Identification of relaxin in the placenta of the ewe

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Summary. Sheep placentomes were collected at the abattoir and the stage of gestation was estimated from the crown–rump length and appearance of the fetus. Samples were extracted and either freeze dried (crude extracts) or fractionated on Sephadex G-50 and CM-cellulose. Relaxin immunoreactivity (RXN-IR) was detected in all samples by a pig relaxin RIA and diluted in parallel with the standard curve. Two patterns of RXN-IR were seen after Sephadex G50 purification: (a) a single main peak of RXN-IR eluting at a position similar to pig relaxin; or (b) a 3-peak pattern with additional higher (void volume) and lower (≈ 1000) molecular weight peaks. These peaks were all found with 4 different and specific antisera. The 6000 molecular weight peak eluted at a similar position to pig relaxin on CM cellulose and inhibited electrically stimulated rat uterine contractions in vitro. The amount of relaxin measured in crude extracts of placentomes from different ewes was very variable. Most samples were within the range 0.05–11.2 ng/g wet weight of tissue (3.0 ± 0.45 (s.e.m.), n = 44) but a few contained much higher concentrations (25.5–61.4 ng/g, n = 3). There was no obvious variation in concentration with stage of pregnancy (20 days to term). Samples of intercotyledonary endometrium, allantochorion and whole ovaries from pregnant ewes were also extracted. All contained low concentrations of RXN-IR (0.6 ± 0.13 ng/g, n = 4; 0.6 ± 0.29 ng/g, n = 3; 1.0 ± 0.66 ng/g, n = 7, respectively). We conclude that relaxin-like peptides are present in the pregnant ewe and that, as the placentomes are the largest component by weight, they represent the major source.

Keywords: relaxin; placenta; ewe

Introduction

Relaxin is a peptide hormone with a molecular weight of about 6000 consisting of two chains linked by disulphide bonds. Amino acid sequence analysis has been performed on relaxin obtained from ovarian extracts of pregnant pigs, rats and sharks and this has shown that there is only limited sequence homology among species (for reviews see Bryant-Greenwood, 1982; Sherwood & Downing, 1983). Ideally, studies on the biological actions of relaxin in other species should therefore be based on homologous preparations. Relaxin-like activity has been identified in a variety of mammals by both immuno- and bioactivity. Immunological studies have generally been based on pig relaxin standards and antisera. Sites of localization of relaxin appear to vary among species and include both the ovary and placenta in the human (O’Byrne et al., 1978; Bigazzi et al., 1982), the endometrial glands in the guinea-pig (Pardo & Larkin, 1982) and the syncytiotrophoblast in the rabbit (Eldridge & Fields, 1985).

Relaxin is of potential clinical importance in ruminants, both to ease labour and to dilate the cervix to facilitate artificial insemination and embryo transfer. Fields et al. (1980) have provided

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substantial evidence that relaxin is present in corpora lutea collected from late pregnant cows, but work on the ewe has been limited. Several studies based on the first relaxin radioimmunoassay which used a relatively impure preparation of pig relaxin suggested that significant quantities of relaxin were present in plasma collected from ewes during the oestrous cycle (Chamley et al., 1975), pregnancy (Bryant & Chamley, 1976a) and lactation (Bryant & Chamley, 1976b). More recently Renegar & Larkin (1985) measured only low levels of relaxin immunoreactivity in the endometrium, placenta, ovary and blood of pregnant ewes, and were unable to demonstrate biological activity.

In this study we report the results of a series of experiments in which we have attempted to isolate and characterize relaxin in sheep.

**Materials and Methods**

Collection of tissue. Tissue samples were obtained from the abattoir and frozen within 15 min of death on solid CO₂ or in liquid nitrogen. From non-pregnant ewes whole uteri were collected, and from pregnant ewes whole ovaries, whole placentomes, intercotyledary endometrium and allantochorion were obtained. The stage of pregnancy was estimated from the crown–rump length and external appearance of the fetus (Evans & Sack, 1973). Tissue samples were stored at −80°C before extraction.

