Pattern of medium proteins radiolabelled after culture of Day 13 to 16 pig conceptus tissue with [3H]leucine and absence of chorionic gonadotrophin-like activity*

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Summary. Day 13–16 pig conceptus tissue was cultured for 24 h in medium containing [3H]leucine. The patterns of radioactively labelled proteins that were released into the medium during culture were analysed by SDS–polyacrylamide gel electrophoresis and fluorography. Day-13 conceptuses released two major radiolabelled proteins of M, 23 000 and 26 000 and Day 14–16 conceptuses released these as well as proteins of M, 14 000, 19 000, 44 000, 50 000 and 88 000. Various immunological and biological tests for a human chorionic gonadotrophin-like activity were performed on tissue extracts and on culture medium, but there was no evidence for its presence in the pig conceptus at Day 13–16 of gestation.

Introduction

In mammals the initiation of events leading to successful implantation is thought to require some form of chemical communication between maternal endometrium and developing embryo (Denamur, Kann & Short, 1973; Heap, Flint & Gadsby, 1979). There is considerable evidence for one or more factors with anti-luteolytic properties in sheep embryo homogenates and extracts (Rowson & Moor, 1967; Godkin, Coté & Duby, 1978; Martal, Lacroix, Loudes, Saunier & Wintenberger-Torrès, 1979). At Days 12–13 of pregnancy, when the sheep blastocyst undergoes rapid elongation and maternal recognition occurs, only one major protein was found to be released in culture (Godkin, Bazer, Moffatt, Sessions & Roberts, 1982a). The pig blastocyst undergoes rapid elongation from Day 11 of pregnancy and implantation occurs around Day 18 (Crombie, 1972). Day 16–25 embryo extracts exert a luteotrophic effect (Longenecker & Day, 1972; Ball & Day, 1982a, b), but in this species there is evidence for an active component that is a steroid rather than a protein because it can be absorbed by charcoal and is not denatured by heat (Ball & Day, 1982a, b). This lends support to the proposal that the embryonic anti-luteolytic factor may be oestrogen, which acts by blockade of luteolysis mediated by prostaglandin F-2α (Bazer & Thatcher, 1977; Bazer, Geisert, Thatcher & Roberts, 1982). Preliminary studies have suggested, however, that a gonadotrophin-like substance may be present in the pig placenta and embryonic membranes (Saunders, Ziegik & Flint, 1980; Flint, Saunders & Ziegik, 1982). There is also evidence for chorionic gonadotrophin-like factors in rabbit embryos (Haour & Saxena, 1974; Asch, Fernandez, Magnasco & Pauerstein, 1978) and rodent implantation sites (Wide & Wide, 1979), based on competition for hCG-binding receptors or on immunological cross-reaction with antisera to hCG.

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Moreover, Fishel & Surani (1980) demonstrated the synthesis and release of a major glycoprotein by mouse blastocysts in culture by analysis of both culture medium and blastocyst tissue. The aim of our studies was to determine whether Day 13–16 pig conceptuses synthesize and secrete specific proteins in culture and whether these tissues contain or secrete a protein which is similar, either immunologically or functionally, to hCG. After completion of our experimental work, a study of the molecular size and charge of proteins synthesized by the pig conceptus was published by Godkin, Bazer, Lewis, Geisert & Roberts (1982b), and Masters et al. (1982) reported that high molecular weight glycoproteins are released by pig, sheep and cow blastocysts in culture. Our results on molecular size of synthesized proteins are in general agreement with theirs.

