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# The Role of the Endometrial Oxytocin Receptor in Determining the Length of the Sterile Oestrous Cycle and Ensuring Maintenance of Luteal Function in early Pregnancy in Ruminants

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# The role of the endometrial oxytocin receptor in determining the length of the sterile oestrous cycle and ensuring maintenance of luteal function in early pregnancy in ruminants

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## SUMMARY

The oxytocin receptor, a seven transmembrane domain, G protein-linked receptor molecule, plays a central role in determining the endocrine function of the ruminant uterine endometrium. During non-pregnant cycles the control of this molecule by circulating steroid hormones leads to regression of the corpora lutea. The kinetics of the mechanisms involved determine the time at which luteolysis occurs, and therefore the length of the oestrous cycle. In pregnancy, secretions of the trophoblast block endometrial oxytocin receptor gene expression and lead to luteal maintenance. An understanding of the molecular mechanisms involved in the steroidal control of oxytocin receptor gene expression will provide an explanation for the relative constancy of oestrous cycle lengths in non-pregnant animals. Unravelling the way in which trophoblast products block expression of the oxytocin receptor gene will lead to a better understanding of the reasons for the high rate of embryonic loss in domestic ruminants.

## 1. INTRODUCTION

Ovarian cycle lengths, the time intervals between successive ovulations in animals housed apart from a male, are characteristically constant in polyoestrous mammals which ovulate spontaneously, and where a functional corpus luteum is formed. For instance, mean ovarian cycle length (±s.d.) in rhesus monkeys is 28 ± 0.7 d; in the domestic sow, 21 ± 0.5 d; and in the western grey kangaroo, 35 ± 4.4 d. Where a pseudopregnancy occurs, with the formation of a functional corpus luteum, its length may also be relatively constant: hamster, 9 ± 0.3 d; rat, 13 ± 1.7 d. This constancy in ovarian cycle lengths is remarkable because, as Short (1974) pointed out, pregnancy is the normal reproductive state in most free-living mammals and selection pressures may not generally have been exerted on the length of the sterile ovarian cycle. The mechanisms determining these intervals are ill-understood.

When conception occurs the repetitive oestrous cyclicity of the non-pregnant polyoestrous mammal is generally suspended, luteal regression (luteolysis) is blocked and the corpus luteum formed at the last ovulation continues to secrete progesterone which is required by the uterus to support the growth of the conceptus. The maintenance of luteal function involves the secretion by the developing conceptus of a signal indicating its presence to its mother. In the absence of the signal, the corpus luteum regresses and a new ovulation provides a further opportunity for conception. A limited number of exceptions to this generalization are known, in which gestation length is equal to that of the non-pregnant ovarian cycle (for example the ferret and the dog).

The endocrine mechanisms controlling corpus luteum function during the cycle and in early pregnancy have been studied in most depth in the domestic ruminants, in which cycle lengths are also relatively constant (sheep, 17 ± 0.9 d; cattle, 21 ± 0.8 d). In sheep, cattle and goats the uterine endometrium plays an important role in controlling the corpus luteum through the production of the luteolytic substance, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In non-pregnant ruminants the interaction of circulating oxytocin with the oxytocin receptor stimulates production of $PGF_{2\alpha}$ and initiates the process of luteal regression; the oxytocin involved is secreted by the corpus luteum itself. Concentrations of the oxytocin receptor in the endometrium are low during the greater part of the cycle, rising only towards the end of the luteal, and during the follicular phases; the constancy of oestrous cycle length reflects the kinetics of oxytocin receptor induction and

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repression. In pregnancy the signal produced by the conceptus suppresses expression of the oxytocin receptor in the endometrium, thereby preventing episodic $PGF_{2\alpha}$ production and blocking luteolysis. This article reviews the evidence that the mechanisms controlling ovarian hormone secretion in pregnant and non-pregnant ruminants involve modulation of endometrial concentrations of the oxytocin receptor, and that ovarian cycle length is determined by the kinetics of expression of the oxytocin receptor by uterine cells. The oxytocin receptor, a seven transmembrane domain, G protein-linked molecule, has recently been cloned from a human myometrial cDNA library and partial sequence information has been obtained for the sheep endometrial form (see Stewart *et al.* 1993; and references therein).

## 2. THE OXYTOCIN RECEPTOR AND LUTEOLYSIS IN NON-PREGNANT CYCLES

The ovarian cycle consists of two phases, a follicular phase preceding ovulation, and a luteal phase following it. The length of the cycle is determined principally by the length of the luteal phase; in species in which follicular development occurs during the luteal phase, the follicular phase may only account for 20% of the length of the cycle. Therefore in examining factors determining overall ovarian cycle length, mechanisms influencing the length of the luteal phase must be given emphasis. The length of the luteal phase is determined by the time of luteal regression.

In sheep, in which the process has been examined in most detail, luteolysis results from the episodic secretion of $PGF_{2\alpha}$ by the uterus (McCracken *et al.* 1972; Thorburn *et al.* 1973; Barcikowski *et al.* 1974; figure 1, this paper). The principal source of $PGF_{2\alpha}$ in the uterus at luteolysis is the endometrium (Caldwell *et al.* 1968). $PGF_{2\alpha}$ entering the uterine vein gains access to the ovarian artery and thence to the corpus luteum by a process generally attributed to counter current distribution of the lipoidal $PGF_{2\alpha}$ molecule between the two vessels (McCracken *et al.* 1971). In domestic ruminants the ovarian artery follows a tortuous course over, and is in close apposition to, the uterine vein and this anatomical relationship facilitates the transfer of lipoidal solutes between the uterine vein and the ovarian artery (Ginther 1981). Detailed characterization of the pattern of pulsatile release of $PGF_{2\alpha}$ during luteolysis shows that pulses are most frequently secreted over a 48 h period at intervals of 6–8 h. $PGF_{2\alpha}$ is more effective in causing luteolysis when administered in divided doses, the most effective time interval between doses being 6–8 h (Schramm *et al.* 1983). Pulsatile release of $PGF_{2\alpha}$ by the endometrium may be more effective than continuous secretion because luteolysis involves activation of luteal protein kinase C (Wiltbank *et al.* 1991; Abayasekara *et al.* 1993), an enzyme that can be down-regulated by prolonged exposure of a tissue to agonists (Hu, Azhar & Hoffman 1992). As expected, prolonged exposure to raised concentrations of $PGF_{2\alpha}$ leads to refractoriness (Lamsa *et al.* 1992).

In seeking to explain the pulsatile nature of $PGF_{2\alpha}$ secretion, attention has focussed on the hormone oxytocin (Roberts *et al.* 1975; Silvia & Raw 1993; see reviews by Flint *et al.* 1990*a*; Silvia *et al.* 1991, and references therein; figure 2, this paper). Oxytocin stimulates endometrial $PGF_{2\alpha}$ secretion in most domestic ruminants as a result of the activation of phospholipase C, following interaction with the endometrial oxytocin receptor; other phospholipases are probably also involved (Flint *et al.* 1986). The time course of the response of the endometrium to oxytocin in terms of increased phosphatidyl inositol metabolism is consistent with that of the activation of secretion of $PGF_{2\alpha}$ *in vivo*. Immunization against oxytocin blocks luteal regression. Measurement of oxytocin and the pulmonary metabolite of $PGF_{2\alpha}$ (13,14-dihydro-15-keto $PGF_{2\alpha}$, concentrations of which change in parallel with uterine $PGF_{2\alpha}$ secretion) in the peripheral circulation during luteal regression shows that in general oxytocin is secreted synchronously with each episode of $PGF_{2\alpha}$ (figure 1). Detailed analysis of the temporal relationship between oxytocin and $PGF_{2\alpha}$ at the start of each episode of secretion suggests that each spike is initiated by a rise in the concentration of $PGF_{2\alpha}$ (Moore *et al.* 1986); however, oxytocin secreted by the neurohypophysis, which is quantitatively minor but is also episodic, may be the initial trigger (McCracken *et al.* 1984).

