- β subunit, has demonstrated the presence of an hCG-like material in the guinea-pig placenta on Days 16, 21, 31 and 46 of pregnancy and electron microscope studies suggest that this material is probably synthesized in the spongy syncytium.

The studies described in this paper were carried out in an attempt to extract, purify and characterize this hCG-like material present in the guinea-pig placenta.

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Materials and Methods

For use in the ovarian ascorbic acid depletion assay hCG (Primogonyl) and PMSG (Anteron) were purchased from Schering Ag (Berlin, West Germany). Blue dextran, cytochrome-C, chymotrypsinogen, ovalbumin and bovine serum albumin, DEAE-Sephadex A50 and A25 and Sephadex G-100 and G-150 were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Cellogel was purchased from Lab. Sales Ltd (U.S.A.), and Coomassie brilliant blue R250 was a product of Colab Lab. (Chicago, U.S.A.).

Extraction and fractionation of placentae. Placentae were collected from 50 guinea-pigs at Day 26 of gestation. A crude placental extract was prepared by a modification of the method of Lee, Wong, Lee & Ma (1977) in which the pH of the homogenate was adjusted with HCl to 5.3 and 4.3 instead of pH 4.0 and 3.0 respectively.

Purification of hCG-like material. All steps of purification were carried out according to the method of Bahl (1969) and were done at 4°C unless specified otherwise. Eluates from columns were monitored for protein content by measuring absorbance at 280 nm. Pooled peaks were assayed for hCG-like activity using radioimmunoassay.

Radioimmunoassay. An hCG- β subunit double-antibody radioimmunoassay was used to monitor column eluates for hCG-like activity. The rabbit antiserum used (F98) was produced with a multisite intradermal injection technique against the hCG- β subunit preparation CR115 (R. E. Canfield, New York). This antiserum was used at a final dilution of 1:125 000 and showed cross-reactivity with other human protein hormones as follows: CG, 6.67%; CG- α , <0.2%; FSH, 0.2%; FSH- α , 0.27%; FSH- β , <0.2%; LH, 5.0%; LH- α , 0.36%; LH- β , 0.8%; TSH, 1.0%; GH, <0.2%; placental lactogen, <0.2%; prolactin, <0.2%. The hCG- β subunit preparation CR115 was used for iodination and, after demonstrating parallelism to inhibition curves obtained with the purified hCG- β preparation CR115, the Second International hCG Standard (MRC 61/6, NIBSC, London) was used as the reference material. Serial dilutions of human pregnancy serum, guinea-pig plasma at mid-pregnancy and placental extracts from the guinea-pig showed no significant deviation from parallelism to the hCG- β standard curves: the assay sensitivity was <0.12 m.i.u./tube. The inter-assay coefficient of variation was 5.1%.

Isoelectric focussing on cellulose acetate membranes. This was performed according to the method of Ambler & Walker (1979) using an ampholyte gradient of pH 3.5–10. Proteins focussing at different pH points were eluted with 0.9% (w/v) NaCl and assayed for hCG-like activity.

Bioassay. Biological activity was estimated using a modification of the ovarian ascorbic acid depletion assay as described by Parlow (1961). A four-point design was used and the Second International hCG Standard was used as the reference preparation. The relative potency, fiducial limits of error at $P = 0.95$ and index of precision (λ) of each assay were calculated according to the method of Gaddum (1953).

Separation of subunits. The separation of the subunits of the purified guinea-pig hCG-like protein (GF-1) was carried out according to the method of Morgan & Canfield (1971).

Stokes' radius and approximate molecular weight determinations. The Stokes' radius and approximate molecular weight determinations were carried out by gel filtration on Sephadex G-150 according to the method of Ryan, Jiang & Hanlon (1970). A solution of 5 ml containing 15 mg of each of (1) cytochrome-C (M_r 12 500, Stokes' radius 1.64 nm), (2) bovine serum albumin (M_r 67 000, 3.55 nm), (3) ovalbumin (M_r 43 000, 3.05 nm) and (4) chymotrypsinogen A (M_r 25 000, 2.09 nm) was applied to a column of Sephadex G-150 (2 $\times$ 100 cm) previously equilibrated with 0.04 M-sodium phosphate buffer, pH 7.5 at 4°C. Samples (10 mg) of native GF-1, GF-1 α and GF-1 β in 5 ml buffer were each chromatographed separately on the same column under identical conditions.

Gel electrophoresis. The separation was performed using 7% cross-linked polyacrylamide gels with a running pH of 6.8 at 30 mA for 12 h according to the method of Swank & Munkres (1971). Gels were run under reducing (8 M-urea in gel mixture) and non-reducing conditions.

Results

Purification

The use of pH and ammonium sulphate fractionation yielded 10.3 g of a freeze-dried crude extract (CPE-26) with an immunoassay potency of 7080 i.u./mg. Ion exchange chromatography of CPE-26 on a DEAE-Sephadex A50 column yielded several peaks designated I–XI as shown in the elution profile presented in Text-fig. 1. Peaks V to IX showed the presence of hCG-like material by radioimmunoassay. The greatest immunoassay potency was present in pooled peak VIII which yielded 1.8 g of a freeze-dried material (designated IC-1) with an immunological potency of 12 800 i.u./mg.