Materials and Methods

Materials

Purified hCG (10 800 i.u./mg) was a gift of Dr V. C. Stevens, Ohio State University, U.S.A. Tissue culture supplies were purchased from Flow Labs, Irvine, U.K. Streptomycin sulphate and Crystapen were from Glaxo Labs Ltd, Greenford, U.K. Fetal calf serum was from Seralab Ltd, Crawley Down, U.K. Acrylamide, N,N,N,N-tetramethylethlenediamine, 2-mercaptoethanol, polyvinyl alcohol, Freund’s incomplete adjuvant, and bovine serum albumin (BSA) (fraction V) were from Sigma, Poole, U.K. Sodium dodecyl sulphate (SDS), glycine, silver nitrate, glutaraldehyde, formaldehyde and methanol were from BDH Biochemicals, Poole, U.K. X-OMAT S film was from Kodak Ltd, Hemel Hempstead, U.K. Sephadex G-50 and activated CNBr Sepharose 4B were from Pharmacia Ltd, Uppsala, Sweden. L-[4,5-3H]leucine (sp. act. 130 Ci/mmol), 14C-labelled methylated protein molecular weight standards for electrophoresis and Na125I were obtained from Amersham International, Amersham, U.K. Medium 199, donkey anti-rabbit immunoglobulin precipitating antibody and Freund’s complete adjuvant were purchased from Wellcome Laboratories, Beckenham, U.K. Fluorescein isothiocyanate (FITC)-labelled antirabbit immunoglobulin antiserum was from Miles Ltd, Slough, U.K. OCT embedding compound for cryostat sectioning was from R. A. Lamb Ltd, London, U.K. All other chemicals were of the best reagent grade obtainable commercially.

Animal tissues

Uterine tracts were removed from sows between Days 13 and 16 of pregnancy (onset of oestrus = Day 0) after slaughter at a local abattoir. The tracts were flushed with sterile prewarmed (37°C) Eagle’s Minimum Essential Medium (MEM) and conceptuses were collected into sterile Petri dishes. The tissue was washed with at least two changes of prewarmed medium before distribution into multiwell Petri dishes for culture. For testing in radioimmunoassay and radioreceptor assay, conceptus tissue was collected in phosphate-buffered saline (PBS) containing 0·9% (w/v) NaCl in 0·05 M-sodium phosphate, pH 7·5. It was then sedimented and stored frozen at −20°C until extraction with Triton X-100 (1% v/v).

Tissue containing gonadotrophin receptors for use in radioreceptor assay was prepared by previously described methods (Catt, Dufau & Tsuruara, 1972; Stewart, Allen & Moor, 1976; Garverick, Polge & Flint, 1982). Corpora lutea from sows between Days 6 and 18 of pregnancy were dissected free of connective tissue and were homogenized (10% w/v) in Buffer A (10 mM-sodium phosphate, 5 mM-MgCl$_2$, 15 mM-sodium azide, 0·1 M-sucrose, pH 7·4) using a Polytron homogenizer. The particulate material sedimenting between 200 and 10 000 g was taken up in Buffer A containing Triton X-100 (1% v/v) and insoluble material was removed by centrifugation at 150 000 g. The supernatant containing solubilized receptors was freeze-dried and stored at −20°C. Rat Leydig cell receptors were prepared as described by Stewart et al. (1976).
In-vitro culture of conceptus explants

Eagle's Minimum Essential Medium without leucine (MEM-leu) was supplemented with penicillin (Crystapen, 200 i.u./ml), streptomycin (0.02% w/v) and 0.1% (w/v) polyvinyl alcohol. Conceptus explants were cultured using a method adapted from Rice, Ackland & Heap (1981). Tissue was placed on pieces of filter paper (Millipore THWP 102F0) on stainless-steel grids or directly into wells containing 1–2 ml medium. After addition of [3H]leucine (10–50 μCi) cultures were placed in an incubator jar, the atmosphere was replaced with humidified air containing 5% CO₂, and incubation was at 37°C for 24 h. The medium was then aspirated, centrifuged at 10 000 g, and stored at −20°C. The blastocyst tissue was washed extensively with PBS and stored at −20°C. The medium was subsequently thawed and subjected to gel filtration on small columns (0.5 × 20 cm) of Sephadex G-50 to remove unincorporated radioactivity and salts. After elution with distilled water the protein-containing fraction was freeze-dried.

Iodination of hCG

Purified hCG was iodinated by a chloramine T method (Stewart et al., 1976), purified by gel filtration on Sephadex G-50 (fine), and stored in 50 mM-sodium phosphate, 1% BSA (w/v), pH 7.4 at 4°C. The 125I-labelled hCG had an apparent specific radioactivity of 10–30 μCi/μg. Measurement of specific radioactivity by self-displacement (Catt, Ketelslegers & Dufau, 1976) gave values approximately 3-fold higher in two determinations. The active fraction as determined by incubation with excess tissue was about 40%.

