# Polymorphism of ACTH Released by Adenohypophysis of Fetal Rat during Perinatal Period

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=국문초록=

## 주산기 태아 흰쥐의 뇌하수체 전엽에서 분비되는 ACTH의 다형현상

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흰쥐의 태아에서 ACTH의 분비양상을 알아보기 위하여, 태아의 제태일수에 따라 혈장 및 뇌하수체 전엽에서 여러 분자형태의 ACTH를 방사면역측정법으로 측정하였다.

태아의 혈장 ACTH농도는 제태 19일에서 가장 높았으며 그후 계속 감소하여 출생후 1주에서 가장 낮은 값을 보였다. 출생 1주후부터 ACTH농도는 다시 증가하기 시작하여 출생후 21일에서는 거의 성체의 값에 도달하였다. 측정된 ACTH는 chromatogram 상에서 항상 3가지 peak가 나타났다. 즉 "big"형("big" ACTH, MW≈44,000), "intermediate"형("intermediate" ACTH, MW≈13,000) 및 "little"형("little" ACTH, MW≈4,500)으로 구분되었다.

임신말기(제태기간 17일에서 21일 사이)에서 태아 혈장의 ACTH는 "little"형의 비율이 증가한 반면 "big"형의 비율은 감소하였다. 그러나 뇌하수체 전엽에서 분비된 ACTH는 3가지 형이 같은 비율이었다.

뇌하수체 전엽에서 분리한 "big"형의 ACTH를 시험관내에서 trypsin을 처리한 결과 "intermediate"형과 "little"형이 출현하였다. 이 결과로 미루어 태아 흰쥐의 뇌하수체에서 분비된 ACTH가 순환도중 다른 형으로 전환될 수 있음이 시사된다.

## INTRODUCTION

Until now few studies were devoted to the ACTH levels and the polymorphism of ACTH in the plasma of the rats during the perinatal period. We had previously shown the presence of ACTH in the anterior and neurointermediate lobes of the fetal pituitary gland with immunocytochemical (Chatelain *et al.*, 1979; Dupouy *et al.*, 1979), biological and immunological techniques (Chatelain & Dupouy, 1981) and in the fetal plasma with a direct immunoassay of ACTH on unextracted plasma (Chatelain *et al.*, 1980).

The ACTH values measured with this procedure showed higher values at every stage of pregnancy with a peak on day 18 (Chatelain *et al.*, 1980). Moreover, chromatography on Sephadex G-50 of acid extracts of anterior and neurointermediate lobes revealed three main immunoreactive forms of "big", "intermediate" and "little" ACTH whose relative proportions changes during the perinatal pe-

riod (Chatelain & Dupouy, 1985).

There were many studies about the polymorphism of immunoreactive ACTH in the plasma of adults investigated almost exclusively by gel-filtration technique. The chromatography profiles of ACTH were very different according to species; only one peak of "intermediate" and "little" ACTH was found respectively in the plasma of the mouse (Coslovsky *et al.*, 1975) and the rat (Jackson & Lowry, 1980) whereas in the human plasma the main ACTH form was either "big" (Thoren *et al.*, 1981) or "little" (Yalow & Berson, 1971 & 1973; Yalow, 1974; Yamada *et al.*, 1978; Ratter *et al.*, 1980). However, both "big" and "little" peaks of immunoreactive ACTH were reported in the plasma of some patients with disorders of the hypothalamic pituitary adrenal axis (Yalow & Berson, 1971 & 1973; Yalow, 1974; Orth & Nicholson, 1977a & 1977b; Yamada *et al.*, 1978; Ratter *et al.*, 1980; Thoren *et al.*, 1981). By using reversed-phase HPLC, Schoneshofer *et al* (1981) showed the presence of many immunoreactive ACTH molecules whose molecular mass was lower than that of the (1-39) ACTH ones in normal human plasma. The first aim of the present study was to reinvestigate the changes in plasma immunoreactive levels in rat fetuses and newborns on extracted plasma in order to avoid the interference with substances which were present in plasma. The second aim was to study if the different molecular forms of ACTH present in the pituitary gland could also be revealed in the plasma (*in vivo* study) and in the effluent perifusate of anterior lobes (*in vitro* study) of fetuses and to follow the evolution of these different molecular forms of immunoreactive ACTH during pregnancy.