The episodic secretion of oxytocin in synchrony with $PGF_{2\alpha}$ reflects secretion of oxytocin by the corpus luteum (Flint & Sheldrick 1982). The corpora lutea of all ruminants so far investigated express the oxytocin gene (Wathes & Swann 1982; Ivell & Richter 1984; Jones & Flint 1988; Flint *et al.* 1991), and synthesis involves the oxytocin-neurophysin prohormone, as in the neurohypophysis (see Sheldrick & Flint 1989, and references therein). In the corpus luteum, as in the neurohypophysis, post-translational processing and storage of the secreted product occur in secretory granules, although secretory granules in the corpus luteum are larger than those in the neurohypophysis (Theodosis *et al.* 1986). Steroidogenic luteal cells are of two types, large and small, and are thought to arise by luteinization of follicular granulosa and theca cells respectively (Hansel *et al.* 1991; Wiltbank *et al.* 1991); synthesis and secretion of oxytocin is limited to large luteal cells in both sheep and cattle, and concentrations of oxytocin in the corpus luteum are approximately one-quarter those in the posterior pituitary. However, a larger proportion of stored oxytocin is available for secretion as a single pulse in the corpus luteum than is the case in the neurohypophysis and for this reason, and by virtue of its size, the corpus luteum may represent a source of larger quantities of oxytocin than the posterior pituitary. As in the posterior pituitary luteal secretion of oxytocin is accompanied by secretion of neurophysin, and as expected on the basis of the structure of the prohormone the two compounds are released in a 1:1 molar stoichiometry (Watkins *et al.* 1984).

Secretion of oxytocin by the corpus luteum is stimulated by $PGF_{2\alpha}$, including $PGF_{2\alpha}$ reaching the ovary from the uterine vein (Flint & Sheldrick 1982;




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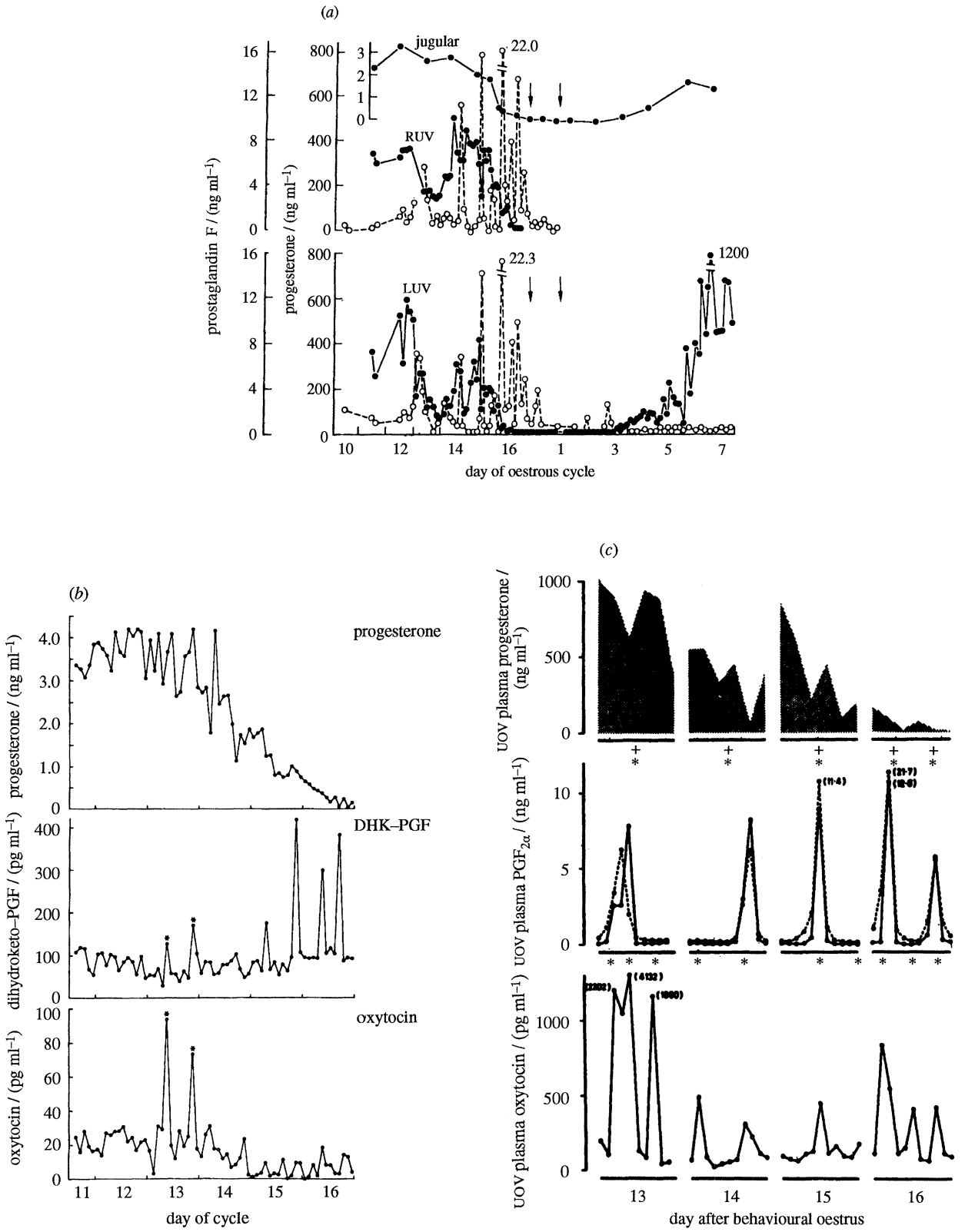


Figure 1. Episodes of prostaglandin and oxytocin secretion at luteolysis in sheep. (*a*) Data of Thorburn *et al.* (1973); concentrations of prostaglandin $F_{2\alpha}$ (open circles) and progesterone (closed circles) in right and left uterine venous plasma (RUV and LUV respectively) in one animal between day 10 of one oestrous cycle and day 7 of the next. Progesterone concentrations in jugular venous plasma are also shown. Vertical arrows indicate times of oestrus. (*b*) Data of Flint & Sheldrick (1983); jugular venous plasma concentrations of progesterone (top panel), 13,14-dihydro-15-keto $PGF_{2\alpha}$ (middle) and oxytocin (lower) in one ewe between days 11 and 16 after oestrous. Samples were taken every 2 h, and synchronous episodes are indicated by *. (*c*) Data of Hooper *et al.* (1986); concentrations of progesterone (top panel), $PGF_{2\alpha}$ (middle) and oxytocin (lower) in uterine venous plasma from one ewe during 12 h periods on days 13–16 after oestrus. $PGF_{2\alpha}$ concentrations in the right (hatched line) and left uterine veins (solid line) are shown separately. * and +, pulses identified by the authors in the left and right uterine veins respectively.

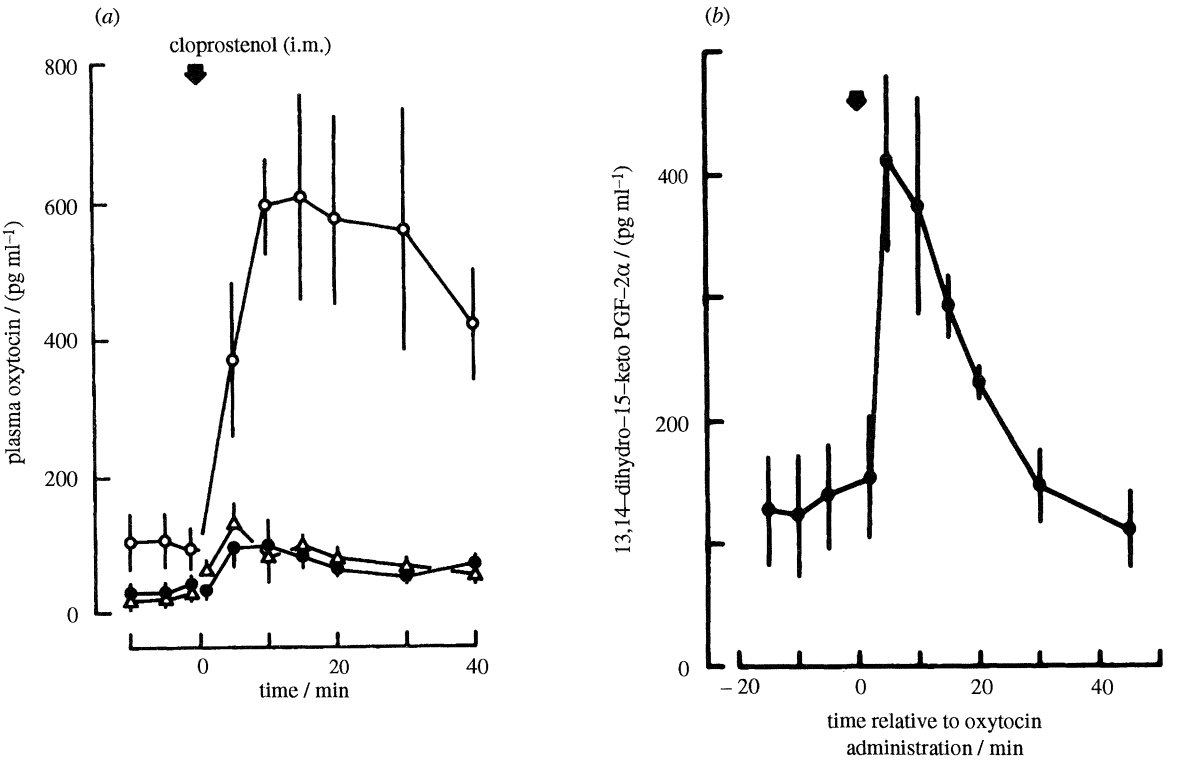


Figure 2. Oxytocin secretion is stimulated by a $PGF_{2\alpha}$ analogue, and $PGF_{2\alpha}$ secretion is stimulated by oxytocin. (*a*) Oxytocin concentrations in ovarian venous (open circles), carotid arterial (filled circles) and jugular venous (open triangles) plasma in mid-cycle ewes treated with a luteolytic dose of cloprostenol. From Flint & Sheldrick (1982). (*b*) Concentrations of 13,14-dihydro-15-keto $PGF_{2\alpha}$, pulmonary metabolite of $PGF_{2\alpha}$, in jugular venous plasma before and after treatment with 1 μg oxytocin, in steroid-pretreated ovariectomized ewes. From Flint *et al.* (1986). In both cases $t = 0$ indicates time of treatment.

