J. steroid Biochem. Vol. 22, No. 6, pp. 803–808, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

POSTNATAL DEVELOPMENT OF CHOLESTEROL ESTER HYDROLASE ACTIVITY IN THE RAT ADRENAL

SUSAN J. HENNING and GLENN M. GENOVESE
Department of Biology, University of Houston, Houston, TX 77004, U.S.A.

(*Received* 23 *July* 1984)

Summary—In certain circumstances the activity of cholesterol ester hydrolase (CEH) activity is believed to be rate-limiting for corticosterone production by the adrenal. The principal aim of the current study was to determine whether the activity of CEH displays a developmental increase in the infant rat which could, in part, account for the marked increase in serum corticosterone which begins at the end of the second postnatal week. The data show that the specific activity of CEH (units/mg cytosolic protein) during development is actually a mirror image of the pattern seen for serum corticosterone, i.e. CEH activities are high when serum corticosterone concentrations are low and then fall when serum corticosterone is rising. Even when total activities of CEH in the adrenal were calculated, there was no increase in parallel with the initial rise of serum corticosterone. At each age studied, stressed pups displayed significant increases of serum corticosterone; however, their CEH activities were no different from those in the non-stressed littermates. It is concluded that the activity of CEH is not the rate-determining factor for the developmental surge of basal concentrations of serum corticosterone nor for stress-induced elevation of corticosterone during the developmental period. A second aim of the current study was to address the more general question of whether steroidogenesis in the developing adrenal is limited by substrate supply. Measurement of the cholesterol content of adrenal mitochondria showed no ontogenic increase, suggesting that substrate supply, from any source, is not rate-limiting for steroidogenesis at these ages.

INTRODUCTION

It has been demonstrated previously that, in infant rats, circulating concentrations of corticosterone are almost undetectable at 6–12 days of age and then increase markedly, peaking at approx 24 days [1–4]. The developmental increase in serum concentrations of this hormone cannot be due to decreased turnover because the half-life of corticosterone has been shown to slowly decline during the postnatal period [5]. Thus, to account for the developmental rise of serum corticosterone, it is reasonable to propose increased synthesis and secretion by the adrenal gland. Although there have been several studies of steroidogenic enzymes in developing adrenals [6–8], none of the enzymes investigated have shown a developmental pattern that would account for increased synthesis beginning between days 12 and 14.

In the adult rat it has been shown that the rate of adrenal steroidogenesis may be limited by the supply of substrate, cholesterol [9]. The adult adrenal has abundant lipid droplets containing cholesterol esters. Cholesterol is released by the action of cholesterol ester hydrolase (CEH) located in the adrenal cytosol [10]. The activity of CEH is increased following stress, and this is believed to be important in allowing increased production of corticosterone [11]. The effect of stress on adrenal CEH activity cannot be mimicked by administration of ACTH [11]. The effector of CEH changes during stress is thus presumed to be some other neuroendocrine factor, possibly γ_3-MSH [12, 13]. In the infant rat, circulating concentrations of ACTH have been shown to be unchanged from 1 through 21 days of age [14]. Thus, to account for the dramatic increase in circulating corticosterone between days 12 and 21, an ACTH-independent parameter such as CEH activity becomes an attractive possibility. Moreover, as cytoplasmic lipid droplets are abundant in adrenal cytoplasm of rats aged $4\frac{1}{2}$ through 14 days [15], mobilization of the cholesterol esters stored in these droplets would provide a likely mode of regulation of the steroidogenic pathway. The specific hypothesis investigated in the current study was that during post-natal development, the activity of CEH in adrenal cytosol would increase just prior to or in parallel with serum concentrations of corticosterone. An additional aim was to determine the age at which this enzyme begins to display stress responsiveness.