Receptor assay

Binding of 125I-labelled hCG to rat testis Leydig cell preparations was measured by the method of Catt et al. (1976). Bound hormone was separated from free hormone by dilution followed by centrifugation at 900 g. Non-specific binding (2–5%) was determined in the presence of a large excess of human LH (about 10 i.u./tube) from human pituitary fraction CM-1 (Stockell Hartree, 1966). Receptor number and affinity were determined by equilibration of receptors (3 h at 37°C) with increasing concentrations of unlabelled hormone in the presence of a constant amount of 125I-labelled hCG (0.5–1.0 ng). Scatchard (1949) or weighted logit-log (Davis & Rodbard, 1979) transforms of the data yielded the number of binding sites and the equilibrium dissociation constant (K_d) of binding. The 125I radioactivity was counted by a gamma spectrometer (75% efficiency). In studies of binding of 125I-labelled hCG to solubilized receptor preparations, separation of bound from free hormone was by double precipitation with polyethylene glycol (Dufau, Charreau & Catt, 1973).

Down-regulation of hCG-binding receptors

Adult male rats (60–70 days of age) were allocated to Groups A, B or C (4 animals/group). Each rat was injected s.c. with Buffer B alone (0.1% v/v Triton X-100 in PBS) (Group A); 25 i.u. hCG in Buffer B (Group B); or detergent extract of Day-16 conceptuses (approximately 0.5 embryo equivalent) in Buffer B (Group C). Rats were killed at 25 or 50 h after injection. Both tests from each animal were used to prepare separate Leydig cell receptor preparations and specific binding of 125I-labelled hCG was measured.

Preparation of anti-hCG immunoglobulin

An antiserum to hCG (Organon lot 375, potency 2790 i.u./mg) was raised in rabbits by the method of Herbert (1978). The serum immunoglobulin fraction (IgG) was isolated by sodium
sulphate fractionation (Heide & Schwick, 1978), extensively dialysed against distilled water, clarified by centrifugation, freeze-dried and stored at −20°C.

Radioimmunoassay

Radioimmunoassay (RIA) for cross-reactivity with hCG was by the double-antibody procedure developed for the World Health Organization matched RIA reagent programme. Rabbit antiserum to hCG and 125I-labelled hCG were used with donkey anti-rabbit gamma globulin as second antibody. The assay buffer containing 1% (w/v) BSA in PBS was modified in these studies to include Triton X-100 at a final assay concentration of 0-03% (v/v). Labelled antigen, antiserum and test solution were incubated for 12 h at 4°C in a total volume of 0.2 ml. Second antibody (1:30 dilution) in buffer (0.1 ml) containing 8% (w/v) dextran and normal rabbit serum (1:200 dilution) was then added and incubation continued for 6 h at 4°C followed by addition of 1 ml buffer, centrifugation, removal of soluble material and determination of radioactivity in the precipitate. Non-specific binding was 1-4% and the lower limit of detection of the assay was 0.5 ng 125I-labelled hCG.

Immunofinity chromatography

An affinity chromatography matrix for hCG was prepared by coupling anti-hCG IgG to activated CNBr-Sepharose 4B according to the manufacturer’s instructions. The gel was washed extensively by a batch procedure with: (1) equilibration buffer—PBS containing 1% (w/v) BSA and 0-1% (v/v) Triton X-100, pH 7-4; (2) elution buffer—0-1 M-propionic acid, pH 3; (3) buffer to remove hydrophobically adsorbed material—50 mM-sodium phosphate, pH 7-4, 1 M-NaCl, 1% (w/v) BSA; (4) 6% (w/v) BSA in PBS. The gel was then packed (0.5 ml per column) into small siliconized glass columns (0.5 × 10 cm) and stored at 4°C. The columns remained active for over 3 months and could each bind more than 25 ng 125I-labelled hCG.