## MATERIALS AND METHODS

**Animals**: Experiments were performed on Wistar rats bred in the laboratory. Females were mated with a male for one night. The next day was taken as day 0 of pregnancy if spermatozoa were found in the vaginal smears. Pregnant females were transferred into individual cages 24 h before being sacrificed. The day of parturition was designed as day 0 postpartum and newborns were weaned on day 21.

**Blood Collection**: Adult non pregnant female rats and newborns from week 1 to week 4 were killed in the morning between 8.00 and 9.00 by a blow on the head. Blood samples were collected from carotid arteries in polyethylene tubes containing EDTA (20 μl 5% for 1 ml blood) and aprotinin (500 units for 1 ml blood) (Antagosan, Institut Behring, Laboratoires Hoechst). Fetal blood was similarly collected at the trunk level and kept in cooled polyethylene tubes. To obtain enough fetal plasma it was necessary to pool the blood from several fetuses according to gestational age. The blood samples were immediately centrifuged for 10 min at 5,000 g and 4°C. Plasma samples were then stored at −20°C until assayed for immunoreactive ACTH or chromatography.

**Extraction Procedure for ACTH**: ACTH was extracted from the plasma according to a modification of Goverde's technique (1981). Silicic acid powder (Silicic Acid 100 mesh-Mallinckrodt-USA) was previously activated at 700°C for 48h; 0.2 ml of distilled water containing 40 mg of silicic acid was added to 0.5 ml plasma samples. After 10 min agitation the tubes were decanted for 15 min at 4°C and subsequently centrifuged at

5,000 g for 10 min. The supernatant was discarded and the pellet washed with 2 ml cold distilled water; the suspension was centrifuged again at 5,000 g for 10min. The supernatant was discarded again and the pellet washed with 2 ml 1 N HCl. After centrifugation the supernatant was discarded and ACTH eluted with 1 ml of acetone-distilled water (50 : 50, V/V). After 10 min agitation the tubes were decanted for 15 min at 4°C again and centrifuged at 5,000 g for 10 min. After centrifugation 0.95 ml of the supernatants was evaporated in a speed-vac concentrator (Savant) to dryness and reconstituted with 0.610 ml of 0.02 M veronal buffer pH 8.2 supplemented with 0.2% 2-mercaptoethanol (Calbiochem) and 0.3% human serum albumin (HSA). The recovery of human $^{125}$I-ACTH (1-39) added to the plasma as tracer was over 72%.

**Perifusion System:** The perifusion apparatus has previously been described (Chatelain & Dupouy, in press). Ten or 15 anterior lobes from 19 and 21 day-old fetuses and 20 whole pituitaries from 17 day-old fetuses were mixed with a suspension of Biogel $P_2$ (Bio Rad, Richmond, USA) into 1 ml syringe and perifused with Krebs-Ringer-Bicarbonate buffer pH 7.4 supplemented with 0.2% glucose (KRBG) and 0.1% bovine serum albumin (BSA) (Sigma, St Louis, USA). Both the temperature (37°C) and the flow rate (10ml/h) were maintained constant throught the experiment. The pituitary glands were stimulated with an extract of adult hypothalamus (0.4 hypothalamus-equivalent was dissolved in 1 ml KRBG-BSA). The effluent perifusate was collected into 1 ml fractions in which ACTH was assayed by radioimmunoassay. The fractions corresponding to the peak of ACTH released were stored at −20°C until they were chromatographied.

**Sephadex Chromatography:** The chromatography was performed according to Ratter et al. (1980). Plasma samples as well as perifusate ones were thawed and acidified to pH 1.5 by addition of 1.6% glycine in 1 N HCL (0.2 ml/ml; v : v). The pre-acidified samples (2 ml) were chromatographied at 4°C on Sephadex G-50 fine (Pharmacia, Uppsala, Sweden). The columns (Pharmacia K 9/60-60×0.9 cm) have previously been equilibrated and eluted with 1% formic acid (Merck) containing 100 mg polypep/100 ml (Sigma, polypep p 5115). The flow rate was 10∼14 ml/h and 1 ml eluate fractions were collected into polyethylene tubes containing 10 μl of 10% mannitol to facilitate the reconstitution of the fractions. Indeed before ACTH radioimmunoassay these fractions were evaporated in a speed-vac concentrator to dryness and then reconstituted with 0.610 ml of 0.02 M veronal buffer containing phenol red as ph-indicator (20 μl for 100 ml buffer). The tubes were shaken with a vortex and the pH adjusted to 8.2 with 5 N NaOH (approximatively 5 μl/tube).