EXPERIMENTAL

Materials

Cholesteryl oleate (oleate-1-^{14}C) and [^{3}H]oleic acid were obtained from New England Nuclear (Boston, MA). The purity of the cholesteryl [^{14}C]oleate and the [^{3}H]oleic acid were checked by thin layer chromatography using hexane–diethyl ether–glacial acetic acid (90:10:1, by vol) as solvent. Nonlabelled cholesteryl oleate and oleic acid were obtained from Sigma Chemical Company (St Louis, MO). For assay of cholesterol in adrenal mitochondria, [1,2-^{3}H(N)]-cholesterol was obtained from New England Nuclear (Boston, MA) and silica gel TLC plates (Si250F) were from Baker Chemical Co. (Phillipsburg, NJ).

Animals

Timed-pregnant Sprague–Dawley rats [Charles River Crl:CD (SD)BR] were received from Charles River Breeding Laboratories (Wilmington, MA) on day 15 of gestation. They were housed individually in opaque polystyrene cages and provided with food (Rodent laboratory chow 5001; Ralston Purina, St Louis, MO) and water *ad libitum*. Animal quarters were air-conditioned (22 ± 1°C) and maintained on a 12-h light/dark cycle with lights on at 0500 h. On the due date, cages were checked every 2 h for births. The birth date was regarded as day 0. Approximately 24 h postpartum, litters were culled to 8 pups. Pups were housed with their dams until 25 days of age, when weaning was completed by removal of the dams.

Procedures

The studies of CEH at 9, 16, 21 and 24 days employed 6 litters of rat pups. To ensure basal levels of corticosterone, the 6 cages were isolated overnight prior to each experimental day. At the time of isolation, pups were weighed and in each litter a pair of pups with close body weights was marked for use the next day. For sacrifice, cages were quietly removed from the isolation room one at a time. In each litter at each age, one of the marked animals was sacrificed immediately, i.e. in a non-stressed condition. The second marked pup was purposefully stressed by i.p. injection of 0.1 ml physiological saline and was sacrificed 10 min later. At all ages, sacrifice was completed between 0830 and 0930 h. For all animals, trunk blood was collected and adrenals were removed and placed in ice-cold physiological saline. The blood was allowed to clot at room temperature for 30 min then centrifuged at 3000 *g* to obtain serum. Sera were frozen (−10°C) for future corticosterone assay. Adrenals were trimmed of fat, blotted, weighed and then used immediately for assay of cytosolic CEH activity. At 9 days of age, adrenal weights from individual animals were insufficient for the standard assay. Therefore, at this age in the original series, adrenals from 2 pups were pooled. An additional 6 stressed and non-stressed pups at 9 days were therefore utilized to maintain $n = 6$.

At 35 days of age the procedure was basically the same as described above, except that pups from 5 litters were used. On the day before the experiment, the pups were weight-paired and housed 2 per cage, giving a total of 10 cages. On the day of the experiment, each cage was then treated as described above, i.e. 1 pup was killed immediately (non-stressed) and the other was killed 10 min after i.p. injection (purposefully stressed). Thus, for each group at this age, $n = 10$ with 2 pups from each of the 5 litters.

Assay of CEH activity

Adrenal cytosol was prepared as described by Pederson and Brownie[11] using 1 ml homogenization medium for every 5 mg adrenal tissue. A most important aspect of this method is that it eliminates all endogenous cholesterol esters, thus avoiding the dilution of the [^{14}C]cholesteryl oleate with variable amounts of unlabelled substrate [11]. In our protocol, this was achieved by overlaying the adrenal homogenates with corn oil prior to centrifugation in thin-wall polyallomer tubes (30 min at 250,000 *g*). After centrifugation, the lipid-free cytosol was removed by piercing the tube (approx 2 mm above the pellet) was a 23 g needle attached to a 5 ml syringe. The corn oil prevented the lipid layer from breaking up as the lower aqueous layer was removed.