Text-fig. 1. Chromatography of crude guinea-pig material on DEAE-Sephadex 50. Sample in 0.02 M-Tris-phosphate buffer, pH 8.7, was applied to the column (5 × 100 cm) in 0.04 M-Tris-phosphate buffer and eluted in a step-wise discontinuous gradient. Elution was started with 0.04 M-Tris-phosphate buffer, pH 8.7, and was changed to 0.1 M-NaCl (a) and 0.2 M-NaCl (b) in 0.04 M-Tris-phosphate buffer, pH 8.7, and finally 0.2 M-NaCl (c) in 0.04 M-Tris-phosphate, pH 9. Absorbance of every 25th fraction is shown.

Rechromatography of preparation IC-1 on a 2.5 × 100 cm DEAE-Sephadex A50 column, using a 0.1–0.2 M-NaCl linear salt gradient in 400 ml 40 mM-Tris-phosphate pH 8.3, yielded a single large peak. Pooling and freeze-drying of the fractions within this peak gave a yield of 1.1 g of a preparation (designated IC-2) with an immunological potency of 15 700 i.u./mg. Preparation IC-2 was subjected to further purification by gel filtration on a 2.5 × 100 cm Sephadex G-100 column in 0.05 M-sodium phosphate buffer pH 7.5. A highly purified material (designated GF-1) eluted as a single peak and when pooled produced a yield of 1.0 g of freeze-dried material with an immunoassay potency of 21 000 i.u./mg.

An aqueous solution of GF-1 (1 mg/ml) had an optical density of 0.38 at 280 nm in a 1-cm cell.

Isoelectric focussing

Preparation GF-1 focussed in 6 bands between pH 4.4 and 5.7 on a cellulose acetate membrane and after elution each of these bands was immunoactive. The isoelectric focussing points of these bands and their estimated immunological potencies are shown in Table 1 and are compared with data from isoelectric focussing studies of a number of hCG preparations.

Table 1. A comparison of the isoelectric points of a purified guinea-pig placental extract (GF-1) and different preparations of hCG and the immunological potencies associated with eluates of GF-1 isoelectric bands 1–6

	Isoelectric band number					
	1	2	3	4	5	6
(a) <i>Isoelectric points</i>						
GF-1; this study	4.4	4.6	4.9	5.1	5.5	5.7
hCG; Brossmer <i>et al.</i> (1971)	4.0	4.2	4.5	4.6	4.9	5.2
hCG; Graesslin <i>et al.</i> (1972)	3.8	4.3	4.5	4.9	5.1	—
hCG; Merz <i>et al.</i> (1974)	4.0	4.2	4.6	4.7	4.9	5.2
hCG; Qazi <i>et al.</i> (1974)		3.73 – 4.35				
(b) <i>Immunoassay potency</i>						
GF-1 (i.u./l); this study	20	31	59	28	30	8

Bioassay

A pilot assay showed that 15 and 45 i.u. of the 2nd International Standard hCG preparation were suitable for use as standard potencies in the four-point ascorbic acid depletion assay. On the basis of its immunological potency, preparation GF-1 was assayed at two dose ranges of 0.6, 1.8 and 5.4 µg and 1.1, 3.3 and 9.9 µg per rat respectively in separate assays. The index of precision for these assays was 0.11 and 0.9 and the estimates (and 95% confidence limits) of relative biological potency were 22 585 (18 500–27 583) and 25 545 (22 454–29 273) respectively, giving a mean biological potency of 24 064 i.u./mg.

Separation of subunits

The elution pattern of 400 mg GF-1 material treated with 10 M-urea, from a 2.5 × 100 cm DEAE–Sephadex A25 column is shown in Text-fig. 2. In the hCG-β RIA the first emergent peak had a potency of < 30 i.u./mg compared to a potency of 15 000 i.u./mg in the second emergent peak and the peaks were therefore tentatively designated as α and β subunits. Incubation of the two peaks together for 3 h at room temperature resulted in 63% recombination as determined by gel filtration and immunoelectrophoresis.

Stokes' radius and molecular weight

Gel filtration of GF-1 on Sephadex G-150 gave a value of 3.40 nm for the Stokes' radius and 59 000 for the molecular weight. The α-subunit of GF-1 had a Stokes' radius of 2.38 nm and a molecular weight of 30 500. The corresponding values for the β-subunit of GF-1 were 3.15 nm and 49 000.

Polyacrylamide gel electrophoresis of A, crude guinea-pig material; B, GF-1; and C, subunits of GF-1. Migration is towards the anode at the bottom. Gels A and B were run under non-reducing conditions and Gel C under reducing conditions (8 M-urea).

