

PN-ABC-868

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: December 27, 1988

MEMORANDUM

TO: AID/PPC/CEIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-101 From 6/30/88 to 12/31/88

"Isolation of a lutetotropic substance produced by the blasotcyst
and early pregnancy diaganosis in cattle"

Attachment

C5-101

AID Grant No. DPE-5544-G-SS-6046-00.

Interim progress report for period of 6/30/88-12/31/88.

Dec. 31, 1988.

Title: Isolation of a luteotropic substance produced by the blastocyst and early pregnancy diagnosis in cattle.

Objectives for second six months:

1. Purification of bCG by eliminating higher molecular weight substances using acidic extracts.
2. Analysis of the lower molecular weight fractions by using reverse phase HPLC.

Summary:

To determine if luteotropic activity is present in the bovine placental granules, fetal cotyledons from fetuses of 50-100 days of gestation were used. Enriched granules were prepared using a Percoll gradient. Active substances were obtained from the granules by freeze-thawing. The extracts thus obtained were then eluted on a Sephadex S-300 Column. Higher molecular weight substances were eliminated by using acidic extracts. The low molecular weight fraction was further analysed using reverse phase HPLC (acetonitrile:water gradient). It was found that the elution of this substance at 45% acetonitrile resulted in a 100 fold increase in luteotropic activity in the bioassay compared to Sephadex fraction. The small molecular weight substance was heat stable and not extracted to the organic phase when partitioned between ethanol:chloroform.

Methods:

Acidic extract: Granules (preparation described in previous report) were heated to 97C for 5 min in 2 N acetic acid immediately after collecting from the Percoll gradient. The granules lysate was centrifuged at 100,000 g for 30 min at 4C and the supernatant was lyophilized.

HPLC reverse phase chromatography:

The acidic granules extract was fractionated on a C-18 reverse phase column (Econosphere, 5 micron, Alltech, Avondale, PA) with a linear gradient of 0-80% acetonitrile containing 0.1% trifluoroacetic acid. Fractions of 1ml were collected and lyophilized.

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DEC 27 1988

Results:

Fig.1. shows SDS polyacrylamide gel electrophoresis comparing cytosolic extract, total granule extract (b) and the acid granule extract (c). Different protein profiles were obtained using these purification steps. The total and acidic extracts both showed a larger variety of low molecular weight proteins than did cytosol. In the total extract there was an enrichment of the protein of Mr 29,000 and 35,000 whereas the acidic extract exhibited proteins of Mr 31,000 and 32,000.

The acidic granules extract was fractionated using HPLC C-18 columns, lyophilized and analyzed in the bioassay, i.e., the enhancement of progesterone production by dispersed bovine luteal cells as described in the previous report. Fractions 17-20 enhanced progesterone production to 3.4 times more than in the control (Fig.2). HCG by itself increased progesterone secretion 3 fold.

As shown in table 1, the acidic granule extract was 10 times more active than the total granule extract. The C-18 peak was 470 times higher than that for the total granule extract.

The h.p.l.c-active fractions were extracted by phase partition of methanol:chloroform (2:1 v/v). No enhancement of progesterone secretion when cells were coincubated with the chloroform phase.

Objectives for the next six months:

1. Continued purification of the bCG.
2. Hybridization of the placental mRNA using cDNA probes for hCG.

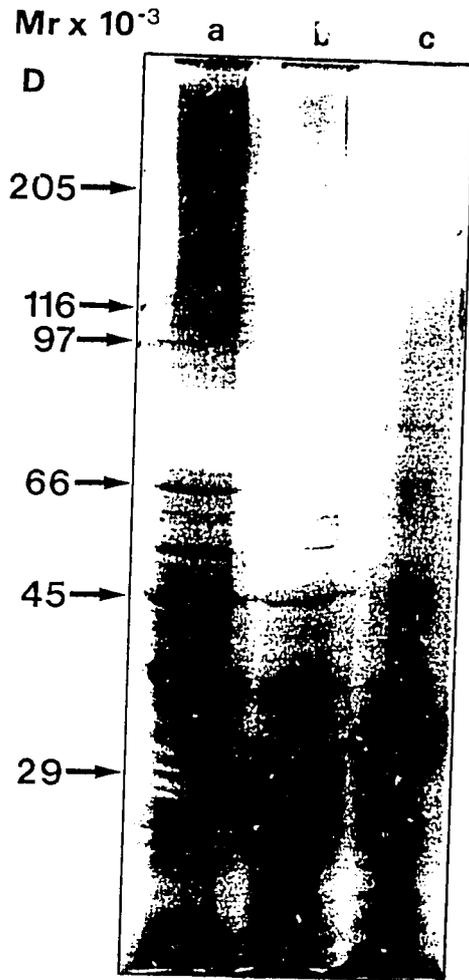


FIG. 1

C₁₈-HPLC

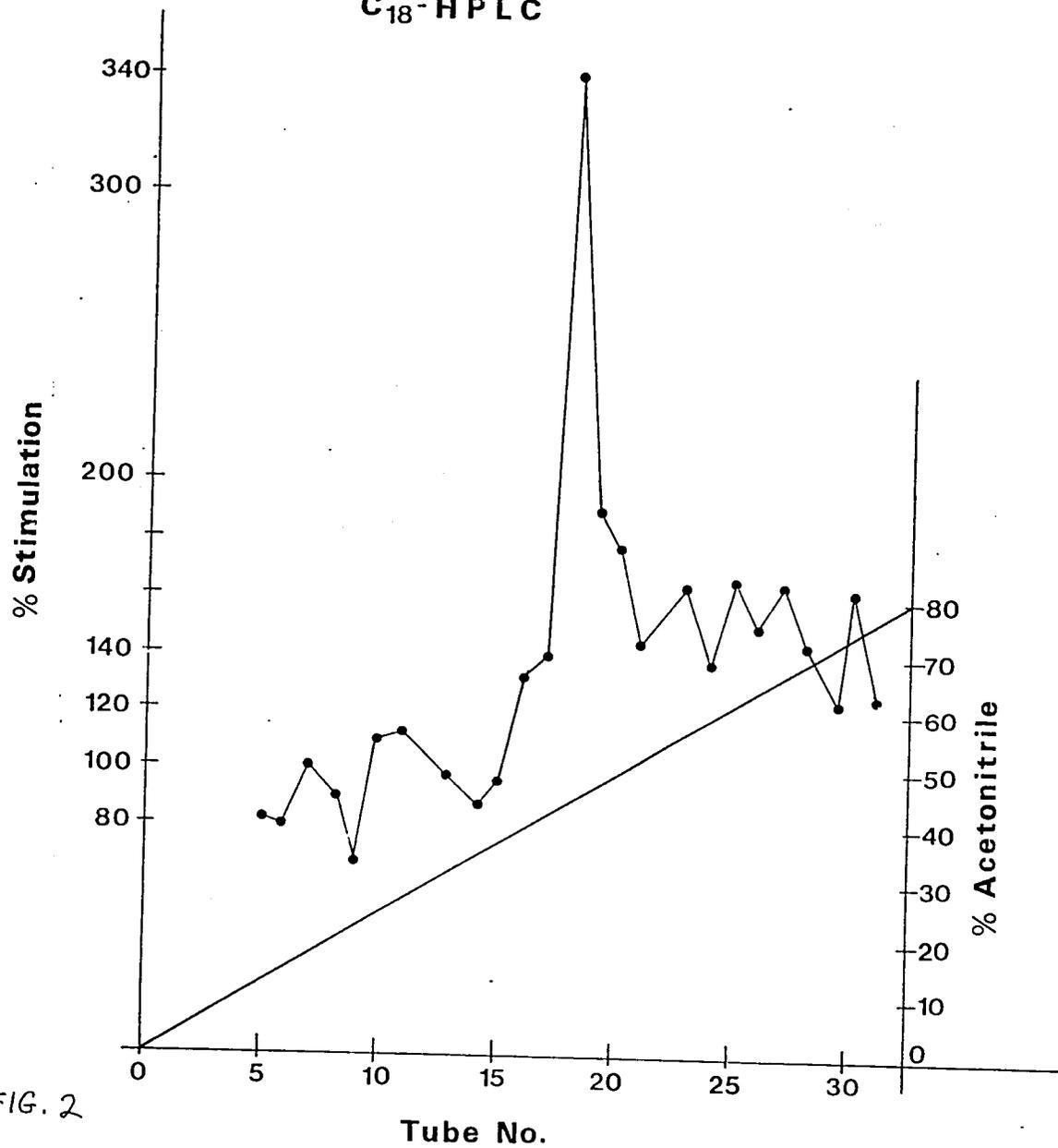


FIG. 2

Tube No.

| Column | Progesterone ng/ μ g granule protein/200000 cells/2h |
|---|---|
| Total granule extract | 0.8 |
| Sephacryl S-300 high molecular weight peak | 3.84 |
| Acidic granule extract | 10.2 |
| C-18 h.p.l.c. (45% acetonitrile) | 470 |

Table 1. Progesterone synthesis by bovine luteal cells in the presence of granular fractionated extracts.