Heap *et al.* 1989; figure 2). This response to $PGF_{2\alpha}$ is relatively rapid, maximum rates of secretion being reached within 10 min of treatment. Large luteal cells express the $PGF_{2\alpha}$ receptor and respond to $PGF_{2\alpha}$ with increased production of inositol phosphates and diacylglycerol, and activation of protein kinase C, although it is uncertain whether these second messengers are involved in the mechanism of activated oxytocin secretion (Fitz *et al.* 1982; McCann & Flint 1990; Wiltbank *et al.* 1991).

The tissue concentration of oxytocin in the corpus luteum is maximal during the mid-luteal phase, although concentrations of the oxytocin-neurophysin prohormone mRNA peak within 3–5 d after ovulation in both cattle and sheep before declining to low levels during the remainder of the luteal phase (see Flint *et al.* 1990*a*, and references therein). This decline in prohormone mRNA, which as we shall see has important implications for pregnancy maintenance, is thought to involve the transcription factor COUP-TF (Wehrenberg *et al.* 1992). The delay between peak mRNA and peak oxytocin may reflect the time required for post-translational processing of prohormone. Continuous secretion of oxytocin results in elevated oxytocin levels in the systemic circulation and these levels reflect the concentration of oxytocin in the tissue. Episodic secretion of oxytocin by the corpus luteum at the end of the luteal phase of the cycle is therefore superimposed on a declining basal circulating concentration. The transient nature of the expression of the oxytocin gene in the corpus luteum has profound consequences in pregnancy as we shall see later.

Because oxytocin secretion by the corpus luteum is stimulated by $PGF_{2\alpha}$, and $PGF_{2\alpha}$ secretion by the endometrium is stimulated by oxytocin, the possibility exists of a positive feedback relationship between these tissues such that a small rise in the secretion of either hormone is amplified following secretion of the other (Flint & Sheldrick 1983). This process has been suggested to underlie the rapid rise in the rate of secretion of both oxytocin and $PGF_{2\alpha}$ characteristic of the episodic secretion of these hormones during luteolysis; each episode of secretion of $PGF_{2\alpha}$ may be driven by this positive feedback loop. Cessation of secretion at the termination of each episode may in principle be due to one or more of the following: depletion of available oxytocin from the corpus luteum; depletion of a precursor of $PGF_{2\alpha}$ from the endometrium; the development of luteal refractoriness to $PGF_{2\alpha}$, or the development of endometrial refractoriness to oxytocin. There is evidence for the involvement of each of these events (see Flint *et al.* 1990*a*).

Although maximal concentrations of oxytocin in the large luteal cell and peak circulating concentrations of oxytocin in the systemic circulation are reached in the mid-luteal phase of the cycle, episodic uterine $PGF_{2\alpha}$ secretion is restricted to the period of luteal regression. Therefore a mechanism must exist to limit uterine responsiveness to oxytocin during the early and mid-luteal phases of the cycle, and




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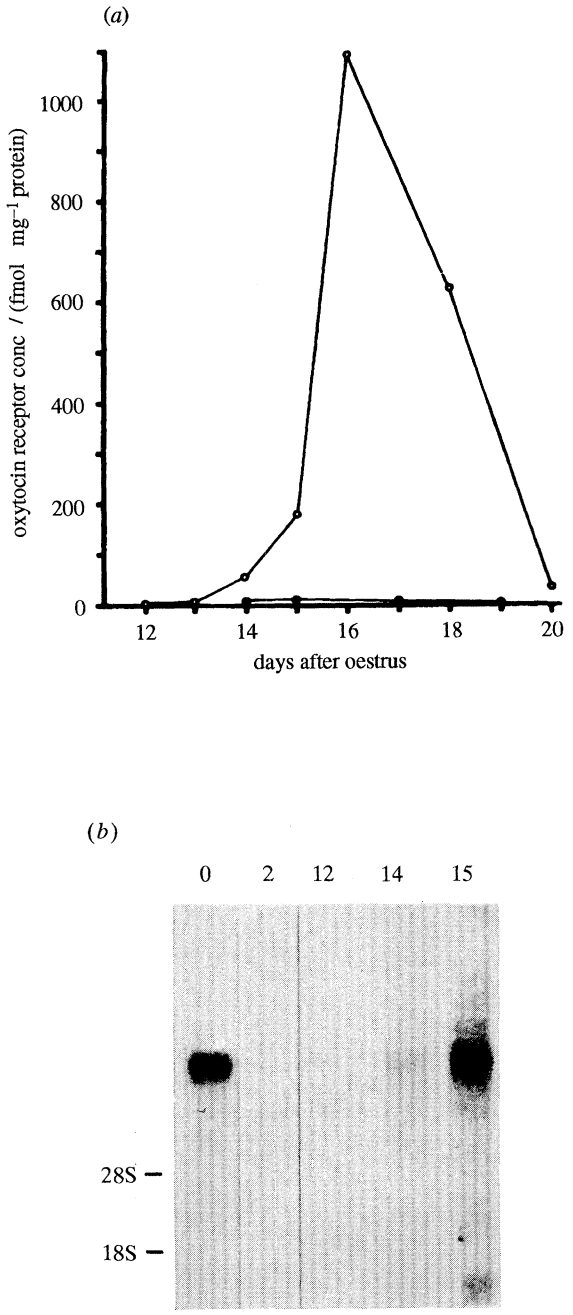


Figure 3. (*a*) Endometrial oxytocin receptor concentrations in pregnant (filled circles) and non-pregnant (open circles) ewes at various times after oestrus. Data shown refer to inter-caruncular endometrium; receptor concentrations in caruncular endometrium were reduced similarly in pregnancy. Values are from Flint & Sheldrick (1986). (*b*) Concentrations of oxytocin receptor mRNA determined by Northern blotting between days 0 and 15 (from Stewart *et al.* 1993).

inhibition of endometrial oxytocin receptor expression (and probably uncoupling of receptor from second messenger systems) appears to be responsible for this process. Concentrations of oxytocin receptor and the mRNA encoding it rise on approximately days 13 or 14 of the sheep cycle at the time of the first episodes of $PGF_{2\alpha}$ secretion; before this time endometrial oxytocin receptor concentrations are low and the uterus is unresponsive to administered oxytocin in terms of $PGF_{2\alpha}$ secretion (Roberts *et al.* 1976; Sheldrick & Flint 1985; Stewart *et al.* 1993; figure 3, this paper). Availability of the oxytocin receptor in the endometrium therefore defines the period of responsiveness of the uterus to oxytocin in terms of prostaglandin secretion and determines the ability of the uterus to cause luteal regression.

Premature administration of a receptor agonist would not be expected to induce premature receptor activation, where the physiological response depends upon receptor induction; this is the case in the oestrous cycle where administration of oxytocin does not generally induce premature luteolysis. An exception may be where oxytocin is administered shortly after ovulation, before uterine oxytocin receptor concentrations have declined to the level characteristic of the luteal phase. However, this is only observed in cattle and goats, and has not been shown conclusively in other domestic ruminants; in cattle, endometrial oxytocin receptor concentrations do not decline below 100 fmol mg$^{-1}$ protein until day 6 after oestrus, whereas in sheep this level is reached by day 4 (figure 3; Jenner *et al.* 1991).

