RELAXIN IN THE BLOOD OF PREPARTURIENT EWES

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SUMMARY

The concentration of relaxin in the circulating blood of ewes has been investigated during the last 2 months of pregnancy.

When assayed on the symphysis pubis of oestrogen-primed mice, the concentration of relaxin present in extracts was less than 0·2 u./ml. serum. The extracts produced no measurable effects on weight or histology of the uterine tract or on distensibility of the cervix of oestrogen-primed mice, whether or not progesterone was also given.

It is concluded that in the sheep the amount of relaxin in the circulating blood during the later stages of gestation is too low to detect, by existing assay procedures, abnormalities in blood concentrations in conditions such as Ring Womb.

Confirmation is provided of an earlier report that the concentration of relaxin in the blood of pregnant mice during the period of rapid interpubic separation is also less than 0·2 u./ml. serum.

INTRODUCTION

Softening and increased distensibility of the cervix and lower uterine segment after treatment with relaxin have been reported in sows and heifers (Graham & Dracy, 1952, 1953; Zarrow, Sikes & Neher, 1954; Zarrow, Neher, Sikes, Brennan & Bullard, 1956), rats (Steinetz, Beach & Kroo, 1959; Cullen & Harkness, 1960), monkeys (Hisaw, 1959) and women (Eichner, Waltner, Goodman & Post, 1956; Stone, Sedlis & Zuckerman, 1958). Reports on the action of relaxin on the human cervix, however, are confusing for other workers (e.g. Slate, 1961) have not obtained increased distensibility. As with other actions of relaxin, priming with oestrogen or the presence of endogenous oestrogen is necessary.

In the course of an investigation into the causes and treatment of Ring Womb in sheep (failure of the cervix to dilate during parturition), one of us (C.B.T.) administered at parturition 1500 u. relaxin (Releasin, Warner Chilcott) to six ewes with this condition and in five instances obtained softening of the previously hard cervix (Hindson & Turner, 1962). Although with one exception this treatment did not result in normal delivery of the lambs, it was felt that if affected cases could be anticipated and relaxin therapy initiated earlier, more dramatic results might be achieved.

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Before investigating further the possible involvement of relaxin in Ring Womb in sheep, it seemed advisable to attempt quantitative estimations of the amount of relaxin produced during gestation and of the normal range of relaxin levels in the circulating blood during the period before parturition.

Only one reference to relaxin estimations in pregnant ewes has been found. Bassett & Phillips (1955, and personal communication) reported, from preliminary tests, that relaxin appeared to be present in the ovaries in about the same concentration as in those of pregnant sows (one of the richest known sources of the hormone) but in low concentration in the blood. These observations were made on a very small number of animals and were largely qualitative.

This paper reports the results of an investigation of the level of relaxin in the circulating blood of ewes during the last part of gestation, and is the first stage of a more extended study of the physiological role of relaxin in sheep.

**Materials and Methods**

*Ewes*

Blood was taken from fifty ewes of mixed breed from flocks in Devon and Cambridge.

*Blood samples and method of extraction*

The samples were taken at various periods during the last 2 months of gestation. Blood was withdrawn into heparinized tubes by venepuncture from the jugular vein, and despatched to Birmingham for extraction. Serum was extracted 24–48 hr. after removal from ewes and the extracts were used immediately in assay experiments. Extraction was by the method of Albert & Money (1946), the final precipitate being taken up in distilled water in a volume equivalent to 1:5 to 1:10 that of the original serum. At first, samples from individual ewes were extracted and tested separately. It soon became apparent that the concentration of relaxin would turn out to be very low, and as the average amount of serum per ewe was only 15–20 ml., in subsequent assays sera from 4 or more ewes were pooled.

*Assay procedure*

The extracts were tested for their effect on the symphysis pubis and on the weight and histology of the reproductive tract and distensibility of the cervix of mice. The mice were intact, virgin females of Parkes strain from mixed litters bred in Birmingham and were approx. 5 weeks old when used. All mice were primed with a single injection of 5 μg. oestradiol cyclopentylpropionate 3–7 days before the start of the test (see Table 1). Vaginal smears were made as a check on the effectiveness of priming.

