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## Adrenal maturation of the sheep fetus during late pregnancy

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**Summary.** The mechanism by which adrenal growth is achieved and steroidogenesis is stimulated in the fetal lamb during late pregnancy has been studied. Fetal plasma ACTH levels remained roughly constant between 14 and 3 days *pre partum* ( $298 \pm 38$  pg/ml), then increased to a mean of  $634 \pm 89$  pg/ml. The rise in ACTH did not occur before the *pre partum* rise in cortisol. The increase in adrenal weight was achieved in three periods, one of DNA duplication between two phases of cellular hypertrophy. Cellular hypertrophy was accompanied by an important biosynthesis of membrane proteins. This development might partly explain the increased production rate of cortisol by the fetal adrenals during late pregnancy. At the same time, the number of ACTH receptors per 2 adrenals increased 5-fold. There was a close correlation between the levels of fetal plasma corticosteroids and the number of ACTH receptors during late pregnancy. It has been suggested that receptor modulation is one of the factors responsible for the enhanced sensitivity of the fetal adrenals to ACTH just before parturition. Preliminary results on the endocrine regulation of that development are also presented.

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### Introduction.

In many mammalian species, an important increase in plasma corticosteroids is a common feature of the fetal endocrine milieu during late pregnancy. In the sheep, this increase in cortisol, necessary for the maturation of some fetal organ systems (adrenal medulla : Comline *et al.*, 1970 ; liver : Liggins, 1969 ; lungs : De Lemos *et al.*, 1970 ; pancreas : Bassett *et al.*, 1973 ; haemopoietic system : Pierce *et al.*, 1976), is also responsible for the onset of parturition (Liggins *et al.*, 1973). The rise in fetal sheep cortisol levels during the last 2 weeks of gestation (Bassett and Thorburn, 1969 ; Liggins *et al.*, 1973) develops simultaneously with an enlargement of the adrenal glands (Comline and Silver, 1961). This *pre-partum* burst of adrenocortical function involves the influence of hypophyseal factors. Indeed, it has been shown that removal of the fetal pituitary abolishes the increase in cortisol (Challis *et al.*, 1977b), whereas perfusion with adrenocorticotropin (ACTH) stimulates the fetal adrenals at about 100 days of pregnancy, resulting in premature parturition (Liggins, 1968). However, under physiological conditions the relationship between ACTH and cortisol during late pregnancy is not clear. On the other hand, accumulating evidence suggests a change in the sensitivity of the fetal adrenal to ACTH near term (Madill and Bassett, 1973 ; Wintour *et al.*, 1975). The

purpose of the present study was to obtain more information on the mechanism by which fetal adrenal growth is achieved and steroidogenesis is stimulated during late pregnancy. We report here the variations in the plasma ACTH levels in the sheep fetus, and the DNA, protein and ACTH receptor concentrations in the fetal adrenal glands during the perinatal period. Preliminary results on the endocrine regulation of these parameters are also presented.

### Material and methods.

*Animals.* — Cross-bred fetuses and lambs (Ile-de-France  $\times$  Romanov-Ile-de-France) from single or multiple pregnancies were used. The day of fertilizing insemination was considered as day 0 of pregnancy. The fetuses were obtained by caesarean section under general anesthesia. The adrenal glands and the blood for cortico-steroid assay were sampled at slaughter.

*Sampling for ACTH assay.* — Vascular silastic catheters were implanted into a fetal artery (carotid or femoral) according to Dawes *et al.* (1972). Samples (2 ml) were taken between 9 h 00 and 12 h 00 at least 24 hrs after surgery in cold tubes containing EDTA as an anticoagulant. The plasma was removed by centrifugation at 1 500 g and 4 °C for 10 min, then stored at  $-20$  °C.