Extraction. Partly thawed tissue samples not exceeding 24 g in weight were extracted on ice by the method of Walsh & Niall (1980). The homogenate was passed through pre-equilibrated Sep-Pak cartridges (Waters Associates, Milford, MA, U.S.A.) and the bound material was eluted with 25 ml 80% acetonitrile (Koch-Light, Haverhill, Suffolk, U.K.) in 0-1% trifluoroacetic acid (Rathburn Chemicals Ltd, Walkerburn, U.K.). The volume was reduced to about 3 ml by rotary evaporation and the extract was then either diluted in distilled water and lyophilized (crude extract) or purified further by gel filtration.

Chromatography. Initial purification was performed using a column of Sephadex G-50 (fine, 90 × 1.6 cm) equilibrated in 0.1 M-ammonium acetate buffer pH 5.0 and run using upward flow at a rate of about 10 ml h⁻¹ at 4°C. Fractions (7.5 ml) were collected, lyophilized, split into several aliquots for analysis, lyophilized again and stored desiccated at −20°C.

Peaks of immunoreactive relaxin-like material from several extractions were pooled and purified further by ion-exchange chromatography using a 6 × 0.8 cm column of CM52 carboxymethyl cellulose (Whatman, Chester, U.K.) equilibrated with 0.05 M-ammonium acetate buffer pH 5.0 at 4°C. Unabsorbed protein was eluted with 0.05 M-ammonium acetate buffer and then a linear gradient was applied by mixing 0.05 and 0.5 M-ammonium acetate buffer with a gradient mixer (Pharmacia Fine Chemicals, Uppsala, Sweden). Initially, 1-ml fractions were collected. These were either pooled in peaks according to the protein profile (monitored by absorbance at 280 nm) or, if no clear peak pattern emerged, they were pooled in batches of 10. The conductivity was measured and the pooled fractions were lyophilized as described above.

Radioimmunoassay. Freeze-dried extracts were reconstituted in 1 ml 0.05 M-sodium barbitone buffer pH 8.5 containing 1% bovine serum albumin (BSA) and were measured in triplicate at 2–3 dilutions in a modified version of the radioimmunoassay based on pig relaxin as described previously (Taverne et al., 1982; Evans et al., 1983). NIH-RXN-P1 was used as the standard and CM purified relaxin (2000–3000 guinea-pig units/mg) was used for iodination. The antiserum P₁AS₁B is used for most of the study was raised in a rabbit against an extract of pig relaxin purified on Sephadex G50 and cross-reacted <0.02% with pig prolactin and <0.1% with pig insulin. The use of this antiserum was corroborated by comparison with the radioimmunoassay results obtained using three additional antiseria. (i) Antiserum PMAB₂ was raised at Bristol using a similar source of antigen and then purified by affinity chromatography. Purified CMa relaxin (200 µg) was coupled to 310 mg CNBr-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) and the resultant relaxin–Sepharose beads were mixed with 0.4 ml PMAB₂ antiserum in 5 ml 0.05 M-barbitone buffer pH 8.5 overnight at 4°C. After removal of the supernatant, the beads were washed and bound antibodies to CMa relaxin were removed using 5 ml 50% (v/v) ethylene glycol in 0.5 M-acetic acid. The antibody solution was dialysed against 0.1 M-Tris HCl pH 7.6 using a CA-30 ultrafiltration unit (Millipore, Bedford, MA, U.S.A.) and stored at −20°C. (ii) Antiserum R6 was also raised against a G-50 fraction, 1000 U/mg (O’Byrne & Steinmetz, 1976) and (iii) Antiserum 1082 was raised against a combination of CM-B, CM-a and CM-a’ (Sherwood et al., 1975). The sensitivity of the main assay was 16 pg per tube and the intra- and inter-assay coefficients of variation were 8.4 and 13.8%. All values are given as ng equivalents of the NIH-RXN-P1 pig relaxin standard and are referred to as relaxin immunoreactivity (RXN-IR).