The columns were calibrated with Blue Dextran 2,000 (Pharmacia), Ovalbumin (Sigma), Cytochrome C (Sigma) and synthetic human (1-39) ACTH (Ciba-Geigy).

**Trypsin Treatment of "big" ACTH Form:** An acid extract (0.1 N HCl) of twenty anterior lobes of 21-day-old fetuses was subjected to gel filtration. Sephadex gel eluates containing "big" ACTH form were pooled, evaporated to dryness, reconstituted with 2 ml of phosphate buffer (0.1 M, pH 7.4) and distributed into three aliquot fractions (0.6 ml). Five or 10 μg of trypsin (Worthington Lyophylized trypsin) were added at room temperature to the first and second fractions which

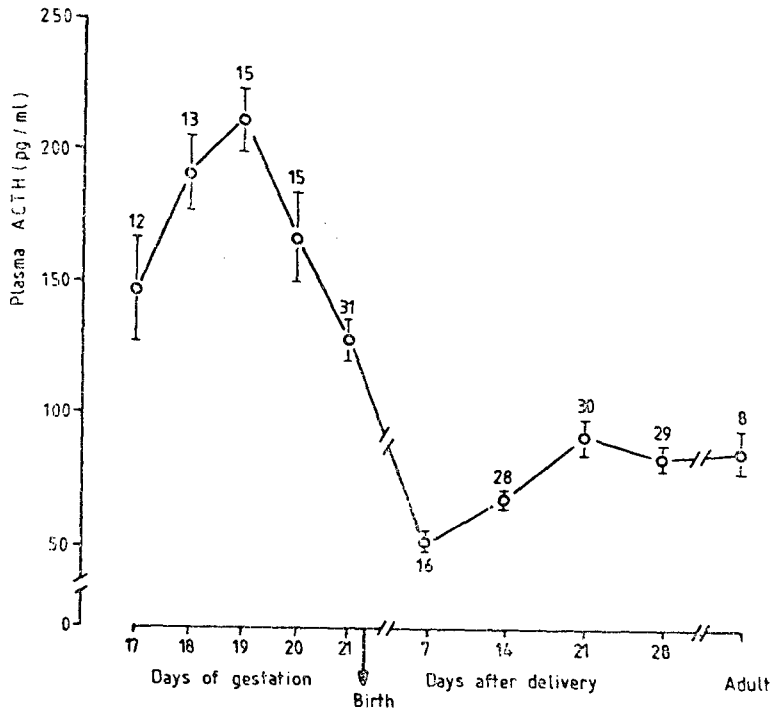


Fig. 1. Plasma levels of immunoreactive ACTH in rat fetuses, newborns and adult non pregnant females. Each point represents the mean±SEM.

were then incubated for 10 sec. The tryptic digestion was stopped by addition of 10 $\mu$g LBI (Lima bean trypsin inhibitor-Millipore Corporation) to each medium and by introducing the tubes into boiling water for 3 min. Ten $\mu$g of trypsin and 10 $\mu$g of LBI were added to the third fraction taken as control and the mixture was immediately boiled for 3 min. All the fractions were refractionated on Sephadex G-50 fine columns as described above.

**ACTH Radioimmunoassay**: ACTH radioimmunoassay was perfomed according to the procedure previously described (Chatelain & Dupouy, in press). ACTH levels were expressed as picograms of synthetic human ACTH (1-39) used as reference standard and samples were assayed in triplicate.

**Statistics**: All the results were expressed as mean±SEM. Calculations of statistical significance of differences between means were performed by the Student "t" test.

## RESULTS

**Plasma ACTH Levels in The Fetus, Newborns and Adults** (Fig. 1): The concentration of immunoreactive ACTH increased in the fetal plasma from day 17 to day 19 and then decreased until term on day 21. It was then nearly 0.6 time as low as on day 19. After birth the plasma ACTH decreased again to reach a low level one week after delivery. From week 1 to week 3 this concentration regularly increased and remained stable until week 4. Plasma ACTH levels were not significantly different from those found in adult non pregnant females (90±8 pg/ml).