Extracts were injected s.c. in a volume of 0.2–0.75 ml. daily (see Table 1); control mice received the priming dose of oestrogen only. In some groups, oestrogen-primed mice were injected with 0.5 or 1.0 mg. progesterone on the same days as the extract, controls receiving oestrogen and progesterone only. On the day following the last injection, mice were autopsied, the symphysis pubis examined *in situ* under magnification and illumination, cervixes of control and experimental mice compared for distensibility by the insertion of a probe of graded diameter, and horns, cervix and vagina weighed and fixed in Bouin's solution for histological examination and histochemical visualization of glycogen.
In an attempt to potentiate the activity of any relaxin present in the extracts, two batches (see Table 1) were each divided into two portions to one of which was added 1% benzopurpurine, a depot agent known to potentiate the activity of relaxin from sow ovaries (Steinetz et al. 1959).

Table 1. Test of activity of extracts of sheep serum on symphysis pubis of mouse

<table>
<thead>
<tr>
<th>Serial no. of extract (no. of ewes sampled in parentheses)</th>
<th>No. of mice injected</th>
<th>No. of daily injections</th>
<th>Interpubic separation (mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4 (1)</td>
<td>5</td>
<td>5</td>
<td>0.25–0.4</td>
</tr>
<tr>
<td>C1 (1)</td>
<td>5</td>
<td>4</td>
<td>0.25</td>
</tr>
<tr>
<td>D5 (1)</td>
<td>5</td>
<td>5</td>
<td>All &lt; 0.4</td>
</tr>
<tr>
<td>C4 (1)</td>
<td>4</td>
<td>5</td>
<td>All &lt; 0.35</td>
</tr>
<tr>
<td>C3 (1)</td>
<td>2</td>
<td>4</td>
<td>All &lt; 0.35</td>
</tr>
<tr>
<td>C2 (1)</td>
<td>2</td>
<td>6</td>
<td>0.3</td>
</tr>
<tr>
<td>C6 (1)</td>
<td>2</td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>C13–15 (3)</td>
<td>2</td>
<td>4</td>
<td>All &lt; 0.35</td>
</tr>
<tr>
<td>C13–15 (3)*</td>
<td>5</td>
<td>4</td>
<td>All &lt; 0.35</td>
</tr>
<tr>
<td>C7–12 (6)</td>
<td>6</td>
<td>5</td>
<td>All &lt; 0.4</td>
</tr>
<tr>
<td>C7–12 (6)*</td>
<td>6</td>
<td>5</td>
<td>All &lt; 0.4</td>
</tr>
<tr>
<td>C17–20 (4)†</td>
<td>3</td>
<td>3</td>
<td>All &lt; 0.35</td>
</tr>
<tr>
<td>C31–50 (12)‡</td>
<td>10</td>
<td>5</td>
<td>0.3–1.2 (mean 0.5)</td>
</tr>
</tbody>
</table>

Final precipitate dissolved in vol. aq. dest. = 0.2 vol. serum extracted; 0.2 ml. injected daily unless otherwise stated. First injection extract given day 4 after priming injection of 5 µg. oestradiol cyclo-

pentylopropionate.

* Precipitate dissolved in 1% benzopurpurine.
† Vol. extract = 0.15 vol. serum; 0.75 ml. injected daily; priming period 8 days.
‡ Vol. extract = 0.1 vol. serum; 0.5 ml. injected daily.

Twenty control mice, littermates to some of the above, received no extract and were killed on appro-

priate days after priming dose of oestrogen.

As a check on the extraction procedures, serum from rabbits 28 days pregnant was extracted by the same method (a) immediately after withdrawal and after standing for more than 24 hr.; (b) with and without the addition of heparin.

Comparison with activity of relaxin from pregnant sow ovaries

Three groups each of eight mice were injected, on each of days 4–7 after a priming injection of 5 µg. oestradiol cyclopentylopropionate, with 0.24, 1 or 2 or 6.0 µg. relaxin (Releasin, Warner Chilcott; the reference standard preparation was used, containing 150 u./mg. by guinea-pig symphysis pubis palpation test).

Comparison with activity of serum from pregnant mice

Blood was collected at autopsy from twenty-eight mice on days 16–19 of pregnancy, the serum extracted and the extract injected into three oestrogen-primed mice (0.2 ml./day on days 4–7 after priming).

RESULTS

Symphysis-relaxing activity of extracts

Effect on symphysis pubis of mouse

In preliminary tests (not included in Table 1) a single injection of 0.2 or 0.5 ml. extract given on the eighth day after priming with oestradiol produced no effect on
the symphysis. As it proved impractical to take up the precipitate in volumes of water less than 1:10 that of the serum, in all subsequent tests 3–6 daily injections were given in the volumes shown in Table 1. It soon became apparent that if any activity was present in the extracts, the mouse symphysis pubis method was not sufficiently sensitive to provide an effective comparative quantitative assay technique. After 4–6 daily injections of 0·2 ml. extract, no difference was detected between the symphysis pubis of treated mice and of those which received oestrogen alone. Increasing the priming interval to 8 days and the amount of extract to 0·75 ml. × 3 days of the more concentrated solution (C17–20, see Table 1) still produced no significant effect. Finally, six daily injections of 0·5 ml. of extract C31-50 (volume 1:10 that of serum extracted) resulted in interpubic separations ranging from 0·3–1·2 mm. (mean 0·5 mm.). Interpubic separations in the twenty oestrogen-primed control mice never exceeded 0·4 mm.