Immunofluorescence

Day 13–16 porcine conceptus tissue was embedded in OCT medium, frozen on solid CO2 and sectioned in a cryostat. The cryostat sections were transferred to acid-washed coverslips and dried under a fan at room temperature for 30 min. The method for detection of immunofluorescence was adapted from Johnson, Holborow & Dorling (1978). Sections were incubated for 1 h at room temperature with hCG antiserum at dilutions of 1:10, 1:100 and 1:1000 in 1% (w/v) BSA in PBS. The sections were washed twice with the above buffer and then incubated for 30 min with FITC-antirabbit serum at 1:20 dilution. The sections were again washed twice with buffer and once with distilled water before mounting in glycerol on microscope slides. Control slides were prepared with normal rabbit serum in place of antiserum and without hCG antiserum. All slides were viewed with a Zeiss epifluorescence microscope.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli, 1970) was performed under reducing conditions on 15% acrylamide slab gels (1.5 mm thick) with a 3% stacking gel. Samples in microfuge tubes were dissolved in 0.25 M-Tris–HCl, 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 15% (v/v) glycerol, pH 6.8. The tubes were immersed in boiling water for 2 min and centrifuged at 10 000 g. Samples of 50–100 µl were applied and gels electrophoresed in buffer containing 0.38 M-Tris, 0.05 M-glycine and 0.1% (w/v) SDS. Molecular weight marker proteins (14C-labelled) were included in every gel run. After electrophoresis, gels were treated with 2,5-diphenyloxazole (PPO) scintillant (Bonner & Laskey, 1974) and dried. Fluorography (Laskey & Mills, 1975) was carried out using Kodak X-OMAT S film, autoradiographs scanned (Helena Quicksan R & D) and results computed using an Apple micro-
computer (Crosby, 1982). For comparison of newly synthesized labelled proteins with all proteins present, fluorographs were reswelled in 10% (v/v) aqueous methanol and protein bands were visualized by ammoniacal silver staining (Poehling & Neuhoff, 1981). Staining results were improved when the recommended time of washes was tripled. Staining of gels was performed after fluorography because silver staining significantly quenches tritium radioactivity (Van Keuren, Goldman & Merril, 1981).

Results

Culture of conceptus tissue explants and incorporation of labelled leucine

Under the conditions used, between 0·1 and 2% of the [3H]leucine in the medium was incorporated into protein by the conceptus explants after culture for 24 h. Some of the variability could be accounted for by differences in the initial condition of the conceptuses after flushing from the uterus and by damage to cells caused by subsequent handling. The patterns obtained after SDSPAGE and fluorography (Plate 1) represent leucine incorporation into proteins by explants that survived successfully in culture. Medium free of unlabelled leucine was used to enhance the incorporation of added labelled leucine which was 5–10-fold higher in leucine-free medium (Pl. 1, Fig. 2). Polyvinyl alcohol was substituted for fetal calf serum (FCS) in the medium to visualize the pattern of secreted proteins by staining and to eliminate the problem of non-specific binding of radioactivity to albumin in the medium. The substitution of polyvinyl alcohol had little effect on the pattern of protein synthesis (results not shown) and has been used previously for culturing mammalian oocytes and spermatozoa (Bavister, 1981). Modified Eagle's medium has been shown to support the development of preimplantation pig embryos in culture (Kuzan & Wright, 1982).

Macroscopically, the explants appeared to form processes or 'buds' stemming from the original tissue. These buds grew, in some cases, to be larger than the original tissue mass and this was taken as an indication of viability in this medium. Numerous proteins were labelled by Day 13–16 conceptus tissue under the conditions used, resulting in a complex gel electrophoretic pattern which was not readily interpretable. However, peaks appeared in similar positions in tissue harvested at different days of pregnancy, and there were some differences in the relative amounts of radioactivity in different peaks (data not shown). The predominant peak at all stages studied had a molecular weight of about 44 000; this is likely to be actin, which is usually the major radiolabelled protein in protein synthesis studies (Bravo & Celis, 1980). The identities of other radioactive peaks are not known. In the region of molecular weight 14 000–30 000 there were generally 3 or 4 minor peaks which corresponded to the major labelled proteins found in the medium after culture and described in the following section. The silver staining pattern of these gels after autoradiography was too crowded and complex to interpret easily. An example of Day 16 conceptus tissue is shown in Pl. 1, Fig. 1. There was no consistent correlation between the staining and radioactivity patterns (Pl. 1, Figs 1 & 2), indicating that many proteins were not turned over within the 24 h of these radio-uptake studies. Similar complex staining patterns were observed with conceptus tissue from other days of pregnancy (results not shown).