**Immunoreactive Forms of ACTH in The Fetal Plasma and in The Perifusate of Fetal**

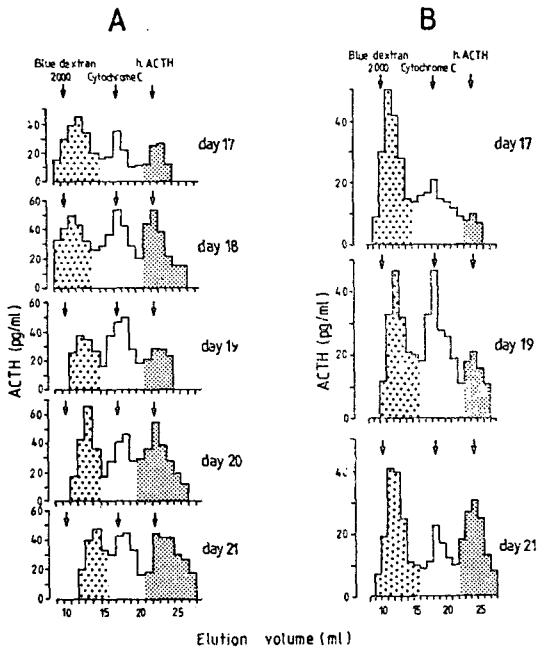


Fig. 2. Sephadex G-50 fine filtration of fetal plasma (A) and effluent perifusate of anterior pituitary lobes of fetuses (B) at different stages of development. Preacidified plasma or effluent perifusate (2 ml) was applied to a column of Sephadex G-50(60 cm×0.9 cm) equilibrated and eluted with acid formic(1%) containing polypep(1 g/1). The concentrations of immunoreactive ACTH in the eluate fractions(1 ml) were expressed as pg of synthetic human ACTH(1-39) per ml. Each graph represents the chromatography of one plasma or effluent perifusate sample. Arrows indicate peaks of elution for Blue Dextran 2,000, cytochrome C and human ACTH(1-39).

**Hypophysis** (Fig. 2 A,B) : Three main immunoreactive forms of ACTH were isolated by chromatography of both fetal plasma samples and perifusates of anterior lobes of fetal hypophysis stimulated by an extract of adult hypothalamus.

The first immunoreactive form which eluted in the void volume of the column as Blue Dextran 2,000 was called "big" ACTH (Fig. 2A, B). According to the determination of Kav on chromatography columns calibrated