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DATE: 9/8/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C 5 - 101
PR - June 30, 1988

Attachment

Grant started on 12/31/87 !!

CS-101

Progress Report

AID Grant No. DPE-5544-G-S5-6046-00

June 30, 1988

Title: Isolation of a luteotropic substance produced by the blastocyst and early pregnancy diagnosis in cattle.

Objective for the first six months:

1. To partially purify hCG by ammonium sulfate precipitation and column chromatography.
2. To develop a new system based on isolation of granules to give substances of high specific activity.

Summary of Results:

To determine if luteotropic activity is present in the bovine placental granules, fetal cotyledons from fetuses of 50-100 days of gestation were used. Enriched granules were prepared using a Percoll gradient. Active substances were obtained from the granules by freeze-thawing. The extracts thus obtained were then eluted on a Sephacryl S-200 column. The resultant fractions were then analysed by two methods: (1) a radioreceptor assay for hCG-like substances and (2) a bioassay using progesterone production by bovine luteal cells. It was found that there were two peaks of activity, one indicative of a high molecular weight substance and the second of a low molecular weight substance. Both peaks gave positive results in the radioreceptor assay and bioassay.

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Description of Techniques:

Granule preparation

Fetal cotyledons were removed from the chorion and tissue was minced with scissors and granules released by stirring vigorously for 2 h at 22°C in MEM (1:1; v/v). The material was filtered through two layers of gauze and centrifuged at 350g for 5 min. The supernatant was further centrifuged at 4800g for 15 min. The pellet was resuspended in a small volume of MEM and loaded on a discontinuous Percoll gradient (1.03, 1.04, 1.06, 1.06 g/ml). After 15 min centrifugation at 4800g, the 1.04 g/ml density layer, which contained the granules, was collected. The granules were then lysed by freezing and thawing and membranes were pelleted by centrifugation (100,000xg, 30 min at 4° C). Supernatant was collected and lyophilized.

Gel filtration

Total granules extracts were fractionated on a Sephacryl S-300 column (100 x 2.5 cm; Pharmacia, Uppsala, Sweden) and eluted, using Tris buffered saline (0.0137 M-Tris-HCl, 0.012 M NaCl and 0.005 M HCl; pH 7.4). Fractions of 4.7ml were collected and lyophilized.

Chorionic gonadotrophin-like activity

Activity of the various fractions obtained throughout purification procedures was determined by a radioreceptor assay (RRA) for hCG using rat testicular tissue, and ¹²⁵I labelled hCG. hCG was radiolabelled with ¹²⁵I by the Iodo-gen method.

Bovine luteal cells were dispersed with collagenase. The trypan blue dye exclusion test indicated that 80% of all cells were viable. The cells (200,000) in 0.5 ml MEM were incubated in 5 replicates with hCG, h.p.l.c. or Sephacryl S-300 chromatographed fractions. After a 2 h incubation at 37°C, in the presence of 5% CO₂, cells were pelleted by centrifugation, and progesterone measured by RIA.

Results:

The total granules extract of the granules was fractionated on a Sephacryl S-300 column, lyophilized and analysed for relative inhibitory binding of ¹²⁵I-labelled hCG in radioreceptor assay. Two main peaks showed displacement of hCG: (a) Fractions 36-41 and (b) smaller molecular weight fractions 65-73. The first peak is equivalent to 110 ng hCG/ 20 µg protein, as detected by RRA, and the low molecular weight peak is equivalent to 55 ng hCG/20 µgm protein. Chorionic gonadotrophin activity of the major peak was not stable after 2 days at -20°C. On the other hand, the lower molecular weight peak was stable to freezing for at least 1 month.

Luteal cell cultures were incubated with Sephacryl S-300 fractions and progesterone synthesis was measured by RIA. hCG (5 ng/ml) increased progesterone synthesis by 65% above control. The low molecular weight fractions (67-78) increased progesterone synthesis by 85%, and the higher molecular weight peak (fractions 45-50) increased it by 57%.

Objective for next six months:

Higher molecular weight substances will be eliminated by using acidic extracts. The low molecular weight fraction will be further analysed using reverse phase HPLC (acetonitrile: water gradient).

will send next report 12/31/88.