*Perfusion of fetuses.* — The fetuses were infused by the intraperitoneal route as follows : after laparotomy under light anesthesia (fluothane, O<sub>2</sub>) the catheter was inserted into the fetal peritoneal cavity through a needle which was then withdrawn. The extremity of the catheter was fixed to the skin of the fetus and two holes were drilled on the tube 1 cm from its distal end. The free end of the catheter was then connected to a perfusion pump. ACTH<sub>1-24</sub> and metyrapone (metopirone, Ciba), an inhibitor of 11 $\beta$ -hydroxylase, were dissolved each day in 4.8 or 10.6 ml of 0.9 p. 100 NaCl solution which was infused at a constant rate over the next 24 hrs.

*ACTH assay.* — Plasma ACTH was determined by radioimmunoassay according to Berson and Yalow (1968) without extraction but with a control of the ACTH degradation during incubation. The ACTH used for standardization and iodination was synthetic ACTH<sub>1-24</sub> (Synacthen, Ciba). The antibody employed (ACTH-K, CEA) was raised in the rabbit using ACTH coupled to BSA. Parallelism of immunoreactivity between the standard ACTH<sub>1-24</sub> and serial dilutions of sheep plasma was observed (not shown). *Other methods.* — DNA, proteins, ACTH receptors and plasma corticosteroids were assayed as described elsewhere (Durand *et al.*, 1978 ; Durand, 1979).

### Results and discussion.

*Plasma ACTH levels.* — The plasma ACTH concentrations in lamb fetuses from 14 days before parturition until 1 hr *post-partum* are shown in figure 1. Despite the small number of values, ACTH levels clearly remained roughly constant between 14 and 3 days *pre-partum*, with a mean value of  $298 \pm 38$  pg/ml. In the last 3 days of gestation, however, ACTH levels rose in most, but not all, fetuses, the mean concentration being  $634 \pm 89$  pg/ml ( $P < 0.01$ ). Finally, plasma ACTH appeared to decrease

promptly after parturition to levels lower than all the other values in late pregnancy. These results are in agreement with other data (Rees *et al.*, 1975 ; Challis *et al.*, 1977a ; Jones *et al.*, 1977a) and confirm that ACTH levels rise only in the very last days of gestation, when cortisol levels are already elevated (Bassett and Thorburn, 1969 ; Liggins *et al.*, 1973). In addition, Jones *et al.* (1977a) have shown that ethanol infused into the fetus during labour, significantly reduces ACTH concentration, but that no changes in fetal plasma cortisol levels are associated with changes in ACTH concentrations. Hence, it appears that the *pre-partum* rise in fetal cortisol levels is not directly correlated with an increase in plasma ACTH.

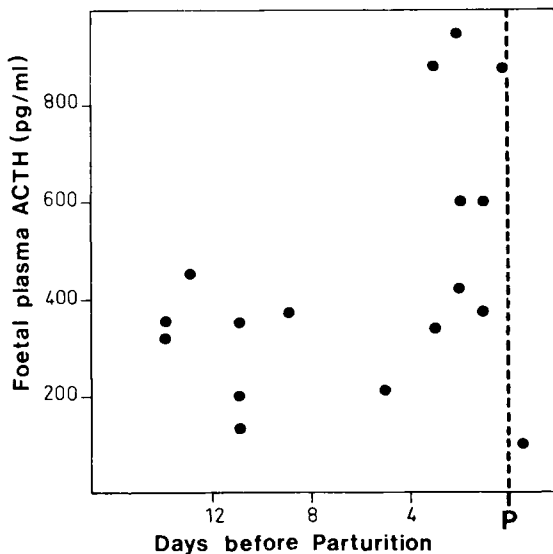


FIG. 1. — Plasma ACTH levels in chronically catheterized lamb fetuses during the last 2 weeks of pregnancy.