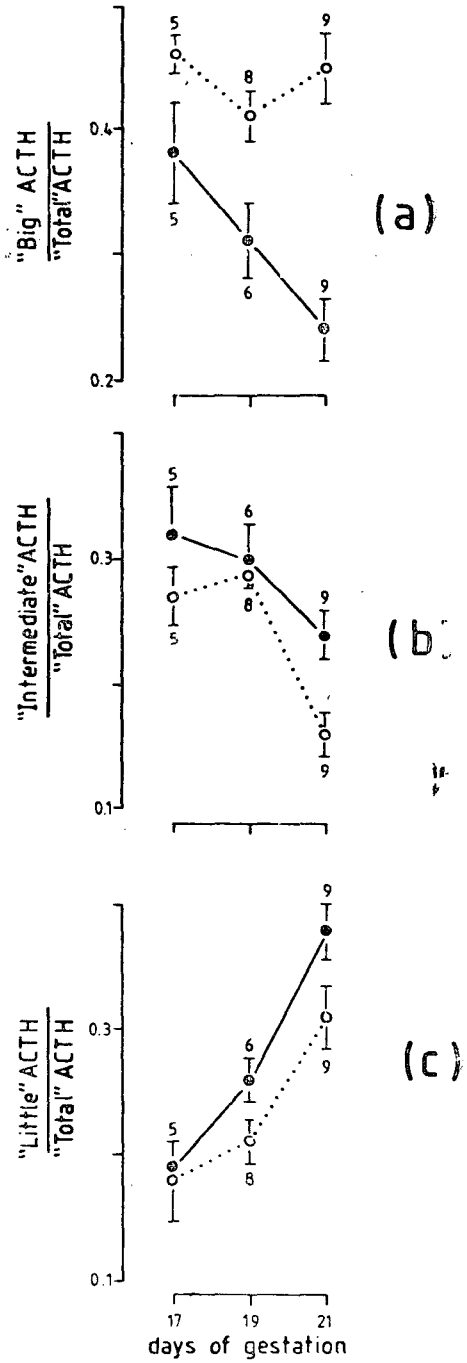


Fig. 3. Variations of the ratio "big" ACTH/"total" ACTH (a), "intermediate" ACTH/"total" ACTH (b), and "little" ACTH/"total" ACTH (c) in the plasma (●—●) and in the perifusate of anterior lobes of pituitary glands (○···○) of fetuses collected on days 17, 19 and 21 of gestation. Each point represents the mean±SEM; figures denote number of chromatograms.

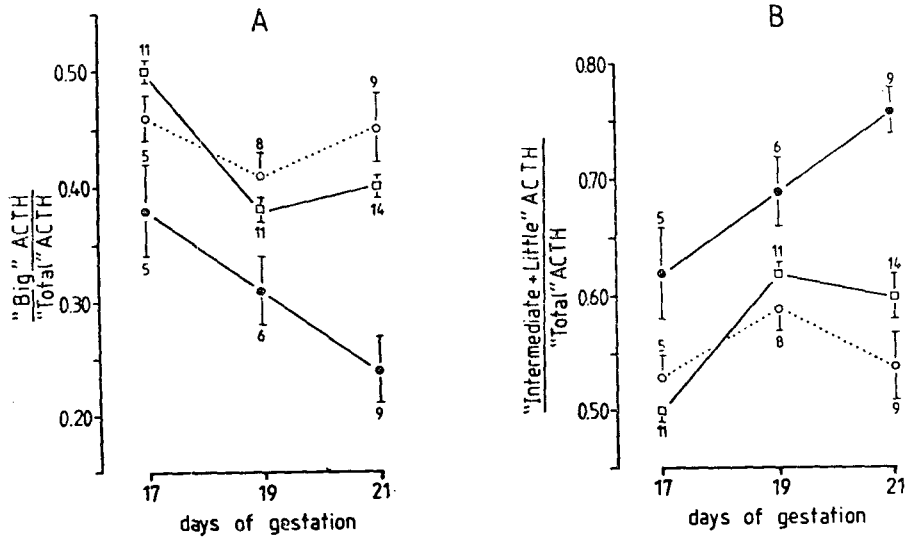


Fig. 4. Variations of the ratio "big" ACTH/"total" ACTH (A) and "intermediate"+"little" ACTH/"total" ACTH (B) in the plasma(●—●), in the perifusate of anterior lobes of pituitary glands (○···○) and in the anterior lobes (□—□) of fetuses collected on days 17, 19 and 21 of gestation. Each point represents the mean±SEM; figures denote number of chromatograms.

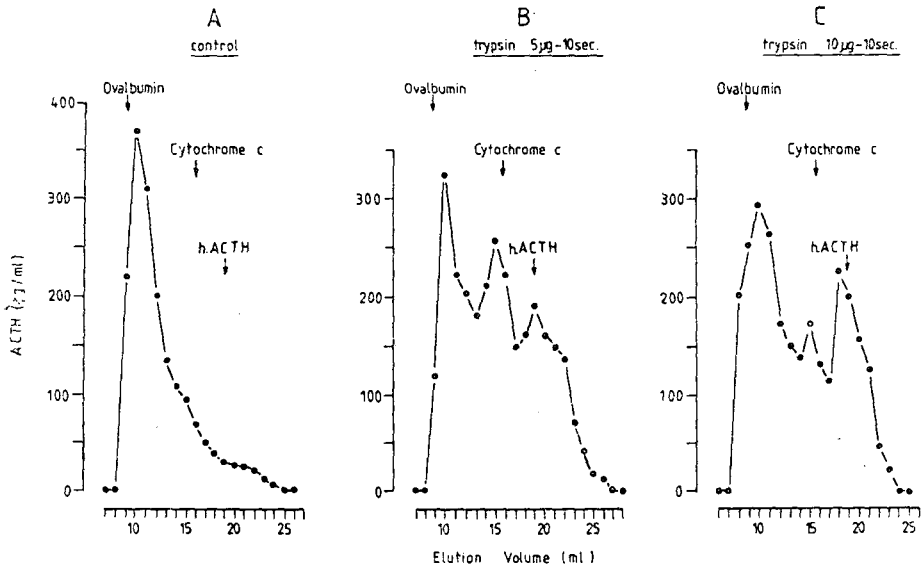


Fig. 5. Sephadex G-50 fine refiltration of the "big" immunoreactive form of ACTH isolated from an acid extract of 20 pituitaries of 21-day-old fetuses, before (A) and 10 sec after a controlled digestion performed with 5 μg (B) or 10 μg (C) of trypsin.

with ovalbumin, myoglobin, cytochrome C and synthetic human (1-39) ACTH the apparent molecular weight of this form was about 45, 000.