The conditions for assay of cytosolic CEH were the same as those used by Pederson and Brownie[11] except for reaction time and amount of tissue (see Results). In this method cholesteryl [^{14}C]oleate is used as substrate. The reaction is stopped by the addition of chloroform–methanol (2:1, v/v). After addition of alkali, the [^{14}C]oleic acid released in the reaction is extracted from the substrate which remains in the organic phase. The extraction efficiency is determined by addition of [^{3}H]oleic acid. The protein content of each cytosol sample was determined by the method of Lowry *et al.*[16] using bovine serum albumin as standard. The activity of CEH was expressed as units per mg cytosol protein where 1 unit = 1 nmol oleic acid released per min.

Assay of serum corticosterone

Serum corticosterone was determined by a competitive protein-binding assay as described previously [17] with two modifications. The pH of the sodium phosphate buffer was changed from 7.2 to 7.9 and the amount of dextran used to make dextran-coated charcoal was increased from 375 mg per 100 ml phosphate buffer to 1000 mg. These changes greatly improved the stability of binding.

Mitochondrial cholesterol content

Adrenal mitochondria were prepared by differential centrifugations in 0.25 M sucrose [18]. The final mitochondrial pellet was resuspended in 1.65 ml of 15 mM triethanolamine buffer, pH 7.3. Twenty μl of [^{3}H]cholesterol (54.8 Ci/mmol; 100,000 dpm/20 μl) was added to estimate recovery. After incubation at 23°C for 30 min to allow the [^{3}H]cholesterol to equilibrate with endogenous pools, an aliquot (0.8 ml) was extracted with chloroform–methanol (2:1, v/v). The organic phase was evaporated to dryness under N_2 and then dissolved in 20 μl ethanol and subjected to thin-layer chromatography (TLC) in petroleum ether–diethyl ether–glacial acetic acid (75:25:2, by vol) according to the method of Simpson and Boyd[19]. The spot corresponding to cholesterol was scraped from the TLC plate and eluted with ethanol. For color development, 25 μl of the ethanol extract was added to 0.45 ml of an 18:1 (v/v) mixture of concentrated H_2SO_4–concentrated HCl. The absorption at 350 nm was read after 30 min at room temperature. Stan-

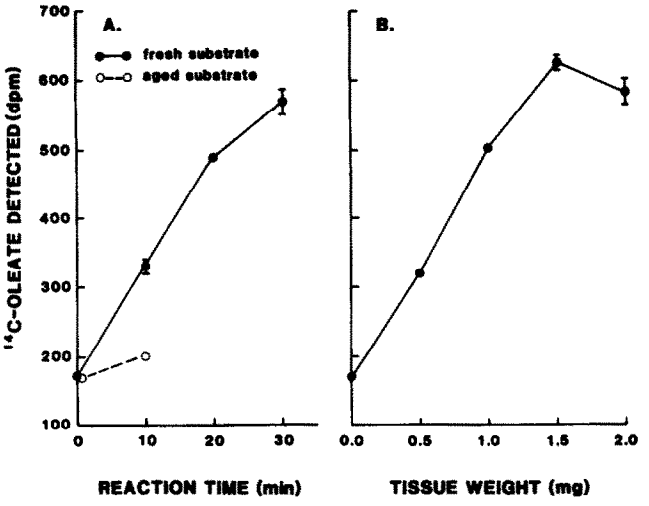

Fig. 1. Linearity of the CEH reaction with time (panel A) and tissue (panel B). The number of assays (n) for each point range from 2–4. Values are given as means ± SE when $n > 2$, means ± range when $n = 2$. Lack of error bars indicates that the SE or range is smaller than the symbol. Panel A shows data obtained using either a fresh substrate emulsion (●——●) or a substrate emulsion that had been allowed to stand 30 min at 37°C prior to use (○---○).

dards containing 0, 2.5, 5.0 and 7.5 ng cholesterol were subjected to identical treatment (including TLC). Recovery of [^{3}H]cholesterol was typically 80%. Values for endogenous cholesterol were corrected using the individual recovery figures. The protein content was determined on a separate aliquot of the mitochondrial suspension using the method of Lowry *et al.*[16].