The central role of the endometrial oxytocin receptor in this respect is supported by the results of three experiments in which oxytocin receptor levels or activity were manipulated artificially. In the first (Hixon & Flint 1987) pharmacological doses of oestradiol-17β were administered on days 9 and 10 of the ovarian cycle; this compound is known to cause premature luteal regression. This treatment induced endometrial oxytocin receptor synthesis (within 24 h), the onset of episodes of prostaglandin secretion (within 36 h) and luteolysis (commencing at 42 ± 3 h). Induction of phosphatidyl inositol metabolism in response to oxytocin occurred shortly after the first appearance of raised oxytocin receptor levels. This experiment not only explains the luteolytic effect of administered oestrogen but also shows that premature induction of the oxytocin receptor is associated with premature onset of episodic $PGF_{2\alpha}$ secretion.

The opposite effect was observed in experiments in which oxytocin was administered continuously from day 12 or 13 (before the onset of luteolysis) to inhibit the appearance of the oxytocin receptor (Flint & Sheldrick 1985; Sheldrick & Flint 1990) and render the endometrium refractory to circulating oxytocin. In this case down-regulation of the oxytocin receptor resulted in a delay in luteal regression and the absence of episodic secretion of $PGF_{2\alpha}$ at the expected time; in fact in these experiments luteolysis was delayed for up to 55 d. An identical effect occurs in cattle (Gilbert *et al.* 1989; Howard *et al.* 1990; Lutz *et al.* 1991).

In the third approach to this question an oxytocin receptor antagonist (1-deamino-2-D-Tyr(oEt)-4-Thr-8-Orn-oxytocin) was shown to block episodic prostaglandin secretion and delay luteolysis in sheep, when administered between days 13 and 16 (Jenkin 1992). Thus manipulations that induce the oxytocin receptor cause premature uterine secretion of $PGF_{2\alpha}$ and luteolysis; those that inhibit oxytocin receptor activation, or block receptor function, delay or prevent these processes.




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It is now clear from experiments in which ovariectomized ewes have been treated with oestrogen and progesterone in doses designed to mimic those circulating during the ovarian cycle, that expression of the oxytocin receptor in the uterus is controlled by circulating levels of progesterone (McCracken *et al.* 1981; Vallet *et al.* 1990; Vallet & Lamming 1991; Beard *et al.* 1994). The effect of progesterone is, however, biphasic; on administration to an ovariectomized ewe progesterone initially reduces endometrial oxytocin receptor concentrations to those observed during the mid-luteal phase of the ovarian cycle (figure 4). After 10 d (in sheep; 12 d in cattle) this inhibition is withdrawn and endometrial oxytocin concentrations rise to the high levels characteristic of luteal regression. The clearest demonstration of these changes is obtained by administration of oestrogen before treatment with progesterone, to mimic oestrogen released at ovulation, and additional pretreatment with progestagen before oestrogen is given, to mimic the previous luteal phase. Further administration of oestrogen following chronic progesterone treatment results not in additional oxytocin receptor induction but in enhanced linkage of the receptor to the post-receptor events resulting in prostaglandin secretion. The bi-phasic effect of progesterone therefore explains the pattern of endometrial oxytocin receptor expression during the ovarian cycle and this may in turn determine the length of the luteal phase.

This suggestion is supported by the effects of progesterone administered immediately after oestrus, before endogenous progesterone production has reached a significant level: this treatment shortens the luteal phase (Woody *et al.* 1967; Ginther 1968)

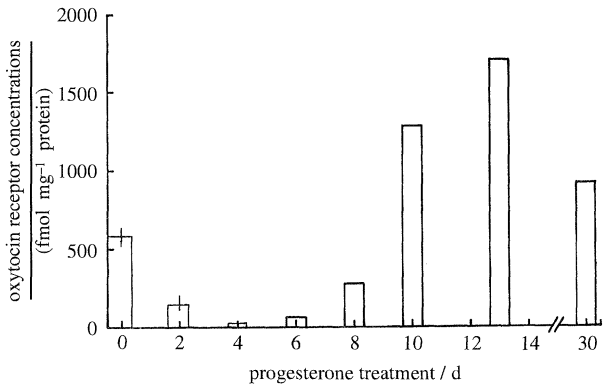


Figure 4. Induction of endometrial oxytocin receptor by prolonged treatment with progesterone. Ovariectomized ewes received progestagen (fluorogestone acetate) by vaginal pessary for 10 d, followed by oestradiol-17β, 25 μg administered intramuscularly twice daily for 2 d, and thereafter were treated with progesterone, 10 mg intramuscularly twice daily for the periods indicated. On the morning after the last injection, animals were slaughtered and endometrium (caruncular and intercaruncular together) prepared for oxytocin receptor assay. Data from Beard & Lamming (1994) (days 0–4), and Vallet *et al.* (1991) (days 6–30). Note timing of responses may differ slightly between the two studies, as steroid treatment regimes differed.

presumably by starting the uterine 'clock' prematurely. Furthermore, treatment with the progesterone receptor antagonist RU486 during the first half of the cycle delays the onset of uterine $PGF_{2\alpha}$ secretion and luteolysis (Morgan *et al.* 1993). It may be therefore that a threshold progesterone concentration exists, and that only when this threshold is reached following formation of the corpus luteum does the 'clock' start. Differences between species in rates of corpus luteum formation (together with differences in the length of the progesterone inhibitory period, see above) may then underlie differences in cycle lengths; thus in sheep (cycle length 17 d) luteinization is relatively rapid, whereas in cattle (cycle length 21 d), it occurs more slowly (see data of Sheldrick & Flint 1983; Lamming *et al.* 1989, 1991). Circulating progesterone concentrations reach 1 ng ml$^{-1}$ (an arbitrary figure for purposes of comparison) on day 2 after oestrus in sheep, but the same level is not reached until day 4 in cattle. It should be noted, however, that the luteolytic effects of progesterone secreted for 8 d or more may be mediated by other molecular changes, in addition to a rise in uterine oxytocin receptor content; chronic exposure to progesterone also increases uterine concentrations of phospholipase C and the mRNA coding for prostaglandin synthase (Raw & Silvia 1991; Raw *et al.* 1988), both of which are involved in $PGF_{2\alpha}$ production.

Consistent with this conclusion is the observation that treatment of ovariectomized ewes with progesterone and oestrogen restores the frequency of $PGF_{2\alpha}$ pulses but not the amplitude of secretory episodes; an additional ovarian factor (presumably oxytocin) is required to generate episodes characteristic of the intact ewe (Silvia & Raw 1993). The biphasic nature of the response of the endometrium to progesterone can be explained in terms of steroid receptor function. Progesterone and oestrogens interact, in their actions on target tissues, through effects on the availability both of their own and each other's receptors. Thus oestrogens promote tissue responses to progesterone by increasing tissue concentrations of progesterone receptor and conversely progesterone reduces oestrogen receptor availability. Both these functions are involved in controlling oxytocin receptor gene expression, but in addition the progesterone receptor is down-regulated by progesterone, over a period of days, and it is this response which is thought to be important in determining the time of oxytocin receptor expression (Ott *et al.* 1993). Thus progesterone, by down-regulating its receptor, makes the tissue refractory to progesterone itself; as a result the inhibitory action on oestrogen receptor synthesis characteristic of the progesterone-dominated uterus is withdrawn and the oestrogen receptor activates oxytocin receptor gene expression. These interactions explain the dependence of oxytocin receptor gene expression on the ratio of progesterone to oestrogen in the circulation (Beard & Lamming 1994) and they emphasize the importance of waves of follicular growth and oestrogen secretion in the control of cycle length: the final wave of follicular growth coincides with the onset of refractoriness to progesterone,




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so that both oestrogen and oestrogen receptor are available to induce oxytocin receptor synthesis.

## 3. THE OXYTOCIN RECEPTOR IN EARLY PREGNANCY

In all spontaneously ovulating mammals with repetitive ovarian cycles luteolysis is blocked following conception. Corpus luteum function must be maintained in early pregnancy to provide the high circulating progesterone concentrations necessary for stimulation of a secretory endometrium; endometrial secretions are required for normal conceptus growth, particularly before implantation. Thus an early endocrine adaptation to pregnancy is the inhibition of luteolysis and this process has been termed the maternal recognition of pregnancy (Short 1969). In those animals, for instance domestic ruminants, in which the embryo remains unattached to the uterine endometrium until after the second week of pregnancy the time of the maternal recognition of pregnancy can be determined by flushing embryos from, or transferring them to, the uterus. Thus the time when embryos can be flushed from the uterus with no prolongation of corpus luteum function beyond the point at which luteolysis would occur in a non-pregnant cycle defines the day of the maternal recognition of pregnancy (Moor & Rowson 1966*a*). This is also the last day upon which the corpus luteum can be rescued by transferring embryos to the uterus (Moor & Rowson 1966*b*). The process of the maternal recognition of pregnancy involves different mechanisms in different groups of animals and cannot always be defined in this way, particularly when it occurs after the blastocyst implants in the endometrium and so cannot be flushed from the uterus (Flint *et al.* 1990*b*).