Patterns of radioactively labelled proteins released into culture medium by conceptus tissue

After 24 h of culture with L-[3H]leucine, media from Day 13 conceptus explants were shown by SDS-PAGE and fluorography to contain two major peaks of radioactivity at molecular weights of about 23 000 and 26 000 (Text-fig. 1a). In media from Day 14–16 conceptuses these peaks were still present but additional peaks had appeared (Text-figs 1b–d) which included two of lower molecular weight (~ 14 000 and 19 000) and others of higher molecular weight (~ 44 000, 55 000 and 88 000).
A region of radioactivity was also observed at the top of the resolving gel at all stages studied. This may correspond to the large macromolecular complex which is released by Day 16 pig blastocysts (Masters et al., 1982) and by mouse (Fishel & Surani, 1980), sheep (Godkin et al., 1982a) and cow (Masters et al., 1982) blastocysts. Samples containing a high concentration of polyvinyl alcohol (1–5%) caused a localized distortion at the top of the resolving gel. This could be avoided by dilution and so the sample applied to the gels contained <1% polyvinyl alcohol. Clear separation between peaks 4 and 5 was not always obtained using 15% gels, resulting in a broad peak when autoradiographs were scanned (Text-figs 1b & c). In the medium of Day-13 conceptus cultures the radioactive proteins of M, 23 000 and 26 000 were faintly represented in the silver staining pattern of the gels after autoradiography (data not shown). However, there were 3 other more strongly stained bands and several minor bands which were not significantly radioactive. Similarly, in culture media from Day 14–16 conceptuses the radioactive bands were represented in the staining patterns along with additional non-radioactive bands. However, there was poor correlation between major radioactive bands and silver stained bands, and it is possible that many of the stained proteins were products of cell death.

Attempts to demonstrate specific binding of protein in the medium to anti-hCG antibody or to hCG receptors

After culture of Day-16 conceptus tissue explants with L-[3H]leucine, the medium was subjected to gel filtration (to remove unincorporated radioactivity and salts) and freeze-dried as described in 'Materials and Methods'. The freeze-dried material was reconstituted in equilibration buffer (1 ml) and applied to an immunoaffinity column (1·5 × 0·5 cm) of anti-hCG immunoglobulin coupled to Sepharose 4B as described in ‘Materials and Methods’. The first 2 ml of the eluant were collected and reapplied to the column. Further fractions were collected and counted for radioactivity. A control column using 125I-labelled hCG in the same buffer was run in parallel. For culture medium it was found that 94% of the 3H radioactivity applied (102 000 d.p.m.) had not bound to the column after elution with equilibration buffer. In contrast, 70–80% of the 125I-labelled hCG (50 000 d.p.m. total) was bound to the column and could be subsequently eluted with 0·1 M-propionic acid. The unbound 125I-radioactivity could be attributed to degradation products which had formed during storage of the 125I-labelled hCG preparation. No detectable 3H-radioactivity from the culture medium was eluted with 0·1 M-propionic acid. This result indicated that none of the radioactive proteins found in the medium after culture of Day-16 conceptus tissue cross-reacted immunologically with hCG antibody.

A separate freeze-dried preparation of medium proteins was taken up in Buffer A and incubated for 24 h at 4°C with a rat Leydig cell preparation (5 mg protein/ml) containing hCG receptors. A control assay using 125I-labelled hCG was run at the same time. No significant 3H-radioactivity was bound to the rat Leydig cell preparation. In contrast, the 125I-labelled hCG was
Text-fig. 1. Patterns of radioactively labelled proteins found in the medium after culture for 24 h of pig conceptus tissue (Days 13–16) with [3H]leucine. Medium was processed as described in 'Materials and Methods' and applied to 15% SDS-polyacrylamide slab gels after reduction with 2-mercaptoethanol. The figure shows representative scans of autoradiographs obtained after electrophoresis followed by fluorography of gels. The summary table gives a key to the major peaks observed, their approximate molecular weights and the day of pregnancy from which the tissue was taken. + indicates presence, − indicates absence and (+) indicates presence of peak as a minor constituent.
specifically bound at a level (12%) usual for the concentration of rat Leydig cells used. This result indicated that none of the radioactive proteins found in the medium after culture of Day-16 conceptus tissue would bind hCG receptors, or that radioactive protein bound was of a specific activity too low to be detected.