Bioassay. Freeze-dried extracts were redissolved in 0.5 ml Krebs–Henseleit buffer and added to an organ bath containing an electrically stimulated uterine strip taken from a pro-oestrous rat. The details of the methodology have been described previously (Bradshaw et al., 1981). The amplitudes of the 10 contractions immediately before and 10 immediately after the addition of extract were measured and the results are expressed as the mean percentage change.
in the amplitude of the contractions between these two time periods. The minimum amount of pig relaxin which has a reproducible effect in this system varies from 50 to 500 ng depending on different uterine strips and batches of relaxin. BSA, at concentrations of up to 2 mg protein per bath, had no effect whereas higher concentrations of BSA tended to stimulate contractions.

Protein determinations. The protein content of each extract was measured by the method of Lowry et al. (1951) using a freeze dried aliquant of each sample, with bovine serum albumin as the standard.

Contamination. To avoid possible problems of contamination of sheep material with pig relaxin the purification steps were all performed in a separate laboratory which was on a different floor from the assay laboratory and there was no transfer of glassware or reagents between the two sites.

Results

Placentomes

Immunoreactivity. Initial studies suggested that placentomes collected during mid-pregnancy contained more relaxin-like material than did corpora lutea, and so these were used as the starting point for most of the work. Several cotyledons (usually 3) from each sheep were extracted and purified on Sephadex G-50 as described above and each fraction was measured by relaxin RIA. This procedure was performed on 19 occasions.

There was considerable variation among extractions but some RXN-IR was measured on all but one occasion, with two elution patterns predominating (Fig. 1). In one there was a single main peak of immunoreactivity which eluted in the region of $M_t$ 6000, whereas in the other there were 3 clear peaks of activity which were termed the high, mid- and low molecular weight peaks. The high peak eluted in the void volume, indicating a molecular weight in excess of 20 000 whereas the low peak ran in a position similar to oxytocin, suggesting an apparent molecular weight of about 1000.

![Graph showing elution profiles on Sephadex G-50](image)

**Fig. 1.** Diagram to show the two different profiles for sheep relaxin found after purification on Sephadex G-50. The elution profile (measured as absorbance at 280 nm) for an extract of 20 g sheep placentome run on a column of Sephadex G-50 in 0.1 M-ammonium acetate pH 5.0 is illustrated. The histogram shows the RXN-IR content of each fraction with: (a) 1 main peak, (b) 3 peaks.

This different pattern could not be related to any differences in tissue handling, but there was a tendency for the 3-peak pattern to predominate in samples collected in later pregnancy (> 120 days' gestation). This pattern of immunoreactivity was confirmed by measuring the same samples from
Fig. 2. Diagram to show the result of measuring the same fractions from an extract of sheep placentome (22·7 g, 45 days' gestation) purified on Sephadex G-50 using 4 different antisera (a) P7AsRb added at 1:12 000; (b) PMAB4 purified on a CMA affinity chromatography column and diluted 1:300 after elution; (c) R6 (O'Byrne & Steinetz, 1976) at 1:32 000; and (d) 1082 (Sherwood et al., 1975) diluted at 1:9000. Results are all expressed in pg pig relaxin equivalents per fraction. The detection limit of each assay (allowing for sample dilution) is shown by the horizontal bar. The arrows show the elution position of three molecular weight markers: BD, blue dextran in the void volume, > 20 000; CC = cytochrome C, 12 300; T = tyrosine, 181.

one Sephadex G-50 run using 3 additional and specific antisera (PMAB4 purified using a CM-a relaxin affinity column, R6 (O'Byrne & Steinetz, 1976) and 1082 (Sherwood et al. 1975). Both the pattern of immunoreactivity (Fig. 2) and the estimated total relaxin concentration in each case were almost identical (all values in ng/g: P7AsRb, 0·45; PMAB4, 0·42; R6, 0·59 and 1082, 0·54). For all of the runs for which complete information was available (n = 15) the percentage of the RXN-IR in each region was calculated and the combined results were as follows: high molecular weight region 23 ± 3·8%, mid-molecular weight region 58 ± 5·7%, low molecular weight region 18 ± 3·4% (mean ± s.e.m.).