However, *in vitro* (Anderson *et al.*, 1972 ; Madill and Bassett, 1973 ; Wintour *et al.*, 1975) and *in vivo* (Liggins *et al.*, 1973 ; Bassett and Thorburn, 1973 ; Jones *et al.*, 1977b) studies have presented evidence for an enhanced responsiveness of the fetal adrenal to ACTH near term ; the course and the mechanisms of this adrenal maturation are poorly documented. Several factors have been suggested as being responsible :

— increased cortical mass (Comline and Silver, 1961 ; Nathanielsz *et al.*, 1972), but this may be a consequence, and not a cause, of the enhanced responsiveness. In addition, it could be achieved either by an increase in the number of cells or by hypertrophy of the adrenal cells ;

— enhanced ACTH uptake by the cortical cells, possibly due to an increase in the number or the affinity of ACTH receptors ;

— maturation of the enzymes implicated in cortisol biosynthesis. Wintour *et al.* (1975) have shown, however, that between days 60 and 90 of gestation the fetal adrenal *in vitro* produces large quantities of cortisol when stimulated by ACTH. This hypothesis is therefore unlikely.

To learn more about this adrenal maturation, we have studied the changes in protein, DNA and ACTH receptor concentrations in the fetal sheep adrenal glands during the perinatal period.

*Changes in DNA and membrane synthesis.* — The table shows that during late pregnancy adrenal weight increased 2.5-fold ( $P < 0.001$ ), and membrane protein content augmented by a factor of 4 ( $P < 0.001$ ). The postnatal variations of these two parameters were not significant. The DNA pattern showed that this development was achieved in only one period of DNA duplication between days 136 and 143 ( $P < 0.05$ ). Nathanielsz *et al.* (1972) and Durand *et al.* (1978) reported that this growth essentially concerned the adrenal cortex; thus, the increase in DNA should correspond roughly to a doubling in the number of cortical cells. The wet weight : DNA ratio (see table 1) gave evidence of two phases of cellular hypertrophy, one between days 124 and 136, the other between day 143 and birth. These two phases were accompanied by increased biosynthesis of total membrane proteins. This membrane fraction consisted not only of plasma membrane but also of smooth endoplasmic reticulum membrane, mitochondria, structures participating in steroid synthesis. The development of that fraction probably enhanced the ability of the cortical cells to synthesize corticosteroids. This, together with the increased cell number, could partly explain the enhanced *pre-partum* production rate of cortisol by the fetal adrenal glands (Liggins *et al.*, 1973).

TABLE 1

*Changes in membrane protein and DNA contents of fetal sheep adrenal glands during late pregnancy and the early neonatal period (mean  $\pm$  SE for at least 7 animals)*

Age	Before birth (days of pregnancy)				After birth (days)	
	124	136	140	144	Birth (0)	3
Wet weight (mg) . . . . .	279 $\pm$ 18	346 $\pm$ 29	376 $\pm$ 26	469 $\pm$ 28	704 $\pm$ 62	695 $\pm$ 27
DNA (mg) . . . . .	0.586 $\pm$ 0.060	0.630 $\pm$ 0.074	0.706 $\pm$ 0.054	0.839 $\pm$ 0.049	0.888 $\pm$ 0.112	0.870 $\pm$ 0.041
Membrane proteins (mg) . . . . .	3.13 $\pm$ 0.25	5.09 $\pm$ 0.61	5.83 $\pm$ 0.28	8.04 $\pm$ 0.49	12.10 $\pm$ 1.08	12.72 $\pm$ 0.80
Wet weight/DNA . . . . .	512 $\pm$ 56	613 $\pm$ 51	535 $\pm$ 31	539 $\pm$ 38	838 $\pm$ 57	791 $\pm$ 48
Protein/DNA . . . . .	5.87 $\pm$ 0.74	8.67 $\pm$ 1.13	8.54 $\pm$ 0.82	9.12 $\pm$ 0.61	14.25 $\pm$ 0.81	14.55 $\pm$ 1.46

(Data from Durand *et al.*, 1978.)