Statistics

Results are reported as mean ± SE. The differences between stressed and non-stressed animals were evaluated by a two-tailed Student's t-test.

RESULTS

Conditions of assay of CEH activity

Since the assay of CEH activity was described for adult adrenals [11], for the current study it was necessary to modify the method, making it sufficiently sensitive to detect CEH activity in the small amounts of adrenal tissue available from infant rats. The simplest way to make an enzyme reaction more sensitive is to allow it to run for a longer time. However, preliminary studies using adult adrenal tissue (Fig. 1A) showed that the reaction was not linear beyond 20 min. The loss of linearity was not a property of the adrenal cytosol, but rather of the substrate emulsion. As can be seen in the figure, substrate emulsion that was allowed to stand for 30 min prior to use gave a substantially slower reaction rate than did substrate which had been freshly prepared. Presumably, with time the emulsion droplets coalesce, causing lowering of the surface area available for the enzyme reaction to occur. It was also found (data not shown) that vortexing the reaction mixture reduced the rate, probably due to loss of emulsion droplets on the sides of the reaction tube. In view of these findings, the standard approaches were: (1) to always use freshly-prepared substrate emulsion; (2) to shake the tubes gently by hand after substrate addition; and (3) to allow the reaction to proceed for only 10 min.

The original assay [11] used adrenal cytosol equivalent to approx 5 mg adrenal tissue in each reaction mixture. The study shown in Fig. 1B shows that this amount of cytosol is probably excessive, because the reaction is linear with tissue amount only up to 1.5 mg. For the developmental studies described below, each reaction tube contained cytosol equivalent to 1 mg adrenal tissue. This made the reaction sufficiently sensitive to allow individual assays on adrenals from single animals of age 16 days and older. As described in the Experimental section for animals aged 9 days, adrenals from 2 pups were pooled to create each cytosol sample.

Developmental studies with CEH

The results for serum corticosterone (Fig. 2A) show that basal (non-stressed) concentrations of the hormone increased substantially between 9 and 21 days of age and then declined through 35 days of age. This developmental pattern for corticosterone is very similar to that described previously [1]. In the stressed animals, serum concentrations of corticosterone were significantly elevated ($P < 0.05$) at all ages.

The specific activity of CEH in adrenal cytosol (Fig. 2B) showed a developmental pattern that was almost the mirror image of that for basal concentrations of serum corticosterone. CEH activity declined between days 9 and 16, i.e. during the beginning of the ontogenic rise of serum corticosterone. On

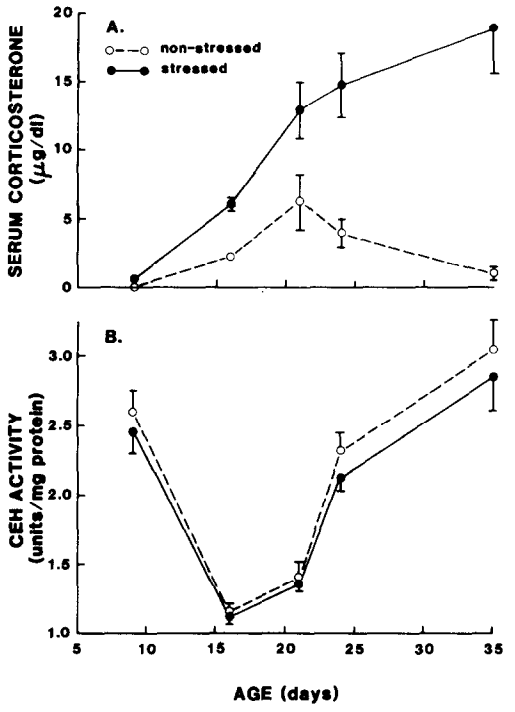

Fig. 2. Serum corticosterone (panel A) and CEH specific activity (panel B) in non-stressed and stressed rats of various ages. Values are given as means ± SE, $n = 6$ at all ages, except day 35 when $n = 10$. Lack of error bars indicates the SE is smaller than the symbol.

the other hand, between days 21 and 35, CEH activity increased while basal concentrations of serum corticosterone decreased. Only between days 16 and 21 did CEH activity increase in parallel with basal concentrations of serum corticosterone. CEH activities in adrenal cytosol from stressed animals were slightly lower than those from non-stressed animals at all ages. However, the differences were not statistically significant ($P > 0.2$).