Text-fig. 2. Separation of the subunits of 400 mg native GF-1 on DEAE-Sephadex A25. The arrow represents the application of the second buffer.

Polyacrylamide gel electrophoresis

Results for polyacrylamide gel electrophoresis are presented in Plate 1. The crude preparation of CPE-26 yielded 3 broad and 5 narrow bands and the purified preparation GF-1 yielded one broad band, and a smaller more slowly migrating band near the cathode. After elution most of the immunoassay potency was present in the larger band (88%; 210 mi.u./ml) when compared to the smaller band (12%; 29 mi.u./ml). Polyacrylamide gel electrophoresis run under reducing conditions (8 M-urea) gave two broad bands and smaller bands near the cathode.

Discussion

The value of 0.38 obtained for the absorbance of an aqueous solution (1 mg/ml) of the purified guinea-pig preparation (GF-1) compares well with the value of 0.388 reported for hCG (Bahl, 1969).

Purified hCG has been reported to give up to 6 bands on isoelectric focussing (Brossmer, Dorner, Hilgenfeldt, Leidenberger & Trude, 1971; Graesslin, Weise & Czygan, 1972; Merz, Hilgenfeldt, Dorner & Brossmer, 1974; Qazi, Mukherjee, Javidi, Pala & Diczfalusy, 1974) and similar results were obtained with the purified guinea-pig preparation (GF-1). Microheterogeneity in hCG is common as reflected by its behaviour in polyacrylamide gel, isoelectric focussing and ion-exchange chromatography (Goverde, Veenkamp & Homan, 1968; Van Hell, Matthijsen & Homan, 1968; Bahl, 1969; Graesslin, Weise & Braendle, 1973; Merz *et al.*, 1974). All these forms have been shown to have an almost identical amino acid composition with variation in their carbohydrate portion, especially the sialic acid content (Bahl, 1973) and it has been suggested that this microheterogeneity could come from the introduction of artefacts during isolation or from the incomplete synthesis of carbohydrate chains. Consideration of the fact that all the 6 bands of GF-1 obtained by isoelectric focussing had an immunoassay potency suggests that the guinea-pig preparation, like the hCG, exhibits microheterogeneity.

Biological potencies of purified hCG have been reported to be between 12 000 and 19 000 i.u./mg (Bahl, 1969; Morgan & Canfield, 1971; Pierce, Bahl, Cornell & Swaminathan, 1971; Bahl, Carlsen & Bellisario, 1973; Morgan, Canfield, Vaitukaitis & Ross, 1974) and these are always higher than the immunological activities of highly purified hCG which are between 5600 and 7000 i.u./mg (Bahl, 1969; Donini, Oliveri, Ricci & Donini, 1973). A similar situation was found when the guinea-pig preparation (GF-1) was used in these assays.

The reported molecular weights for hCG calculated from gel filtration data are 83 000 (Donini *et al.*, 1973), 59 000 (Bahl, 1969) and 67 000 (Canfield, Morgan, Kammerman, Bell & Agosto, 1971). The estimate of Bahl (1969) compares favourably with the results of the present study for GF-1. However, this method measured molecular dimensions and is known to give falsely high values for glycoproteins with appreciable carbohydrate content (Andrews, 1970). For this reason it was believed that the measure of Stokes' radius (a dimensional measure) would provide a more realistic estimate; the value of 3.40 nm (34 Å) for GF-1 compared well with the value of 33 Å reported for hCG (Mori, 1970). The values of Stokes' radii for the two subunits of GF-1 (23.8 Å and 31.5 Å) are remarkably similar to the values of 23.3 Å and 30.2 Å reported for the α - and β -subunits by Morgan & Canfield (1971).

Purified GF-1 run under non-reducing conditions gave a broad band and a small more slowly moving band near the cathode. Because this latter band was not observed in the electrophoretic pattern of the crude extract, but does have immunological activity, it is possible that this band was either too dilute to be detected in the original extract or had arisen as a consequence of purification procedures. It is known that the electrical charges of glycoproteins are affected by terminal sialic acid residues and this can affect the electrophoretic mobility. Thus these two bands may be due to two populations of GF-1 differing in their sialic acid content.

Morgan & Canfield (1971) demonstrated the heterogeneity of the two hCG subunits by using polyacrylamide gel electrophoresis. They obtained two unequal broad bands and designated the broader, cathode-positioned band as the α -subunit, and the smaller anode band as the β -subunit. Similar results were obtained with the guinea-pig preparation GF-1 and we were also able to obtain a 63% recombination of the α - and β -subunits.

In the results discussed above, it has been shown that the purified guinea-pig preparation (GF-1) and hCG are similar in terms of their behaviour during ion-exchange chromatography, polyacrylamide gel electrophoresis and isoelectric focussing and they have similar Stokes' radii. Both have two non-covalently linked subunits, and display microheterogeneity. In addition to such similar physicochemical properties, GF-1 also possessed high biological activity in the rat ovarian ascorbic acid depletion assay and also a high immunological potency in the specific hCG- β immunoassay.

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