The extract remaining from the mid-molecular weight peak was pooled and run on CM-cellulose. This procedure was repeated four times, using 15–25 mg protein per run. No clear pattern of protein peaks as monitored by absorbance at 280 nm was seen but the RIA results were consistent, showing a single peak of immunoreactivity which eluted at about 12 mMHO (Fig. 3). This RXN-IR, as well as that in crude extracts, diluted in parallel with the pig relaxin standard curve (Fig. 4). Results obtained from one batch of 100-day placentomes purified in this way are given in Table 1, showing that a 17-fold increase in the relaxin content was achieved. The yield varied considerably between experiments and the highest relaxin content recorded in the course of the work was much higher than the figures shown here (170 ng/mg protein in one Sephadex G-50 fraction, see Table 3).

The RXN-IR measurements in crude extracts of single placentomes from 44 ewes at all stages of pregnancy are shown in Table 2 and are compared with the concentrations found in non-gravid whole uterus from ewes in the luteal phase. Whilst the amounts present were clearly higher in the placentomes, there was no obvious trend throughout pregnancy and there was considerable variation among animals (placentomes) at each stage.

The relaxin concentrations in these individual crude extracts were compared with those measured in the material purified on Sephadex G-50. For the 8 Sephadex G-50 runs for which the stage of pregnancy was known, the immunoreactivity in each fraction was summed and divided by the weight of starting material. The overall relaxin concentrations in ng/g were in the same range as that found in the unpurified material (1·6 ± 0·5 ng/g (mean ± s.e.m.), n = 7, Day 87 to term, plus one high value of 32·3 ng/g at Day 90.
Bioactivity. Bioactivity was assessed in: (a) crude extracts pooled from 5 individual placentomes, (b) individual G-50 fractions, and (c) CM-cellulose fractions. For crude extracts a clear but reversible inhibition of contractions was found on 3 out of 4 occasions (Fig. 5). For the G-50 fractions a clear region of inhibitory activity was associated with the mid-molecular weight peak in 3 experiments (see Table 3) and the low molecular weight peak in one experiment. For the remaining 4 G-50 runs tested stimulation was observed preventing a clear assessment of the results. Bioactivity was not found after CM-cellulose purification. However, this can be explained by the limited amount of purified material available to test as the maximum amount of CM-cellulose purified RXN-IR added to the bath was 6.4 ng. Although an effect with the G-50 fractions was occasionally seen with concentrations as low as 1 ng pig relaxin equivalents, clear results were usually only found with amounts in excess of 10 ng. These figures should not be compared directly with the 50–500 ng pig relaxin needed to give consistent inhibition in this system because neither the RIA or bioassay employed homologous material and so relative concentrations are likely to be misleading.
Fig. 4. Standard curve for the pig relaxin RIA. The dilution curves for a crude extract of sheep placentome (starting concentration 0.3 ng RXN-IR per mg protein) and for the RXN-IR peak of material purified on CM-cellulose (starting concentration 3.0 ng RXN per mg protein) are also shown.

Table 1. Relaxin concentrations in sheep placentomes at different stages of purification

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Relaxin conc. (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.15</td>
</tr>
<tr>
<td>Sephadex G-50 (mid molecular weight peak)</td>
<td>0.62</td>
</tr>
<tr>
<td>CM-cellulose purified</td>
<td>2.55</td>
</tr>
</tbody>
</table>

Sources of variation. The concentration of relaxin in different placentomes showed considerable variation (see Table 2). Whilst some of this was probably physiological it seemed likely that differences also arose during collection and processing. Two possible factors were investigated: (1) method of freezing, and, (2) length of storage. Individual placentomes were either frozen on solid CO₂ (n = 22) or in liquid nitrogen (n = 25) before storage at −80°C. The mean relaxin values for the two groups were 3.8 ± 1.78 and 4.9 ± 2.59 ng/g, which were not significantly different.