It could be argued that total activity of CEH in the adrenal pair of each pup has more physiological relevance than the specific activity of the enzyme. When expressed in this way (Fig. 3A), the activity of the enzyme declined only slightly between 9 and 16 days of age and then showed a steady increase through 35 days. This pattern primarily reflects adrenal growth which parallels that of body weight. Thus, when total CEH activity was expressed per unit body weight (Fig. 3B), values remained relatively constant from 9 through 21 days of age, increased between 21 and 24 days, then plateaued to 35 days. Here again (Figs 3A and 3B), there were no differences between values obtained from stressed animals as compared with non-stressed animals ($P > 0.2$).

Mitochondrial content of cholesterol

To address the more general question of whether steroidogenesis in the developing adrenal is limited by substrate supply, we measured the cholesterol content of adrenal mitochondria from rats aged 9, 15 and 21 days. For each measurement we pooled adrenals as follows: at 9 days of age, 27 animals were required to give sufficient tissue; at 15 days, 18 animals and at 21 days, 9 animals. The values obtained (means ± SE for 3 pools of tissue at each age) were 16.2 ± 1.7, 15.3 ± 1.7 and 15.5 ± 1.4 ($n = 3$) μg cholesterol/mg mitochondrial protein, respectively, indicating that there is no developmental increase in this parameter. Values at all three ages are approximately half those reported for basal conditions in adult rats [9].

DISCUSSION

The surge of serum corticosterone that occurs in the neonatal rat at the end of the second postnatal week appears to be responsible for coordination of biochemical changes in a number of tissues including salivary gland, gastric mucosa, pancreas, small intestine and liver [20]. Thus, understanding the biochemical basis of the ontogenic increase of corticosterone production has considerable importance to mammalian development. The simplest explanation for increased adrenal corticosteroidogenesis would be increased release of ACTH from the pituitary. However, the available data indicate that circulating concentrations of ACTH remain relatively constant in the rat from age 1 through 21 days [14]. As indicated in the Introduction, the possibility of corticosterone production during development being controlled by the activity of CEH was an attractive possibility because this would be an ACTH-independent mechanism [11–13]. However, the data in Figs 2 and 3 indicate that CEH activity is not rate-limiting for corticosterone production during

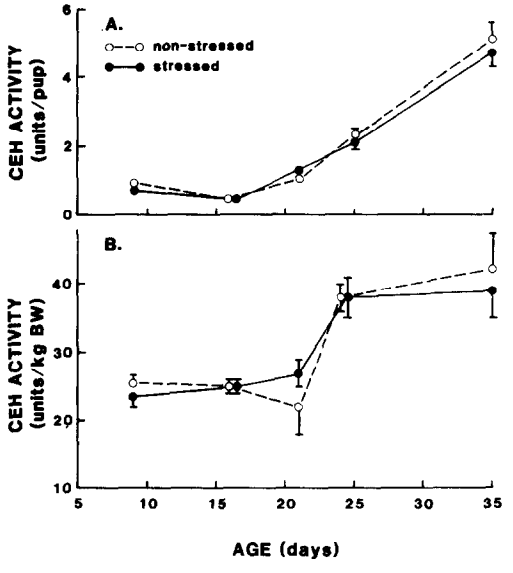

Fig. 3. Total CEH activity expressed per animal (panel A) and per unit body weight (panel B). Data are from the same animals shown in Fig. 2. Lack of error bars indicates SE is smaller than symbol.

development. The specific activity of the enzyme (Fig. 2) is, in fact, very much higher at 9 days (i.e. prior to the corticosterone surge) than at 16 and 21 days (i.e. during the corticosterone surge). Likewise, the total activity of the enzyme (Fig. 3) does not increase between 9 and 16 days and therefore cannot account for increased corticosterone production.