In domestic ruminants inhibition of luteal regression involves inhibition of release of luteolytic episodes of $PGF_{2\alpha}$ from the uterus (Thorburn *et al.* 1973; Barcikowski *et al.* 1974; Nett *et al.* 1976; Peterson *et al.* 1976). This is achieved by the secretion, by the trophoblast of the developing conceptus, of a protein or group of proteins variously termed trophoblastin, trophoblast protein-1 or (more recently) trophoblast interferon (IFN-τ; Rowson & Moor 1967; Martal *et al.* 1979; Godkin *et al.* 1982; Bazer *et al.* 1986; Knickerbocker *et al.* 1986). Evidence will be reviewed which suggests that this protein prevents the induction of endometrial oxytocin receptor following exposure to progesterone for 10 or more days in pregnant animals.

IFN-τ is a 172 amino acid Type 1 interferon related to IFN-ω (previously known as IFN-$\alpha_{II}$; Imakawa *et al.* 1987; Stewart *et al.* 1987; Roberts *et al.* 1992). Northern analysis of RNA extracted from the blastocyst, or *in situ* hybridization with trophoblast tissue, shows that IFN-τ genes are transcribed between days 10 and 21 in the sheep (see Flint *et al.* 1991, and references therein; days 12–25 in the cow; Farin *et al.* 1990). IFN-τ expression is initiated when the blastocysts are between 1 and 5 mm in diameter. *In vitro* translation of blastocyst RNA confirms that IFN-τ RNA is present at high concentrations in the tissue on day 16 (figure 5), and administration of synthetic IFNs blocks episodic $PGF_{2\alpha}$ secretion and prolongs the lifespan of the corpus luteum (Plante *et al.* 1988; Stewart *et al.* 1989*a*; Martal *et al.* 1990; Garverick *et al.* 1992; Parkinson *et al.* 1992). Removal of IFNs from blastocyst-conditioned medium by immunoadsorption inactivates it (Vallet *et al.* 1988).

Southern analysis of genomic DNA has identified at least three genes which hybridize to IFN-τ cDNA probes and this, as well as the cloning and sequencing of several distinct IFN-τ cDNAs, indicates that there are a number of isoforms (Flint *et al.* 1991; Roberts *et al.* 1991). This is confirmed by the heterogeneity of proteins observed on two-dimensional electrophoresis following incubation of blastocysts with labelled amino acids. IFN-τ isoforms are highly active antiviral agents but it is not known how the different members of the IFN-τ family compare in terms of antiviral activity or effect on prostaglandin synthesis, nor is it known which forms predominate at different stages of early pregnancy (though there is evidence for differential expression; Nephew *et al.* 1993).

Interferons produced by the trophoblast bind to endometrial receptors to elicit a number of intracellular events including the induction and repression of

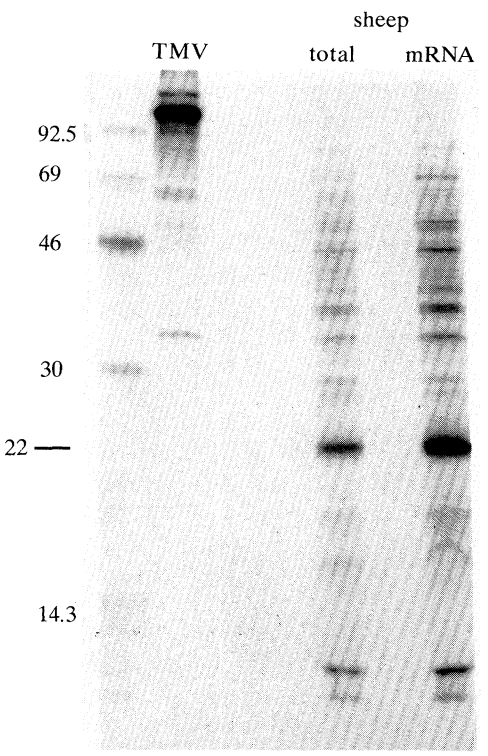


Figure 5. *In vitro* translation of ovine blastocyst RNA. RNA extracted from a blastocyst on day 16 after oestrus was translated *in vitro* using rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine, before and after purification of poly $A^{(+)}$ -RNA. Tobacco mosaic virus RNA (TMV) was used as a quality control standard and molecular masses of products were determined by gel electrophoresis and autoradiography using molecular mass markers ($M_r$). The major product of $M_r = 22\,000$ represents trophoblast Type I interferon before post-translational processing (i.e. including the N-terminal signal peptide). From Stewart *et al.* (1989*b*).




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the synthesis of specific proteins. Among the proteins induced is an endometrial $\beta_2$-microglobulin (Vallet *et al.* 1991) which may be related to changes in expression of MHC antigens on endometrial cells in early pregnancy. Among those repressed is the oxytocin receptor, and this action results in the inhibition of responsiveness of the endometrium to oxytocin; this is manifested by the almost total absence of the oxytocin receptor or its mRNA from the endometrium between days 14 and 19 of pregnancy in sheep (figure 3; also demonstrated on days 16–18, in the cow). In both species oxytocin receptor concentrations during pregnancy are less than 4% of the maximum concentrations observed in cyclic animals at a comparable stage after oestrus (Roberts *et al.* 1976; Sheldrick & Flint 1985; Jenner *et al.* 1991).

The inhibitory effect of IFN-τ on expression of the endometrial oxytocin receptor can be demonstrated directly by infusing pure (e.g. recombinant) IFN into the uteri of cyclic ewes, or into the uteri of ovariectomized ewes pre-treated with steroid hormones to induce oxytocin receptor synthesis (Flint *et al.* 1991; Vallet & Lamming 1991; figure 6, this paper). In both cases, endometrial oxytocin receptor concentrations are decreased and similar effects follow administration of blastocyst-conditioned medium. Furthermore in tissue from IFN-treated sheep stimulation of uterine inositol phosphate turnover by oxytocin *in vitro* is attenuated (Mirando *et al.* 1990) and the affinity of the oxytocin receptor for its ligand decreases (Mirando *et al.* 1993). Blastocyst-conditioned medium inhibits oestradiol- and oxytocin-stimulated $PGF_{2\alpha}$ secretion in intact ewes (Fincher *et al.* 1986). The demonstration that IFN is effective in steroid-treated ovariectomized animals shows that its action is not exerted via the ovary; this is important because, as discussed above, at least two ovarian hormones (progesterone and oxytocin) are known to affect uterine oxytocin receptor concentrations.

Evidence for an inhibitory role for IFN-τ on oxytocin receptor levels also comes from ewes in which the uterus was surgically prepared to mimic an occasionally-observed congenital abnormality which