**Attempts to demonstrate presence of chorionic gonadotrophin (CG) in 13–16-day conceptus tissue**

If pig conceptuses contain significant levels of CG-like activity, then injection of conceptus tissue extracts into rats should result in down regulation of testis LH/hCG receptors (Sharpe, 1976). Injection (s.c.) of a small dose of hCG (25 i.u. per rat) resulted in a 50% reduction of the level of $^{125}$I-labelled hCG binding to rat testis Leydig cell preparations after 50 h. The affinity of binding of $^{125}$I-labelled hCG to these preparations was $1.3 \times 10^{-10}$ M as determined from weighted logit-log plots of saturation analysis data. In contrast, $^{125}$I-labelled hCG binding to receptors from rats treated with Day-16 blastocyst extract was not significantly different from controls at 24 or 50 h after injection. This result indicates that the extract from a single Day-16 conceptus contained <25 i.u. (2 µg) of CG-like activity in vivo.

To test for the presence of immunological cross-reactivity with hCG, Day 13–16 pig conceptus tissue was extracted with Triton X-100 (1% v/v) at two concentrations (10 and 100 mg tissue wet weight/ml). The extracts were incubated with anti-hCG antiserum and $^{125}$I-labelled hCG as described in 'Materials and Methods'. No significant inhibition of binding of $^{125}$I-labelled hCG to this antiserum was observed with any of 6 conceptus extracts at the lower concentration or from 4 extracts at the higher concentration. These results indicate that there was no detectable material cross-reacting immunologically with hCG in extracts equivalent to 3 or 4 conceptuses of the same age between Days 13 and 16. Although the avidity of the antiserum for hCG (ED$_{50}$ = 4.3 ng) was unaffected by the presence of Triton (assay concentration of 0.6% v/v), the precision was reduced because of the increased friability of the sedimented pellets when detergent was present. Displacement of at least 20% of control values would be required for statistical significance. None of the extracts displaced $^{125}$I-labelled hCG binding more than 16%. The lower limit for detection by the assay was equivalent to 0.5 ng hCG per conceptus.

An additional test for immunological cross-reactivity with hCG was performed with fluorescent antibodies. Squashed cell preparations or cryostat sections of Day 13–16 conceptus tissue were incubated with anti-hCG antiserum and FITC-antirabbit immunoglobulin antibody. No significant differences between controls and those treated with anti-hCG were detected in the fluorescence patterns. There was generally a high level of non-specifically bound fluorescence in these sections. This was reduced but not eliminated by 'blocking' with excess BSA or with normal rabbit serum and by preabsorption of antiserum with porcine liver, spleen and brain cell preparations. By this method there was no detectable immunological cross-reactivity with hCG in Day 13–16 conceptus tissue.

To test for the presence of hCG receptor binding activity, pig conceptus tissue extracts were incubated with solubilized pig corpus luteum preparations containing hCG binding sites. The results are shown in Table 1. Extracts with Triton X-100 (1% v/v) were prepared at concentrations of 10 and 100 mg tissue wet weight/ml, and these extracts as well as 1:5 and 1:25 dilutions (in Buffer A containing 0.1% (v/v) Triton X-100) were tested for displacement of receptor-bound hCG. No significant inhibition of specific binding of $^{125}$I-labelled hCG to solubilized receptors was observed. Artefactual enhancement of $^{125}$I-labelled hCG binding was seen at the highest extract concentration tested. This was due to an effect on the separation of bound from free ligand when a very large concentration of conceptus protein was present. A similar enhancement of binding could be obtained by an equivalent increase in the concentration of the bovine gamma globulin carrier protein. The lower limit of detection (about 1-5 ng) of this assay was equivalent to 1 ng hCG-like activity per conceptus. The affinity of the receptor preparation for hCG was about $2 \times 10^{-10}$ M which is comparable to that for the rat testis receptor preparations used in these studies.
Table 1. Tests for hCG receptor binding activity in pig conceptus tissue

<table>
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<tr>
<th>Day</th>
<th>Tissue conc. (mg wet wt/ml)</th>
<th>× Dilution</th>
<th>Bound $^{125}$I-labelled hCG (c.p.m.)</th>
<th>Difference from control (%)</th>
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<td></td>
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<td>Control 12750 ± 18.4</td>
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Triton X-100 (1% v/v) extracts of pig blastocyst tissue were tested for their effect on binding of $^{125}$I-labelled hCG to solubilized pig corpus luteum receptors. $^{125}$I-Labelled hCG (130 000 c.p.m.) was incubated for 3 h at 37°C with a solubilized receptor preparation containing 8 mg protein/ml in 0.5 ml assay volume. Bound was separated from free radioactivity by addition of bovine gamma globulin carrier protein (3 mg/ml) and polyethylene glycol (11% w/v). The pellets were resuspended in 0.5 ml PBS containing 1% BSA (w/v) and 0.1% Triton X-100 (v/v), reprecipitated with polyethylene glycol and counted. By saturation analysis with hCG standards ED$_{50}$ was 7.1 ± 0.95 ng, lower limit of detection was ~1.5 ng and non-specific binding subtracted from the results was 4.6%.