The second immunoreactive form called "little" ACTH which was eluted in the same fractions as human (1-39) ACTH (Fig. 2 A, B) showed an apparent molecular weight of 4, 500.

The third form called "intermediate" ACTH

which eluted between "big" and "little" ACTH in the same fractions as cytochrome C. (Fig. 2 A, B), showed a molecular weight close to 13,000.

**Evolution of The Ratio "big" ACTH/"total" ACTH** (Fig. 3 a): In the fetal plasma, the ratio "big" ACTH/"total" ACTH regularly decreased between day 17 and day 21, it was nearly 1.6 time as low as on day 17. In the perifusate samples of the anterior lobes of the pituitary glands collected on days 17, 19 or 21 of gestation, this last ratio remained high and stable, it was higher in the perifusate than in the plasma; significant difference was noted on days 19 ($p<0.02$) and 21 ($p<0.001$).

**Evolution of The Ratio "intermediate" ACTH/"total" ACTH** (Fig. 3 b): In both fetal plasma and perifusate samples this ratio was higher on days 17 and 19 than on day 21. In the plasma and the perfusate samples it was then respectively 1.3 and 0.17 time as low as on day 17. At term the values were significantly higher ($p<0.02$) in the plasma than in the perifusate samples.

**Evloution of The Ratio "little" ACTH/"total" ACTH** (Fig. 3c): A sharp and regular increase of this ratio was obvious between day 17 and day 21 in both fetal plasma and pituitary perifusate. The values were higher in the plasma than perifusate samples but no significant difference was noted on days 19 ($p>0.05$) and 21 ($p>0.05$). At term this ratio was nearly 1.6 time as high as on day 17.

**Evolution of The Ratio "big" ACTH/"total" ACTH and "intermediate"+"little" ACTH/"total" ACTH** (Fig. 4 A, B): These ratios showed a similar evolution both in perifusate samples and anterior lobes of fetal pituitaries between days 17 and 21, but in the plasma the evolution of these ratios was very different.

**Trypsin Treatment of "big" Immunoreactive Form of ACTH** (Fig. 5): After the treatment of the "big" immunoreactive form of ACTH with trypsin (5 $\mu$g or 10 $\mu$g) for 10 sec, three peaks of immunoreactive ACTH corresponding to "big", "intermediate" and "little" forms were revealed by filtration on Sephadex G-50 fine. With the higher amount of trypsin the peak of little ACTH increased whereas that of the intermediate one decreased.

## DISCUSSION

In the fetal plasma, immunoreactive ACTH levels reached highest values on day 19 of gestation and decreased thereafter until day 21. Such evolution of the plasma ACTH levels could explain that of the corticosterone which also reached a peak on day 19 in both adrenals (Cohen & Brault, 1974; Dupouy & Cohen, 1975; Dupouy & Dubois, 1975; Dupouy *et al.*, 1975) and the plasma (Holt & Oliver, 1968; Cohen, 1973; Cohen & Brault, 1974; Dupouy & Cohen, 1975; Dupouy & Dubois, 1975; Dupouy *et al.*, 1975). The ACTH concentrations reported here were similar to those of Boudouresque et al (1984) but lower than those previously obtained by direct immunoassay of ACTH on unextracted plasma (Chatelain *et al.*, 1980; Dupouy & Chatelain, 1981). During the first week postpartum the sharp decrease of plasma ACTH levels was in agreement with the low corticosteone levels reported in both the adrenals and the plasma of newborns (Cohen, 1976). Such fall in plasma ACTH levels after parturition could reflect a drastic reduction of ACTH release by the hypophysis under