To investigate losses during storage, a number of placentomes were collected from one ewe at 100 days' gestation, frozen on solid CO₂ and stored at −80°C. About 20 g tissue were removed from this stock on 6 occasions at intervals from 4 to 21 weeks after collection, extracted and run on a column of Sephadex G-50. The amount of RXN-IR detectable showed a steady reduction from 4.4 to 0.07 ng/g during this period, representing a loss of about 98% of the activity. Subsequently, samples were always extracted within 3 days of collection.
Table 2. Relaxin concentrations in individual placentomes at different stages of pregnancy in the ewe

<table>
<thead>
<tr>
<th>Stage of pregnancy</th>
<th>No. of animals*</th>
<th>Relaxin concentration (ng/g)†</th>
<th>Mean ± s.e.m.</th>
<th>Range</th>
<th>High values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant</td>
<td>4</td>
<td>0.27 ± 0.07</td>
<td>0.16–0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 21–40</td>
<td>6</td>
<td>3.42 ± 1.74</td>
<td>0.31–11.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 41–60</td>
<td>7 + 1</td>
<td>1.94 ± 0.78</td>
<td>0.05–6.05</td>
<td>61.4</td>
<td></td>
</tr>
<tr>
<td>Days 61–80</td>
<td>10</td>
<td>2.02 ± 0.75</td>
<td>0.28–7.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 81–100</td>
<td>8</td>
<td>1.38 ± 1.19</td>
<td>0.13–9.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 101–120</td>
<td>7 + 1</td>
<td>2.16 ± 1.53</td>
<td>0.13–11.17</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>Days 120–term</td>
<td>6 + 1</td>
<td>0.29 ± 0.06</td>
<td>0.11–0.55</td>
<td>38.3</td>
<td></td>
</tr>
</tbody>
</table>

*In the majority of cases, one placentome per ewe was extracted except in early pregnancy when several placentomes were collected from each animal and pooled.

†Relaxin concentrations are the mean for each age range. However, 3 samples had much higher values and these are shown individually, and are not included in the mean values.

Fig. 5. Tension development *in vitro* in uterine strips obtained from pro-oestrous rats. The strips were electrically stimulated every 45 sec. (a) The effect of adding 250 ng pig relaxin to the bath (RXN). (b) The effect of a crude extract (CE) of sheep placentome containing 40 ng pig relaxin equivalents. WO indicates that the bath was washed out with clean buffer. The bar represents 5 min in each case.
Table 3. A comparison of the relaxin-like bioactivity and immuno-reactivity of an extract of sheep placentome fractionated on Sephadex G-50*

<table>
<thead>
<tr>
<th>Fraction no.†</th>
<th>ng RXN-IR/ fraction</th>
<th>ng RXN-IR/ mg protein</th>
<th>Bioactivity (mean inhibition %)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–8</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>9 BD</td>
<td>6.45</td>
<td>0.7</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>33.0</td>
<td>3.4</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>15.0</td>
<td>2.7</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>10.5</td>
<td>2.2</td>
<td>—</td>
</tr>
<tr>
<td>13 CC</td>
<td>14.1</td>
<td>3.5</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>28.5</td>
<td>8.7</td>
<td>7.5</td>
</tr>
<tr>
<td>15</td>
<td>68.2</td>
<td>25.8</td>
<td>7.7</td>
</tr>
<tr>
<td>16</td>
<td>162.0</td>
<td>75.0</td>
<td>(unreliable trace)</td>
</tr>
<tr>
<td>17</td>
<td>204.0</td>
<td>170.0</td>
<td>17.5</td>
</tr>
<tr>
<td>18</td>
<td>68.4</td>
<td>77.3</td>
<td>8.0</td>
</tr>
<tr>
<td>19</td>
<td>83.1</td>
<td>83.0</td>
<td>7.3</td>
</tr>
<tr>
<td>20</td>
<td>40.2</td>
<td>50.0</td>
<td>13.0</td>
</tr>
<tr>
<td>21</td>
<td>6.9</td>
<td>8.6</td>
<td>—</td>
</tr>
<tr>
<td>22</td>
<td>0.7</td>
<td>1.8</td>
<td>—</td>
</tr>
<tr>
<td>23</td>
<td>0.5</td>
<td>7.9</td>
<td>—</td>
</tr>
<tr>
<td>24 Tyr</td>
<td>0.6</td>
<td>12.7</td>
<td>—</td>
</tr>
<tr>
<td>25</td>
<td>0.5</td>
<td>13.0</td>
<td>—</td>
</tr>
</tbody>
</table>

ND = not detectable.