These findings regarding CEH activity do not eliminate the more general possibility that steroidogenesis in the developing adrenal is limited by substrate supply. Since the cholesterol used for steroidogenesis can be derived from plasma lipoproteins or from *de novo* synthesis, these aspects of cholesterol physiology in the developing adrenal also need to be considered. Rather than assess each of these independently, we measured the mitochondrial content of cholesterol in rat pups at three critical ages of development. Although this is a static measurement, studies in adult rats have shown that at times of elevated corticosterone synthesis (e.g. following ACTH treatment) there is a significant increase in the cholesterol content of adrenal mitochondria [9]. Thus, if steroidogenesis in the developing adrenal were controlled by substrate supply (from any source), we would have expected an increase in the cholesterol content of adrenal mitochondria as age increased from 9 to 21 days. As such an increase was not observed, we conclude that changes in substrate availability are unlikely to account for the developmental surge of circulating corticosterone.

The data for serum corticosterone concentration in stressed animals (Fig. 2) confirm earlier reports that the stress-responsiveness of the infant rat gradually increases with age [2, 21, 22]. This increase has been shown to be due, in part, to increased production of CRF-like activity by the hypothalamus [22] and, in part, to increasing ACTH responsiveness of the adrenal [2, 14] with increasing age of the animals. The present study indicates that elevated activity of CEH is not necessary for the stress-induced elevation of corticosterone during this period of development in the sense that there were no detectable differences in the activities of CEH in stressed pups as compared with non-stressed littermates (Figs 2 and 3). This lack of correlation between CEH activity and corticosterone production in the developing rat is not surprising, because even in the adult rat, there is not a 1:1 relationship between these two parameters. For example, non-stressed serum concentrations of corticosterone show a 7-fold diurnal variation in the absence of any change in CEH activity [11]. Moreover, during the early part of the light cycle, a 1.5-fold stress-induced increase in CEH activity is associated with a 9.5-fold increase in serum corticosterone, whereas at the onset of darkness, a 1.6-fold stress-induced increase in CEH activity is associated with only a 2.4-fold increase in serum corticosterone [11]. Comparison of the 35-day CEH activities (Fig.2) with those of stressed and non-stressed adult rats killed at the same phase of the light/dark cycle [11] shows that our values in 35-day old animals are much closer to the stressed values reported for adults (2.99 ± 0.05 units/mg protein) than the non-stressed values (2.01 ± 0.06 units/mg protein) despite the fact that the mode of stress in the adult study was identical to that used in the current study, i.e. i.p. injection 10 min prior to sacrifice. This suggests the possibility that what develops between day 35 and adulthood is an inhibitor of CEH activity in non-stressed animals. The effect of stress would then be to remove the inhibitor, thus allowing the enzyme activity to return to levels seen in both non-stressed and stressed animals at day 35. Studies designed to investigate this suggestion are currently in progress.

Acknowledgements—This work was supported by grant number R01 HD14094 from the National Institutes of Health. The authors wish to thank Lucy Leeper, Vikram Rao and Helen Blake for their helpful comments on the manuscript.