the negative feedback action of free corticosterone. Indeed, at the end of gestation and in early postpartum days, the corticosterone binding capacity of the plasma decreased (Koch *et al.*, 1967; Nuñez et al., 1971; Martin *et al.*, 1977; Van Baelen *et al.*, 1977; Vallette *et al.*, 1982; Raymoure & Kuhn, 1983; Dupouy & Chate lain, in press) and the concentration of free corticosterone raised after birth (Koch, 1967). A progressive increase in plasma ACTH levels was observed during the last two weeks preceding the weaning on day 21 and from this stage of postnatal development, concentrations similar to those of the adults were noted. In human fetuses and newborns, similar evolution was reported for ACTH; indeed in the fetal plasma immunoreactive ACTH levels were higher between weeks 12 and 34 than between weeks 35 and 42 (Winters *et al.*, 1974). After birth, plasma ACTH levels decreased (Kauppila *et al.*, 1976) to reach the lowest values one week after delivery (Cacciari *et al.*, 1975 & 1976). According to the present data, the circulating ACTH in the fetal blood as well as the ACTH released by the anterior lobes of the fetal hypophysis in vitro at different stages of gestation was present under three molecular forms named "big" "intermediate" and "little" ACTH; they were similar to those previously observed in the whole fetal pituitary gland (Chatelain & Dupouy, 1980; Dupouy & Chatelain, 1981) or in the anterior lobe (Chatelain & Dupouy, in press).

In plasma the proportions between the different immunoreactive forms of ACTH varied during gestation. The ratio "big" ACTH/"total" ACTH gradually decreased whereas the ratio "little" ACTH/"total" ACTH gradually increased as it was also shown in the anterior lobes of the pituitary gland (Chatelain & Dupouy, in press).

Jones and Roebuck (1979) reported a similar evolution of different molecular forms of ACTH in the fetal plasma of monkey, sheep and guinea-pig. The ratios "little" ACTH/"total" ACTH and "intermediate" ACTH/"total" ACTH showed the same evolution in the perifusate samples and in the plasma. However the values were lower in the former than in the latter mainly at term. The ratio of "big" ACTH/"total" ACTH released in vitro was stable at all the investigated stages of gestation whereas in the plasma that ratio was lower and decreased as the gestation progressed.

The proportions between the different molecular forms of immunoreactive ACTH were similar in both the anterior lobes of the pituitary glands and in the perifusate of these lobes. Our data suggested that the fetal hypophysis released, in vitro, all the molecular forms of ACTH it contained, in the same proportions. If the same type of release occured in vivo, that did not explain why the proportions between the three molecular forms of ACTH were so different in the fetal circulation. Our data on the effect of trypsin on "big" ACTH supported the hypothesis of a transformation of that form into smaller forms as "intermediate" and "litte" ACTH. Then the evolution of the different molecular forms of ACTH in the fetal circulation could be related to an enzyme mediated conversion. Such hypothesis calls further investigations. Present data were in agreement with those reported by Jones (1976a & 1976b); the different forms of ACTH with high molecular weights which were identified in the incubation medium of isolated cells from anterior pituitay gland as well as in the plasma of the fetal lamb, were converted by trypsin or

plasmin into the "little" form of ACTH. According to our "in vivo" and "in vitro" studies performed on the rat, the amount of "intermediate" ACTH was greater on days 17 and 19 than on day 21. The question arises about the physiological meaning of this form during perinatal development. The controlled tryptic digestion of the "intermediate" form of ACTH was followed by a continuous loss of ACTH immunoreactivity without appearance of the little form of ACTH (Chatelain unpublished data); the "in vitro" corticosteroidogenic activity of the intermediate ACTH was as important as that of the "little" form when identical quantity of immunoreactive ACTH expressed in ng equivalent human ACTH 1-39 was used (Chatelain & Dupouy, inpress). On day 17, fetal rat adrenals became sensitive to ACTH (Cohen, 1963) and corticosterone concentration in the plasma began to rise (Dupouy & Cohen, 1975). On day 19, the adrenal sensitivity to ACTH was high and corticosterone concentration reached a peak in both adrenals and plasma. After day 19, corticosterone concentrations decreased until parturition (Dupouy & Cohen, 1975). These present date could suggest that "the intermediate" form of immunoreactive ACTH was to a certain extent responsible for the adrenocortical stimulation between days 17 and 19 of gestation. Complementary investigations are progressing to test this hypothesis.

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