Hormonal regulation of the growth of the adrenal cortex in late pregnancy is not well understood. The termination of adrenal growth in lamb fetuses hypophysectomized on day 115 was demonstrated by Liggins (1969), whereas large doses of ACTH anticipated the increase in adrenal weight (Liggins, 1968). Figure 2 shows that the infusion of 0.1 mg/24 h of ACTH into the sheep fetus between days 115 and 120 resulted in a 4-fold increment in adrenal membrane protein content and a 2-fold increase in DNA concentrations. However, as opposed to Liggins' data (1968), simultaneous perfusion of

metyrapone (100, 200 or 440 mg/24 h) with ACTH (0.1 mg/24 h) did not block the enlargement of the glands and the increase in DNA ( $P < 0.01$ ) and membrane protein ( $P < 0.01$ ) contents. Alternatively, the perfusion of 300 mg/24 h of metyrapone to the fetuses between days 139 and 143 resulted in an increment in the membrane protein content of the adrenal glands ( $P < 0.001$ ) without any significant change in DNA concentration (fig. 2). This was probably due to an increase in endogenous ACTH secretion under the influence of the blockade of cortisol biosynthesis. However, physiologically, ACTH rises only at the end of pregnancy, hence we should examine the influence of increased cortical cell sensitivity to ACTH as well as the role of other possible trophic hormones.

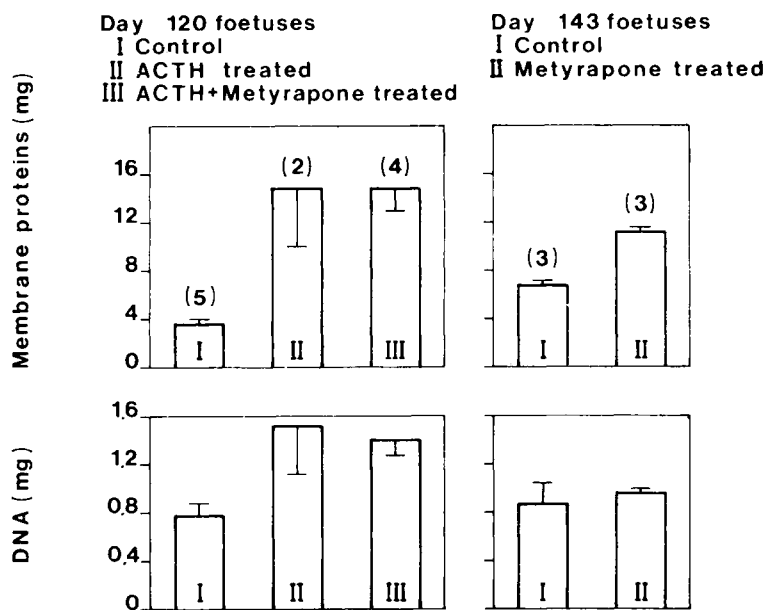


FIG. 2. — Effect of infusion of ACTH, ACTH and metyrapone, or metyrapone alone into the sheep fetus on membrane protein and DNA levels of the fetal adrenals. Left : 115-day old fetuses infused for 5 days with ACTH<sub>1-24</sub> (0.1 mg/24 h) or ACTH<sub>1-24</sub> (0.1 mg/24 h) + metyrapone (100, 200 or 440 mg/24 h). Right : 139-day old fetuses infused for 4 days with 300 mg/24 h of metyrapone. Each point represents the mean  $\pm$  SE for the number of animals indicated in parenthesis.

**ACTH receptor levels in lamb fetuses.** — Figure 3 shows that the number of ACTH receptors per 2 adrenal glands increased 5-fold between day 124 of pregnancy and birth ( $P < 0.001$ ). This increase was biphasic, reflecting not only an increment in cell number, but also an increase in the number of receptors per cell (Durand, 1979). No significant variations in binding affinity were observed during that period. Figure 3 also presents corticosteroid level variations in the blood of these same fetuses and newborn lambs ; the classical rise in cortisol at the end of gestation and the *post-partum* drop were observed. The correlation coefficient of every single point between the

apparent number of ACTH receptors and the corticosteroid levels at the end of pregnancy was  $r = 0.57$ ;  $P < 0.01$  ( $n = 28$ ).