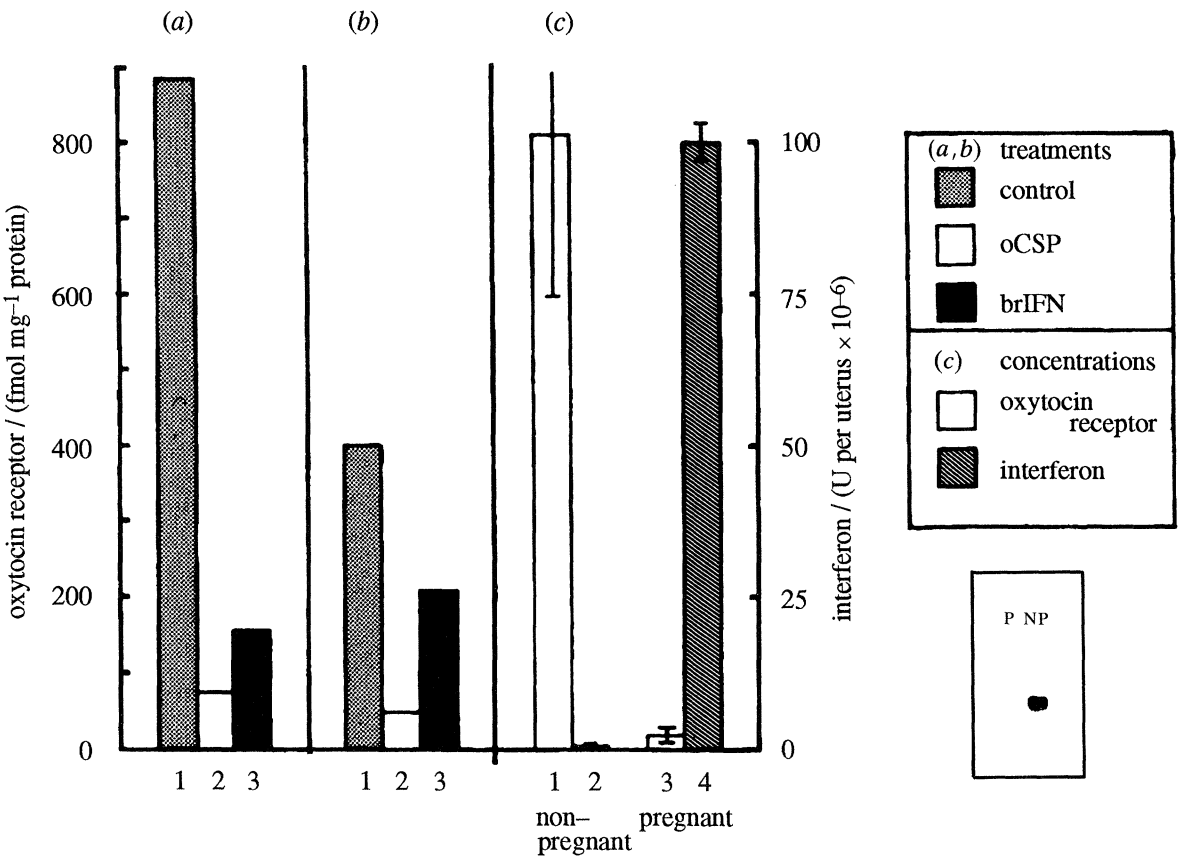


Figure 6. Endometrial oxytocin receptor and uterine interferon concentrations in untreated and ovariectomized ewes, and in pregnant ewes with transected uteri. (*a*) Effect of intrauterine administration of: (1) ovine serum proteins (control; $n = 4$); (2) ovine conceptus secretory proteins ($n = 4$); and (3) bovine recombinant interferon $\alpha_I$ (Ciba-Geigy; $n = 4$) on days 12–14 after oestrus, on oxytocin receptor concentrations in intact ewes. (b) Effect of the treatments in (*a*) on oxytocin receptor concentrations in ovariectomized ewes treated with progesterone for 10 d; treatments administered on days 8–10 after progesterone treatment commenced ($n = 4$ ewes per group). (*c*) Oxytocin receptor concentrations (columns 1 and 3) and interferon concentrations (2 and 4) in non-pregnant (1 and 2) and pregnant (3 and 4) uterine horns, in ewes bearing transected uteri. From Flint *et al.* (1992). Inset shows concentrations of oxytocin receptor mRNA in pregnant (P) and non-pregnant (NP) uterine horns as demonstrated by Northern blotting. Data of Stewart *et al.* (1993).




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results in unilateral pregnancy (O'Shea *et al.* 1974; see also Bazer *et al.* 1979). In these animals on day 16, when oxytocin receptor concentrations are maximal in non-pregnant animals, the level of receptor in the non-pregnant horn is characteristic of that in a non-pregnant, cycling ewe, whereas the concentration in the pregnant horn is characteristic of pregnancy (figure 6). Concentrations of $PGF_{2\alpha}$ in blood draining the two horns reflect oxytocin receptor levels (Payne & Lamming 1994). In contrast, IFN concentrations are elevated in the pregnant horn but low in the non-pregnant horn. The immunoneutralization experiments described above and those involving administration of recombinant IFN indicate that an IFN is the only product of the trophoblast required to maintain corpus luteum function (see Parkinson *et al.* 1991); data from unilaterally pregnant ewes are therefore consistent with the conclusion that it is an IFN produced by the blastocyst which inhibits oxytocin receptor expression. Furthermore, because in this surgical preparation both uterine horns are exposed to the same concentrations of the steroid hormones via the systemic circulation, any inhibitory effect of IFN-τ on oxytocin receptor concentration must be exerted locally rather than through an alternative, unidentified, action on the corpus luteum itself. The direct effects of IFN-τ on oxytocin receptors have been confirmed in endometrial organ culture (Abayasekara *et al.* 1992).

Interferons have a range of actions on target cells, some of which are known to be associated with the induction of resistance to viral infection. Modulatory effects of IFN on prostaglandin synthesis include the activation of phospholipase $A_2$ (Hannigan & Williams 1991), which would be expected to stimulate prostaglandin synthesis by raising intracellular availability of arachidonic acid; indeed in the transected-uterus preparation basal concentrations of $PGF_{2\alpha}$ are raised in blood draining the pregnant horn (Payne & Lamming 1994). Other effects include the stimulation of synthesis of an inhibitor of prostaglandin synthetase (Thatcher *et al.* 1989) and the inhibition of oxytocin receptor expression. There appears to be no direct effect on endometrial concentrations of prostaglandin synthetase itself (Salamonsen *et al.* 1991). Although activation of phospholipase $A_2$ has not been demonstrated in endometrium, the elevated basal concentration of circulating $PGF_{2\alpha}$ observed in pregnancy is consistent with such an action. However, the fact that pulsatile (not basal) secretion of $PGF_{2\alpha}$ is blocked in pregnancy emphasizes the importance of the pathway leading to episodic prostaglandin secretion and is consistent with the proposal that the interaction of oxytocin and its receptor represents an early event in the stimulation of prostaglandin synthesis.

Endometrial steroid receptors are thought to be involved in the control of the oxytocin receptor in non-pregnant cycles and it may be that IFN-τ acts via these receptors during pregnancy. There are two hypotheses for the mechanism of action of IFN-τ: that it either extends the period for which the endometrium is responsive to progesterone (i.e. blocks receptor down-regulation or its consequences) or mimics the action of progesterone (Ott *et al.* 1993). The indications are that it does the former, raising concentrations of the progesterone receptor and reducing that for oestrogen (Mirando *et al.* 1993).

From the above it appears that the roles of endometrial steroid and IFN receptors in the control of expression of the oxytocin receptor are straightforward. In fact interpretation of these experiments is complicated by the heterogeneous distribution of these receptors between endometrial cell types. Oestrogen receptors are localized in luminal epithelial cells and stromal cells of the caruncular and inter-caruncular endometrium and their concentration rises in all these cell types at oestrus (Cherny *et al.* 1991). The oxytocin receptor is also present in luminal epithelial cells (Ayad *et al.* 1991; Riley *et al.* 1993); although detectable in caruncular stromal cells and cells of the secretory epithelia, oxytocin receptor expression in these cells occurs over a shorter interval, and is less marked than in luminal epithelium, in which the receptor is consistently present between the day before oestrus and day 3 of the following oestrous cycle. Prostaglandin synthetase is present in high concentrations in the luminal epithelium of ovariectomized ewes treated with progesterone and oestrogen (Salamonsen *et al.* 1991); it is present in inter-caruncular stromal cells at lower concentrations and is either absent in glandular epithelium (deep cells) or present in very low concentrations (more superficial epithelial cells). Binding of IFN-τ to endometrial cells occurs principally to the luminal epithelium and to glandular epithelium, but is low in endometrial stroma. This latter observation is consistent with the demonstration that IFN released into the uterine lumen by the developing blastocyst is not lost from the uterus in venous blood in large quantities, suggesting that the molecule is too large to pass between cells of the endometrial epithelium. Unfortunately no detailed assessment has been made of the localization of the progesterone receptor in the endometrium. These studies imply that the luminal epithelium, which contains high concentrations of the oestrogen receptor, the oxytocin receptor and prostaglandin synthetase, and is the principal target for IFN-τ, may be the tissue primarily responsible for mediating the endocrine events regulated by these receptors.

It is clear that the oxytocin receptor is not distributed uniformly between all cells of the endometrium and that cyclic changes in endometrial oxytocin receptor concentrations which occur in the glandular epithelium and the endometrial stroma are not necessarily synchronous. In explants of uterine endometrium in organ culture the oxytocin receptor concentration rises rapidly if tissue is removed from ewes during the mid-luteal phase of the cycle, when receptor concentrations are low (Sheldrick *et al.* 1993). Receptor induction is blocked by actinomycin D and therefore presumably requires gene transcription. Thus in at least one endometrial cell type the induction of oxytocin receptor expression may involve changes in gene transcription, rather than changes in receptor post-translational processing or integration into the membrane.