**Effect of injection of whole serum**

One batch of serum was injected without extraction, in a volume of 0·4–0·5 ml. daily for 7 days to oestrogen-primed mice. No effect was detected on the symphysis.

**Effect of addition of depot agent to extracts**

It has been established that the activity on the mouse symphysis pubis of relaxin prepared from ovaries of sows can be potentiated by adding any of a series of repository agents of which benzopurpurine is one of the most efficient (Steinetz et al. 1959). Relaxin extracts from pregnant rabbit serum cannot be potentiated in this way. In the present experiments there was no evidence of increased activity in the two extracts of sheep serum made up in benzopurpurine.

**Test of validity of extraction procedures**

In earlier experiments (Hall & Newton, 1947) in which the concentration of relaxin in the blood of pregnant rabbits was assayed, extraction was always carried out immediately after withdrawal of the blood and no anti-coagulant was added. In the

<table>
<thead>
<tr>
<th>Description of extract</th>
<th>No. of mice injected</th>
<th>Length of interpubic ligament (mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heparin; serum extracted immediately after withdrawal of blood</td>
<td>4</td>
<td>3·0 (in each of 3 mice)</td>
</tr>
<tr>
<td>Heparin added; serum extracted &gt; 24 hr. after withdrawal of blood</td>
<td>3</td>
<td>3·0 (1 mouse)</td>
</tr>
</tbody>
</table>

Table 2. **Effect of addition of heparin to blood samples, and of delay in extraction of serum, on relaxin activity of extracts of pregnant rabbit serum**

All mice received one injection of 5 µg. oestradiol cyclopentylpropionate 3 days before the first of four daily injections of 0·2 ml. extract.

In the present experiment there was an unavoidable delay of at least 24 hr. between withdrawal and extraction, and for this reason heparin was added to the bottles. It was decided, therefore, to investigate whether these two procedures would destroy or
adversely affect the activity of relaxin in the blood of pregnant rabbits. The results are given in Table 2. In both groups, interpubic separation 24 hr. after four daily injections of 0·2 ml. extract was in the expected range (see Hall & Newton, 1947), and no inactivation had resulted from the delay in extraction and addition of heparin.

Comparison with relaxin prepared from ovaries of pregnant sows

The results are given in Table 3. In the group which received the smallest dose, interpubic separations of 0·5 mm. occurred in five mice; of those which received 1·2 µg. (0·18 u.), separations of 0·8 mm. were found in four mice. The response of the first group was very close to that obtained with oestrogen alone. It is apparent, however, that the concentration of relaxin in 0·2 ml. (the volume injected daily) of the sheep serum extract was less than that contained in 1·2 µg. Releasin, i.e. the level of relaxin in the blood of sheep during the last two months of pregnancy appears to be less than 0·2 u./ml. serum.

Table 3. Test of activity of three dose-levels of relaxin from pregnant sow ovaries

<table>
<thead>
<tr>
<th>Daily dose injected</th>
<th>Interpubic separation (mm.)</th>
<th>(mean and range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0·24 µg. (0·036 u.)</td>
<td>0·4 (0·2–0·5)</td>
<td></td>
</tr>
<tr>
<td>1·2 µg. (0·18 u.)</td>
<td>0·5 (0·2–0·8)</td>
<td></td>
</tr>
<tr>
<td>6·0 µg. (0·90 u.)</td>
<td>2·1 (1·0–3·5)</td>
<td></td>
</tr>
</tbody>
</table>

Comparison with extract from serum of pregnant mice

In the four mice which received four daily injections of relaxin extract prepared from serum of pregnant mice, interpubic separations of 0·25–0·5 mm. were recorded. No definite conclusions can be drawn from such a small number of animals, but it appears that very little relaxin is present in the circulating blood of mice during the period of rapid interpubic separation.

Effect on reproductive tract

When it became apparent that the ‘symphysial relaxing’ activity of the extracts would prove to be low, it was decided to investigate other known activities of relaxin.