REFERENCES

1. Henning S. J.: Plasma concentrations of total and free corticosterone during development in the rat. *Am. J. Physiol.* **235** (1978) E451–E456.
2. Schoenfeld N. M., Leathem J. H. and Rabii J.: Maturation of adrenal stress responsiveness in the rat. *Neuroendocrinology* **31** (1980) 101–105.
3. Giannopoulos G.: Levels and subcellular distribution of endogenous corticosterone in rat liver during development. *Steroids* **29** (1977) 539–556.
4. Morishige W. K. and Joun N. S.: Influence of glucocorticoids on postnatal lung development in the rat: possible modulation by thyroid hormone. *Endocrinology* **111** (1982) 1587–1594.
5. Schapiro S., Percin C. J. and Kotichas F. J.: Half-life of plasma corticosterone during development. *Endocrinology* **89** (1971) 284–286.
6. Kolena J., Macho L., Palkovic M. and Poor J.: Steroidogenic enzyme systems in the rat adrenal glands during postnatal development. *Gen. comp. Endocr.* **10** (1968) 247–252.
7. Kolena J., Macho P., Poor J. and Palkovic M.: The activity of enzyme involved in the biogenesis of steroids in the adrenal glands of young rats. *Physiol. Bohemoslov.* **14** (1965) 64–69.
8. Kolena J., Macho L. and Palkovic M.: Studies on the enzymatic systems of rat adrenal in early postnatal period. II. The effect of NADPH on progesterone-4-C^{14} hydroxylation and pentose cycle activity. *Arch int. Physiol. Biochim.* **73** (1965) 587–592.
9. Mahaffee D., Reitz R. C. and Ney R. L.: Mechanism of action of adrenocorticotropic hormone. Role of mitochondrial cholesterol accumulation in the regulation of steroidogenesis. *J. biol. Chem.* **249** (1974) 227–233.
10. Shima S., Mitsunaga M. and Nakao T.: Effect of ACTH on cholesterol dynamics in rat adrenal tissue. *Endocrinology* **90** (1972) 808–814.
11. Pedersen R. C. and Brownie A. C.: Failure of ACTH to mimic the stress-induced activation of rat adrenocortical cholesterol ester hydrolase *in vivo*. *J. steroid Biochem.* **11** (1979) 1393–1400.
12. Farese R. V., Ling N. C., Sabir M. A., Larson R. E. and Trudeau W. L. III: Comparison of effects of adrenocorticotropin and lys-γ3-melanocyte-stimulating hormone on steroidogenesis, adenosine 3′,5′-monophosphate production, and phospholipid metabolism in rat adrenal fasiculata-reticularis cells *in vitro*. *Endocrinology* **112** (1983) 129–132.

13. Pedersen R. C., Brownie A. C. and Ling N.: Pro-adrenocorticotropin/endorphin-derived peptides: co-ordinate action on adrenal steroidogenesis. *Science* **208** (1980) 1044–1045.
14. Guillet R. and Michaelson S. M: Corticotropin responsiveness in the neonatal rat. *Neuroendocrinology* **27** (1978) 119–125.
15. Magalhaes M. M., Breda J. R., Magalhaes M. C. and Reis J.: Postnatal development of the rat adrenal cortex: an ultrastructural morphometric study. *J. ultrastr. Res.* **76** (1981) 215–223.
16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
17. Martin G. R. and Henning S. J: Relative importance of corticosterone and thyroxine in the postnatal development of sucrase and maltase in rat small intestine. *Endocrinology* **111** (1982) 912–918.
18. Wang, H-P. and Kimura T.: Purification and characterization of adrenal cortex mitochondrial cytochrome P-450 specific for cholesterol side chain cleavage activity. *J. biol. Chem.* **251** (1976) 6068–6074.
19. Simpson E. R. and Boyd G. S.: The cholesterol side-chain cleavage system of bovine adrenal cortex. *Eur. J. Biochem.* **2** (1967) 275–285.
20. Henning S. J.: Postnatal development: coordination of feeding, digestion, and metabolism. *Am. J. Physiol.* **241** (1981) G199–G214.
21. Haltmeyer G. C., Denenberg V. H., Thatcher J. and Zarrow M. X.: Response of the adrenal cortex of the neonatal rat after subjection to stress. *Nature* **212** (1966) 1371–1373.
22. Hiroshige T. and Sato T.: Changes in hypothalamic content of corticotropin-releasing activity following stress during neonatal maturation in the rat. *Neuroendocrinology* **7** (1971) 257–270.