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The timing of trophoblast interferon production is particularly important. Northern analysis of blastocyst RNA, *in situ* hybridization and measurement of IFNs in uterine flushings show that trophoblast IFNs are produced during a limited period of time in early pregnancy; in sheep synthesis is initiated between days 8 and 10 and ceases between days 20 and 23 (Stewart *et al.* 1989*b*; Farin *et al.* 1990). This period parallels that originally identified as the time when extracts of blastocyst are antiluteolytic on administration to the uterus (Rowson & Moor 1967). Consideration of other endocrine events occurring in early pregnancy provides an explanation for the times of onset and cessation of IFN-τ synthesis, as follows. As indicated above, continuous exposure of the uterus to progesterone for a period of 10 d is required to induce oxytocin receptor synthesis in the endometrium. Before this time progesterone inhibits oxytocin receptor concentrations. Therefore there is no advantage in the trophoblast producing IFN before day 8–10, as oxytocin receptor concentrations are inhibited by progesterone and the uterus is refractory to oxytocin before this time. After day 20 the corpus luteum contains no oxytocin, oxytocin-neurophysin mRNA having been depleted from the luteal cells during the period following maximal production of the message on days 3–5 after ovulation (Sheldrick & Flint 1983; Ivell *et al.* 1985; Jones & Flint 1988). Therefore, after day 20 the corpus luteum is unable to mount an episode of oxytocin secretion and is incapable of stimulating episodic secretion of $PGF_{2\alpha}$ from the uterus. Interferon production would again be redundant beyond this point. The long period for which the corpus luteum is maintained following withdrawal of oxytocin infusion in experiments designed to down-regulate the endometrial oxytocin receptor, or after ceasing IFN infusion between days 10–20 (see above), confirms that IFN is not required after day 20.

## 4. CONCLUSIONS

Do published data justify the suggestion that oxytocin receptors control cycle length and the survival of the corpus luteum in pregnancy?

In seeking to prove a function for the oxytocin receptor in controlling the length of the oestrous cycle, we should be able to show that premature luteolysis follows premature induction of oxytocin receptor expression and that prolongation of luteal function results when receptor activity is blocked. As reviewed above the evidence supports both these requirements. It should also be demonstrable that artificially reducing circulating concentrations of oxytocin blocks the response to receptor activation; this has been achieved by immunization against oxytocin, which extends luteal function. A mechanism has been proposed for the action of oxytocin, involving activated secretion of $PGF_{2\alpha}$, and episodes of $PGF_{2\alpha}$ and oxytocin secretion have been shown to be synchronous. The stimulatory effects of $PGF_{2\alpha}$ on luteal oxytocin secretion and of oxytocin on uterine $PGF_{2\alpha}$ secretion together explain the pulsatile nature of uterine $PGF_{2\alpha}$ secretion.

If it is accepted that a rise in uterine concentration of oxytocin receptor is responsible for inducing luteal regression, then two further assertions can be made. Firstly, the time at which the oxytocin receptor is induced and second messenger responses to receptor occupancy lead to prostaglandin secretion, determines the length of the oestrous cycle. As reviewed above this time is established by the kinetics of the response of the endometrium to steroid hormones.

Secondly, an agent secreted by the trophoblast which maintains corpus luteum function during the establishment of pregnancy would be expected to inhibit oxytocin receptor function, for instance by blocking receptor induction. Evidence required in support of such an hypothesis would include the demonstration that the trophoblast product reduced endometrial oxytocin receptor concentrations on administration to non-pregnant animals and that it blocked $PGF_{2\alpha}$ release in response to administered oxytocin in these animals. The active compound should also be shown to extend the life of the corpus luteum on administration and this would be further evidence for a role for the receptor in the onset of luteolysis. In fact experimental evidence of each of these kinds is available in support of a role for trophoblast IFN in blocking luteal regression in early pregnancy. Moreover the observations described above resulting from administration of IFN to ovariectomized ewes, and the observed suppression of oxytocin receptor concentrations in the pregnant horn of unilaterally pregnant animals in which the non-pregnant horn expresses high receptor levels, both support a direct effect of trophoblast IFN on the endometrium in the control of receptor levels.

It must be concluded therefore that the length of the luteal phase of the sterile ovarian cycle in domestic ruminants is determined by the time of induction of the oxytocin receptor in the endometrium. Maintenance of the corpus luteum in pregnancy, at a time when luteolysis would normally occur in non-pregnant animals, results from inhibition of endometrial oxytocin receptor expression caused by the direct effect of trophoblast-secreted IFN-τ on endometrial receptors.

In cattle failure of the maternal recognition of pregnancy leads to large numbers of non-pregnant animals being kept when they are expected to be in calf; estimates of the cost of early embryonic loss indicate this syndrome may cost agriculture in the EEC up to £3 bn pa (Lamming *et al.* 1989). A proportion of this cost results from the inappropriate control of the endometrial oxytocin receptor, and if a treatment could be found which led to maintenance of normal pregnancies without securing implantation of genetically abnormal conceptuses, it would be of great benefit.

We started this review by showing that in those mammals for which good data are available the coefficient of variation of non-pregnant ovarian cycle length is low (mean 6% in the seven species described), and we have discussed the mechanism determining ovarian cycle length in one group, the ruminants. To explain the low variation in cycle




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length in any species we would propose that when an endocrine mechanism exists, the timing of which depends on the kinetics of its components, then if the same mechanism operates in the same way in each individual, it would be expected to follow the same time course. Although these mechanisms have been identified only in ruminants, the principles may apply to the determination of ovarian cycle lengths in other taxa; in primates, for example, an ovarian oxytocin receptor may be involved.

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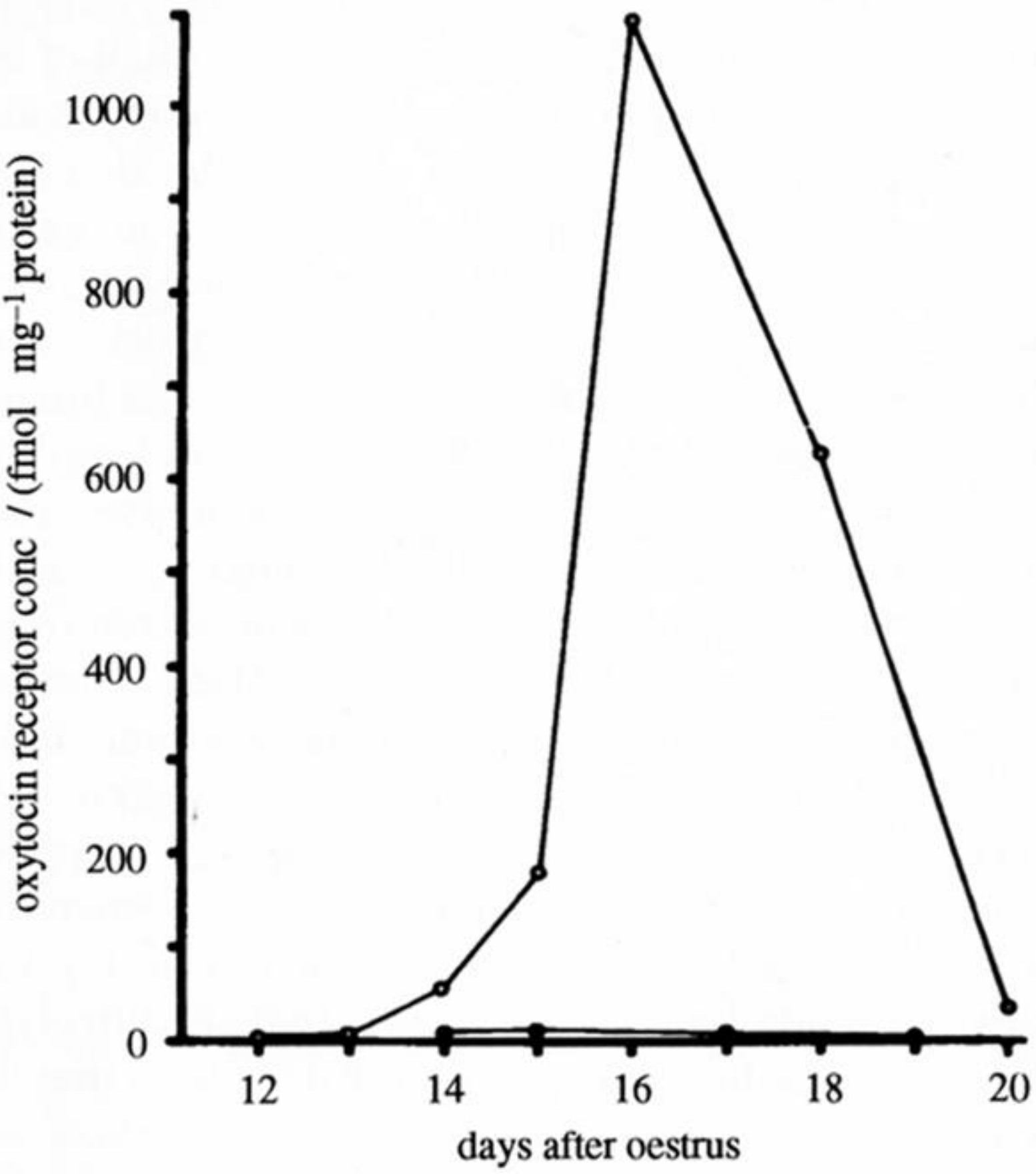
(a)