*Placentomes (24 g) collected from a single animal at ~90 days gestation were used.

†Molecular weight markers: BD = blue dextran in the void volume > 20,000; CC = cytochrome C, 12,300; Tyr = tyrosine, 181.

‡One third of each fraction was measured in a bioassay using a rat uterine strip (see Fig. 5). The results are expressed as the percentage decrease in height between the 10 contractions before and after the addition of each sample.

Fig. 6. Diagram to show the relaxin content of a sample of 11 g intercotyledonary endometrium collected from a ewe at ~125 days' gestation. The extract was run on a column of Sephadex G-50 and the RXN-IR in each fraction was measured. The arrows show the elution position of three molecular weight markers: BD, blue dextran in the void volume, > 20,000; CC = cytochrome C, 12,300; Tyr = tyrosine, 181.
Other tissues

Intercotyledonary endometrium. Tissue was obtained from 2 ewes near term (>125 days' gestation) and extracted as described above. The extract was applied to a column of Sephadex G-50 and each fraction was measured by RXN-RIA. In both cases the 3-peak pattern was seen (Fig. 6).

Total RXN-IR was 0.59 and 0.94 ng/g. Fractions from one column run were measured in the bioassay but only stimulation was observed. Two additional samples of intercotyledonary endometrium were obtained at 75 and 125 days' gestation and the crude extracts contained 0.31 and 0.49 ng RXN-IR/g. The overall concentration of RXN-IR in intercotyledonary tissue was therefore 0.59 ± 0.13 ng/g (mean ± s.e.m., n = 4).

Allantochorion. Three samples of allantochorion were obtained at 50, 75 and 125 days' gestation and the crude extract was measured by RIA. The concentration was 0.64 ± 0.29 ng RXN-IR/g (mean ± s.e.m., n = 3).

Corpus luteum. In early experiments ovaries containing corpora lutea were obtained from pregnant ewes, frozen on solid CO₂, stored at −20°C for between 1 week and 8 months, pooled for all stages of pregnancy and extracted in batches of 13–22 g (mean ovary weight = 1.2 g). The extract was applied to a column of Sephadex G-50 and the fractions measured by RXN-RIA. The peak pattern was very variable with activity detected in high, medium and low molecular weight ranges. The total immunoreactivity (for all molecular weights) was 1.05 ± 0.66 ng/g (mean ± s.e.m., n = 7).

Discussion

This investigation provides evidence that relaxin-like material can be isolated from the placenta of the ewe. The majority of this material has a molecular weight of about 6000, characteristic of relaxin in other species, and it can be purified further by using ion exchange chromatography to give a peak which elutes in a position similar to that of pig relaxin. The immunological activity was confirmed using several antisera to pig relaxin obtained from 3 different sources, including one which had been purified by affinity chromatography using CM-a relaxin. Clear biological activity was found on a number of occasions in both crude extracts and Sephadex G-50 purified material using a uterine strip bioassay. Our inability to obtain uterine inhibition with the CM-cellulose purified extracts can almost certainly be attributed to a lack of sufficient material. It is likely that the inhibition of uterine contractions was caused by a relaxin-like peptide as the effect was similar to that obtained using pig and rat relaxins. The extraction and fractionation procedures should exclude other known myometrial inhibitors such as steroids, prostaglandins, catecholamines and histamine. A non-specific toxic effect is unlikely as the strips recovered their original level of activity following wash-out, and other fractions from the same extracts either had no effect or caused stimulation.