† Mean ± s.d. for 2 replicates.
* Values significantly different from control ($2P < 0.01$).

Discussion

The demonstration that two major proteins of around 23 000 and 26 000 molecular weight are synthesized and released in culture by Day-13 pig conceptuses is of considerable interest because this is the time that rapid elongation occurs. If these proteins are mediators of embryonic elongation they would be of importance to successful development, and of intrinsic interest because of their influence on cell proliferation. Between Days 13 and 16 there is synthesis and release of other proteins of molecular weights 14 000, 19 000, 44 000, 50 000 and 88 000. One or more of these proteins could be involved in maternal recognition of pregnancy or in the events associated with implantation which occurs around Day 18. In studies of sheep blastocysts, Godkin et al. (1982a) observed synthesis and release at Day 13 of a major protein estimated by SDS-PAGE to have a molecular weight of 17 000. Their work suggested that it might be the antiluteolytic factor designated trophoblastin by Martal et al. (1979). Godkin et al. (1982b) reported that the major proteins released by the pig conceptus from Day 10-5 to 12 of pregnancy were acidic and had molecular weights between 20 000 and 25 000. The two major proteins of around 23 000 and 26 000
molecular weight observed in our studies to be synthesized and released by Day-13 pig conceptuses appear to be the same ones described by Godkin et al. (1982b) and confirm their findings. Godkin et al. (1982b) also reported that the dominant proteins synthesized and released from Day 13 to 16 had molecular weights of about 40,000 to 50,000. By Day 15 we also observed proteins of 44,000 and 50,000 molecular weight, but in addition a protein of 19,000 molecular weight was released, and proteins of 14,000 and 88,000 molecular weight appeared by Day 16 of pregnancy. The differences in observations presumably result from the different techniques employed. In agreement with the findings of Masters et al. (1982) we observed synthesis and release by Day 13–16 conceptuses of a high molecular weight component which remained at the top of the resolving gel during electrophoresis.

In these studies we have attempted to determine whether or not a chorionic gonadotrophin (CG) is produced by Day 13–16 pig conceptuses. Although it is well known that CG is produced in early pregnancy by fetal cells of man and other primates (Hodgén, 1980) and by equine trophoblast cells (Allen & Moor, 1972), there is as yet no firm evidence for such a hormone in the pig. Saunders et al. (1980) reported that extracts of pig placenta and embryonic membranes contain a gonadotrophin-like substance which displaces pig LH from pig luteal receptors. However, there is uncertainty as to whether this activity resembles CG from other species because other uterine substances unrelated to pregnancy are also reactive in this assay (Wise, Saunders, Burton & Flint, 1980). Evidence for production of a factor which cross-reacts immunologically with hCG by embryos of rabbits (Asch et al., 1978) and rodents (Wide & Wide, 1979) provided the rationale for the use of hCG antibodies to detect pig CG, but no immunological cross-reaction was observed. This finding does not necessarily prove that pig CG is absent because horse CG (PMSG) has little or no (<0.01%) cross-reactivity with hCG antisera (Christakos & Bahl, 1979). Radioreceptor assays provide a more biological approach to this problem and are less species specific, but again no hCG-like activity was detected in Day 13–16 conceptus tissue using solubilized pig corpus luteum receptors (Table 1) or in the medium after culture of Day-16 conceptus tissue using rat Leydig cell receptors. Finally, a less sensitive biological test for down-regulation of receptors again failed to detect hCG-like activity in Day-16 conceptuses. Although our studies provide evidence that an hCG-like protein is absent in Day 13–16 pig conceptuses, we cannot rule out the possibility that it is produced at a different stage of pregnancy in this species. Nevertheless, the mechanism proposed by Bazer & Thatcher (1977) and Bazer et al. (1982) for establishment and maintenance of pregnancy in the pig does not require a specific luteotrophic factor other than the anti-luteolytic action of oestrogen at Day 13–16.

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References


Pig blastocyst proteins


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