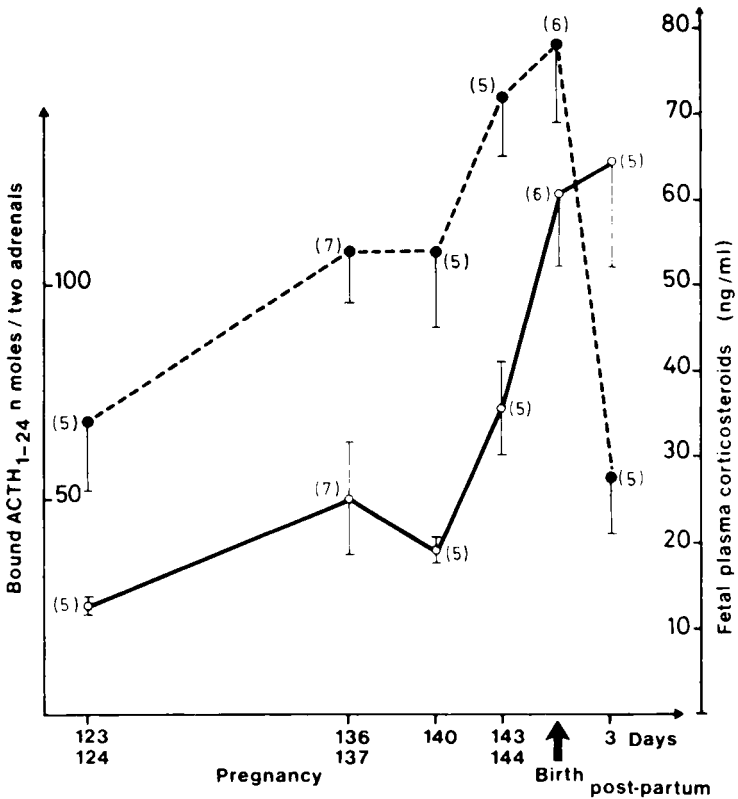


FIG. 3. — Change in the number of ACTH receptors (per 2 adrenals) (o—o) in fetal lamb adrenals at the end of pregnancy, and in lamb adrenals after parturition. ●-● = plasma corticosteroid levels of the same fetuses and lambs. (Data from Durand, 1979.)

Thus, it appears that fetal sheep adrenals undergo fundamental alterations in late gestation which result in a striking increase in ACTH receptors. This increment is closely correlated with the results obtained by Madill and Bassett (1973) and by Wintour *et al.* (1975) in which the response to ACTH was found to develop during the last weeks of gestation. The data summarized above suggest that receptor modulation was one of the factors involved in that maturation. It might be relevant to add that there was a very rapid decrement in corticosteroid levels after birth, despite the high levels of ACTH receptors; this might be due to a change in the sensitivity of the negative feedback between cortisol and ACTH at birth. Indeed, the sensitivity of this mechanism was probably very poor during the last 3 days of gestation, since at that time ACTH and cortisol levels rose parallelly (figs. 1 and 3).

The endocrinological determinism of the gradually developing sensitivity of the adrenals to ACTH remains to be elucidated. The preliminary results presented in figure 4 suggested that self-regulation of the ACTH receptors might be operating in the