(a) Effect on softening and distensibility of cervix

When tested by the insertion of a probe into the cervical canal, no difference in distensibility was recorded between those mice which received the extract and the oestrogen-primed controls. Subjectively, no difference was detected in the ‘consistency’ of the tissues of the cervix in treated and control groups. The addition of 0·5 or 1·0 mg. progesterone given on the same days as the extracts did not affect the results.

(b) Effect on weights of horns and cervix

It has been shown (Jablonski & Velardo, 1957, 1958; Zarrow & Brennan, 1958; Steinetz, Beach, Byre & Kroc, 1957; Hall, 1960a) that relaxin from sow ovaries has
a synergistic effect with oestradiol on the uterine weights of rats and mice. In the present experiments with sheep extracts no synergism with oestradiol or with oestradiol and progesterone was found on the weights (actual or expressed as a ratio of body weight) of horns or cervix.

(c) Effect on histological structure of uterus

Hall (1960a) reported that relaxin from sow ovaries augmented the action of oestradiol in increasing myometrial glycogen and producing oedematous transformation of the endometrial stroma in mice. In the present experiments, extracts of sheep serum did not modify the action of oestradiol on the histological structure of the uterus or on myometrial glycogen.

DISCUSSION

The mouse pubic symphysis method of assay was chosen because in general it provides lower limits of error and a better index of precision than the guinea-pig test, which is more subjective (Kroc, Steinetz & Beach, 1958). From the start, however, it became obvious that the concentration of relaxin in the blood was too low to permit the use of the single dose technique recommended by Steinetz, Beach, Kroc, Stasilli, Nussbaum, Nemitz & Dun (1960), even after a priming interval of 8 days which is the optimum period (Hall, 1948). It was not found practicable to dissolve the precipitate in a volume less than 1:10 that of the original serum. The extracts were administered therefore over a period of 3-6 days, using the same technique as in earlier experiments with rabbit relaxin (Hall & Newton, 1947). Because after 8 days oestrogen alone causes slow progressive intersubic separation (Hall, 1949), the injections were generally started on the fourth day after priming.

Quantitative data for concentrations of relaxin in blood serum during the later stages of pregnancy are available for only a few species. A high concentration has been recorded only in the rabbit—approximately 10-15 u./ml. serum (Marder & Money, 1944; Steinetz et al. 1959). In terms of symphysis-relaxing activity, 1 ml. serum from a rabbit 20-30 days pregnant contains sufficient relaxin to produce, when injected daily s.c. into a spayed, oestrogen-primed mouse, intersubic separation identical both in degree and time interval to that which normally occurs during pregnancy (Hall & Newton, 1947). Steinetz et al. (1959) reported that in mice, although relaxin activity equivalent to 200 u./g. tissue was found in the ovaries, less than 0.1 u./ml. serum appeared in the circulating blood, i.e. about 1:100 that in the blood of rabbits. The present tests confirm this low concentration in the blood of mice. Figures given for other species are: cat, 1.5 u./ml. serum (Steinetz et al. 1959); cow, 0.3 u./ml. (Wada & Yuhara, 1961); women, 2.0 u./ml. (Zarrow, Holmstrom & Salhanick, 1955). Data for concentrations in ovaries or placentae are reviewed by Steinetz et al. (1959) and Hall (1960b). They include 350 u./g. placenta in rabbits and up to 10000 u./g. ovary in sows; no figures seem to be available for blood concentrations in the latter species. Zarrow & Money (1948) produced evidence that relaxin is probably broken down rapidly in the body and is for the most part excreted as a degraded and inactive substance.

The results of the present investigation show that the concentration of relaxin
Relaxin in sheep

(judged by its ‘symphysis-relaxing’ activity) in the blood of the ewe during the last 2 months of gestation is too low to permit quantitative determinations, with the currently available assay methods, of the range of normal variation.

The problem which inspired this investigation involved the activity of relaxin in producing softening and dilatation of the cervix rather than its activity in relaxing the pelvic joints. Unfortunately, no strictly quantitative method is available for assessing this activity. The present tests revealed no difference between the cervices of mice which were given the extracts (with or without the addition of progesterone) and those which received oestrogen alone. It appears therefore that fluctuations from the mean of this activity of the relaxin molecule also cannot be assessed by measurements of its concentration in the blood.

It is concluded that in the sheep, as in the other species for which data are available except the rabbit, the amount of relaxin in the circulating blood during the last stages of pregnancy is too low to determine possible variations from the normal mean in conditions such as Ring Womb. A different approach to the problem will be needed.

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REFERENCES