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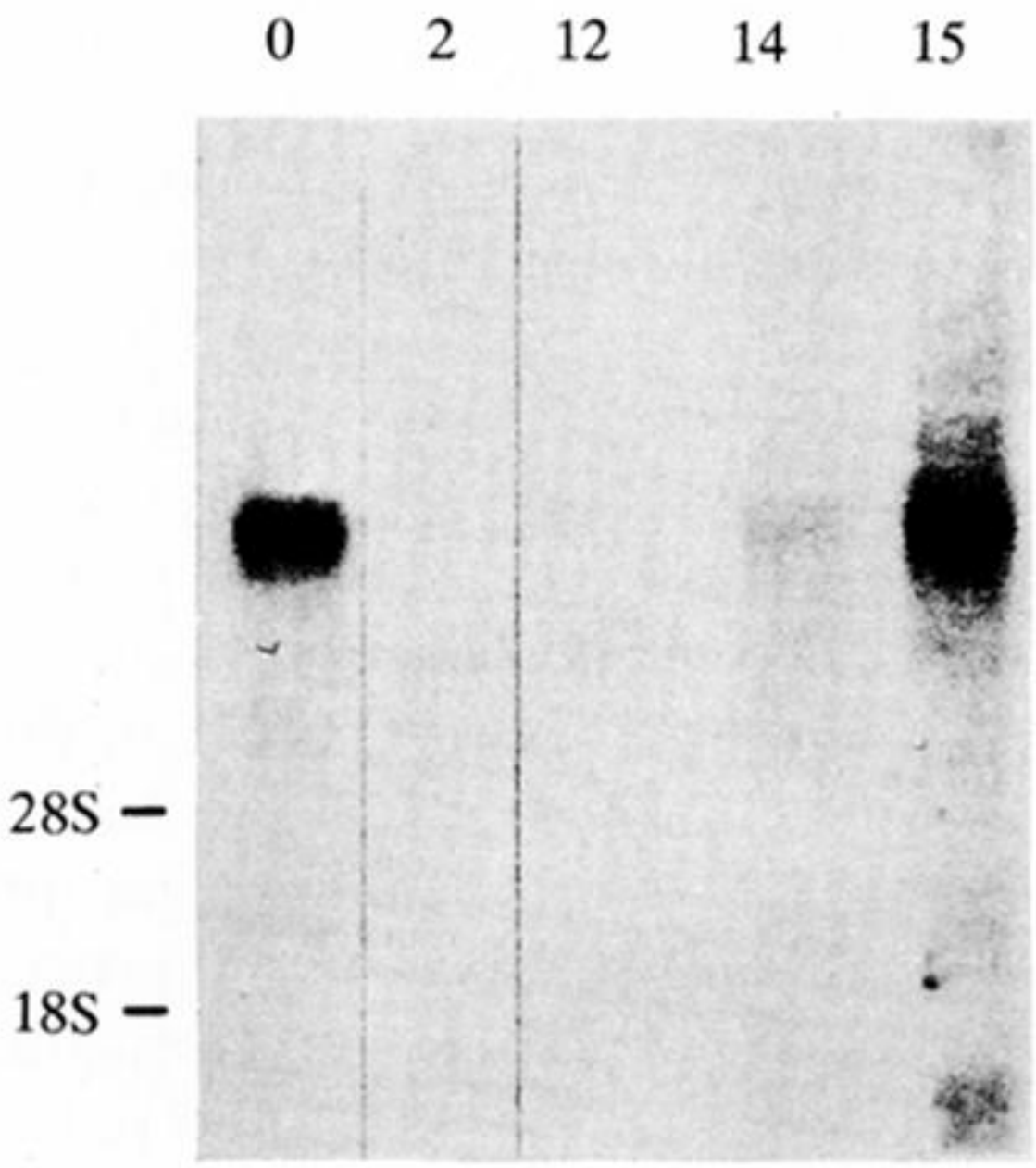


Figure 3. (*a*) Endometrial oxytocin receptor concentrations in pregnant (filled circles) and non-pregnant (open circles) ewes at various times after oestrus. Data shown refer to inter-caruncular endometrium; receptor concentrations in caruncular endometrium were reduced similarly in pregnancy. Values are from Flint & Sheldrick (1986). (*b*) Concentrations of oxytocin receptor mRNA determined by Northern blotting between days 0 and 15 (from Stewart *et al.* 1993).

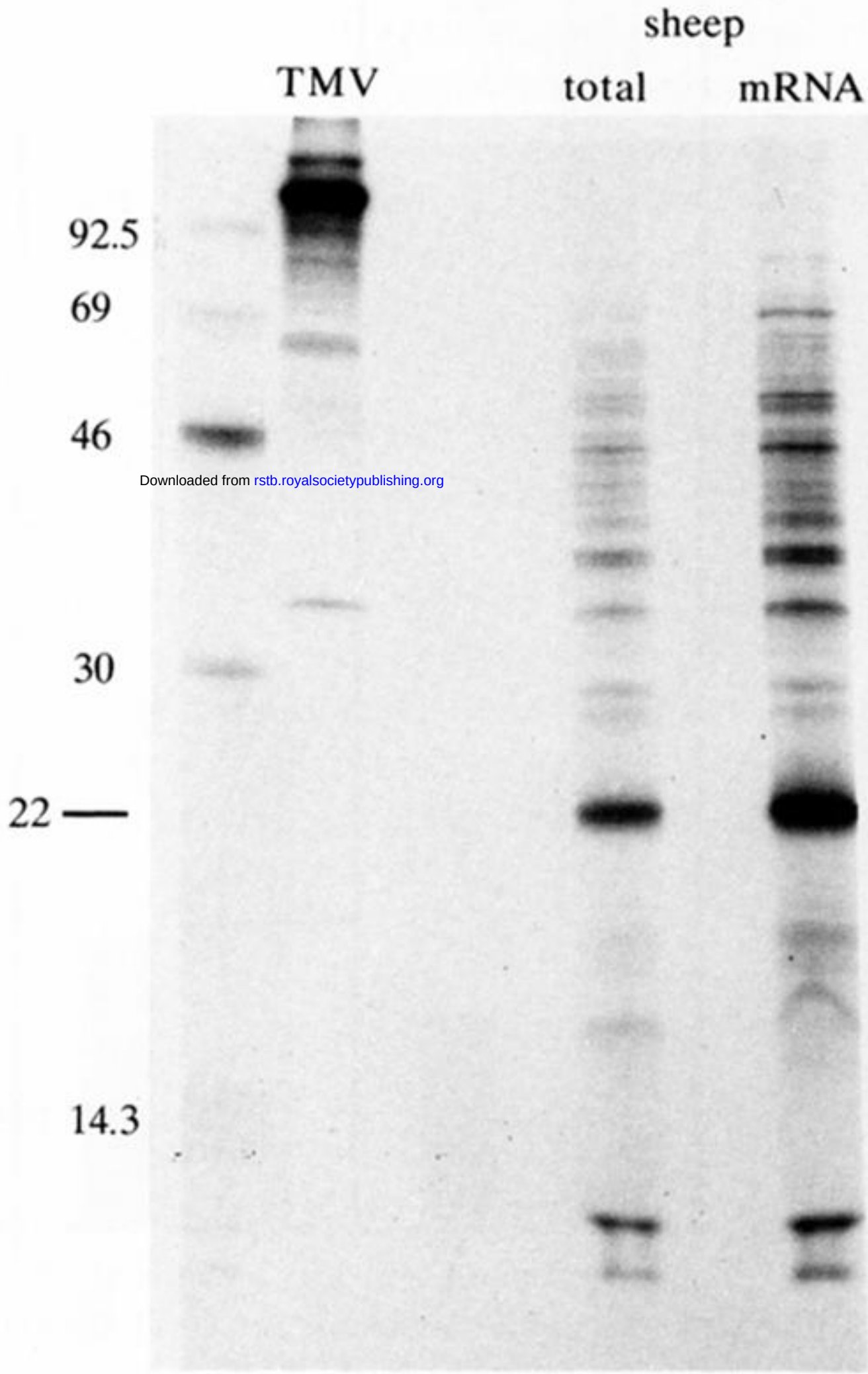




Figure 5. *In vitro* translation of ovine blastocyst RNA. RNA extracted from a blastocyst on day 16 after oestrus was translated *in vitro* using rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine, before and after purification of poly A$^{(+)}$ -RNA. Tobacco mosaic virus RNA (TMV) was used as a quality control standard and molecular masses of products were determined by gel electrophoresis and autoradiography using molecular mass markers ($M_r$). The major product of $M_r = 22\,000$ represents trophoblast Type I interferon before post-translational processing (i.e. including the N-terminal signal peptide). From Stewart *et al.* (1989*b*).