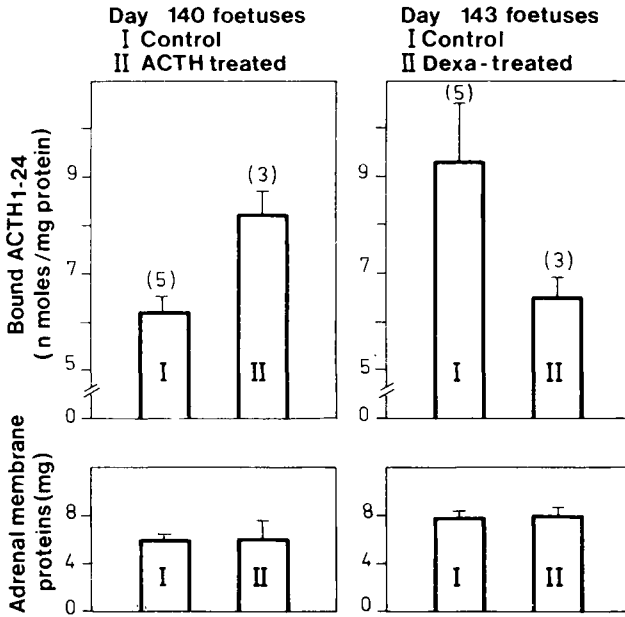


FIG. 4. — Effect of short ACTH infusion into sheep fetuses and of repeated dexamethasone injections to the dam on the number of ACTH receptors in the fetal adrenals. Left : 138-day old fetuses infused for 2 days with ACTH<sub>1-24</sub> (0.1 mg/24 h). Right : dam given a daily i.m. dose of 32 mg of dexamethasone (2 × 16 mg) on days 141 and 142 ; lambing occurred at day 143. Results are expressed in nmoles ACTH<sub>1-24</sub> bound per mg of protein. Each point represents the mean ± SE for the number of animals shown in parenthesis. Adrenal membrane protein content is also shown.

sheep at that period ; a 48 hr ACTH infusion into the fetus between days 138 and 140 increased the apparent number of ACTH receptors, expressed per mg of protein ( $P < 0.01$ ), without changing the membrane protein content of the adrenals. Conversely, repeated injections of dexamethasone to the mother, which momentarily inhibit the fetal pituitary-adrenal axis (Bosc and Fèvre, 1974), reduced the number of ACTH binding sites, but this variation was not significant. Hence, further investigations are needed to specify the role of ACTH. In addition, it has been shown recently that other hormones, such as PGE<sub>2</sub> (Louis *et al.*, 1976) and  $\alpha$ -MSH (Glickman *et al.*, 1979), can increase cortisol secretion by the fetal sheep adrenals. Thus, it appears that ACTH is not the sole factor involved in this regulation, and that synergistic or inhibiting factors actually modulate its effects.

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Clermont-Ferrand/Theix, 17-18 mai 1979.

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**Résumé.** Les modalités de la croissance des surrénales et la régulation de leur stéroïdogenèse ont été étudiées chez le fœtus ovin à la fin de la gestation. Les concentrations de l'ACTH plasmatique, chez le fœtus, restent sensiblement constantes entre 14 et 3 jours *pre partum* ( $298 \pm 38$  pg/ml). Elles s'élèvent alors jusqu'à une valeur moyenne de  $634 \pm 89$  pg/ml. L'augmentation de l'ACTH ne précède pas la montée *pre-partum* du cortisol. L'augmentation pondérale des surrénales s'effectue selon deux phases d'hypertrophie cellulaire séparées par une période de réplication de l'ADN. Ces deux phases d'hypertrophie s'accompagnent d'une importante synthèse de protéines membranaires. Ce développement doit, en partie, rendre compte de l'augmentation de la production de cortisol par les surrénales fœtales en fin de gestation. Parallèlement, le nombre de récepteurs de l'ACTH, exprimé pour les 2 surrénales, est multiplié par 5. Il existe une corrélation étroite entre les niveaux plasmatiques des corticostéroïdes fœtaux et le nombre de récepteurs de l'ACTH, pendant la fin de la gestation. Ces résultats suggèrent que la modulation du nombre des récepteurs est un des facteurs impliqués dans l'augmentation de la sensibilité à l'ACTH des surrénales fœtales juste avant la parturition.

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