Earlier studies concerning the presence of relaxin in the ewe have been equivocal. Hall et al. (1962) used a mouse interpubic ligament assay and found no convincing evidence of relaxin bioactivity in serum collected from ewes in the last 2 months of pregnancy and Renegar & Larkin (1985) could only detect immunoreactivity in 3 of 54 serum samples collected from the uterine vasculature of pregnant ewes. Immunoreactivity was measured in sheep plasma during the oestrous cycle (Chamley et al., 1975), pregnancy (Bryant & Chamley, 1976a) and lactation (Bryant & Chamley, 1976b). Impurities in the porcine relaxin standard used in this work (Bryant, 1972) may have contributed to the high plasma relaxin concentrations reported (e.g. 200 ng/ml during parturition). Alternatively, it is possible that impurities in the antigen and/or the direct assay of the plasma samples allowed for the measurement of higher molecular weight forms of relaxin which could be broken down during the extraction procedure used in the present paper. Renegar & Larkin (1985) have found low levels of relaxin-immunoreactivity in endometrium, placenta and ovaries of ewes at 45 and 140 days of gestation. Concentrations were similar in all tissues and at both times giving an overall concentration of 0.57 ± 0.13 ng pig relaxin equivalents per g tissue.
They found that immunoreactivity was present in most fractions after Sephadex G-50 purification with a peak in the range of $M_w$ 12 000–16 000 but they were unable to demonstrate bioactivity. Renegar & Larkin (1985) used an acid-acetone extraction method whereas we used the method devised by Walsh & Niall (1980) to minimize proteolysis of pig relaxin during its purification.

One puzzling feature of our work was the extreme variability in yield. Whilst many of our concentrations were within the same range reported by Renegar & Larkin (1985) occasionally we obtained much higher values reaching 61 ng/g for placentomes from one ewe collected at Day 45 of gestation. In studies of this kind one must suspect contamination, but we are confident that this was not the case as all the extraction and chromatography work was performed using fresh reagents and glassware in laboratories in which pig relaxin had never been used. Moreover we purified no pig relaxin during the course of this work, and so the most likely alternative is that sheep relaxin is present at higher concentrations than we or others have usually measured, but that it is extremely labile. The work showing the loss of immunoreactivity during storage at $-80\,^\circ\mathrm{C}$ would support this view. Nevertheless, when conditions were apparently optimized to reduce degradation by the use of liquid nitrogen and same day extraction, this did not necessarily result in high yields. In the sow relaxin may normally be stored in granules as a precursor and some degree of proteolysis is probably necessary before the $M_w$ 6000 form of relaxin is produced (M. J. Fields, personal communication). The extraction protocol may therefore need to be modified to improve yields of sheep relaxin.

We frequently found three peaks of RXN-IR which could all be detected with a variety of antisera. In the rat Sherwood et al. (1986) have shown that RXN-IR in the serum on Day 15 is associated with a large molecular weight component (60 000), with a shift towards two smaller components (13 000 and 6500) by Day 19. They attributed this to a change in secretion pattern rather than to peripheral metabolism. In addition we found a peak with an apparent molecular weight of $\sim 1000$, similar to that reported by Fields et al. (1980) from extracts of corpus luteum from late pregnant cows. This relaxin-like material from cows had an apparent molecular weight of about 1400 and was bioactive in the mouse uterine activity and interpubic ligament separation bioassays. Although it seems unlikely that a peptide of this size should retain both the bio- and immunoactivity of the 6000 molecular weight form of relaxin, the similarity in the results of these two studies on ruminants suggests that the subject merits further investigation. All this work indicates that relaxin can be produced in various forms and it remains a possibility that these might exhibit different biological activities.

The cells which synthesize relaxin in the ewe have still not been identified and the results of both this study and that of Renegar & Larkin (1985) suggest that activity is present in placentomes, endometrium and corpus luteum. A dual uterine and ovarian source has also been indicated in the human (O'Byrne et al., 1978; Bigazzi et al., 1982). The much greater weight of placentomes compared with luteal tissue during gestation suggests that the placenta is likely to be the main source of sheep relaxin.

The role of relaxin in sheep is also speculative. Pig relaxin is a potent inhibitor of myometrial contractions in ovariectomized oestrogen-primed ewes (Porter et al., 1981), suggesting that the hormone could contribute in the ewe to maintaining uterine quiescence. Other possibilities are remodelling of the connective tissue framework of the uterus during pregnancy and distension of the cervix at parturition (for review, see Porter, 1979). Some of these queries may be resolved by improved purification techniques or gene isolation leading to the production of sufficient sheep relaxin to develop homologous assay techniques.

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Relaxin in the ewe

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