Factors Influencing the Development of Urea-Synthesizing Enzymes in Rat Liver

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(Received 24 November 1967)

1. The activities of enzymes of the urea cycle, carbamoyl phosphate synthetase, ornithine transcarbamoylase, argininosuccinate synthetase, argininosuccinase (the last two comprising the arginine synthetase system) and arginase, were measured in the liver during development of the rat. All five enzymes exhibited relatively low activities in foetal liver and a rapid postnatal increase was found. The rate-limiting enzyme of urea synthesis in the rat, the condensing enzyme of the arginine synthetase system, showed the lowest activity at birth and the most rapid postnatal increase, a fivefold increase within 24 hr. after birth. A second increase of activity was noted after the tenth day. These results suggest that the postnatal increase of arginine synthetase activity initiates the ability for urea synthesis in the rat. 2. Some factors influencing the development of the rate-limiting arginine synthetase system were studied in more detail. (a) Intraperitoneal administration of puromycin inhibited the postnatal increase of the enzyme activity. (b) Starvation of newborn animals for 24 hr. after birth had no effect on the postnatal development of the enzyme. (c) Bilateral adrenalectomy at birth caused a marked diminution in the postnatal increase of the enzyme activity and injections of triamcinolone were effective in preventing the effect of adrenalectomy. (d) Administration of triamcinolone alone had a marked stimulatory effect on the postnatal development of this enzyme. (e) Premature and postmature birth had virtually no effect on the developmental pattern of the arginine synthetase activity, suggesting that the increase of this enzyme activity after birth is not initiated by the birth process.

The enzymes involved in urea synthesis in the liver, carbamoyl phosphate synthetase (EC 2.7.2.2), ornithine transcarbamoylase (EC 2.1.3.3), argininosuccinate synthetase (EC 6.3.4.5), argininosuccinase (EC 4.3.2.1) and arginase (EC 3.5.3.1), have very low activities in foetal rat liver and show a rapid postnatal increase (Kennan & Cohen, 1959; Rähä & Suihkonen, 1966). The overall capacity of liver slices to synthesize urea is almost absent from foetal rats and guinea pigs and increases rapidly to adult capacity after birth (Rähä & Kretchmer, 1965).

In the present investigation we studied the developmental pattern of all five enzymes involved in urea synthesis in the liver of rats. The rate-limiting reaction in the urea cycle in rat liver is that catalysed by argininosuccinate synthetase (Brown & Cohen, 1959). The development of this enzyme was found to correspond best with the development of the overall urea-producing capacity of liver slices, and thus factors influencing its postnatal development were studied in more detail.

The effects of premature and postmature delivery were investigated in the rat. Adrenalectomy and

puromycin injections at birth significantly inhibited the postnatal increase in enzyme activity and injections of triamcinolone diacetate had a marked stimulating effect.

MATERIAL AND METHODS

Animals. The animals used were rats from our own Sprague-Dawley strain. The ages of the fetuses were calculated from the mating date. Males were kept together with the females for only 24 hr. and thus the gestational ages could be calculated within a possible deviation of ± 12 hr.

Premature rats were obtained by section 24 hr. before normal delivery, on the twentyfirst day of gestation. Some pregnant females mated on the same day served as controls and delivered spontaneously about 24 hr. later on the twentysecond day. The premature rats were dried and kept warm and several of the litter were decapitated for assay of enzyme activity.

Gestation period was prolonged in some of the pregnant rats by daily injections of 5 mg. of progesterone (Orion, Helsinki, Finland). Pregnancy was interrupted on the twentythird and twentyfourth day of gestation by section. Premature and postmature animals were kept at 30° without feeding for 24 hr. They survived in good condition.

Foetuses delivered before the twentyfirst day did not survive and foetuses left *in utero* after the twentyfourth day died *in utero*.

To test whether progesterone would affect the liver arginine synthetase activity of the foetus, some pregnant rats received 5mg. of progesterone daily from the fifteenth to the twentyfirst day of gestation. The foetuses were delivered by section and enzyme was assayed from the liver. No increase in activity was observed.

Adrenalectomy and treatment with triamcinolone. Bilateral adrenalectomy was performed on newborn rats. The rats were anaesthetized by hypothermia on ice. The adrenals were removed by suction through a small incision in the dorsal muscle wall. The operation was started immediately after birth. The animals were rewarmed under an electric lamp and given back to the mother together with the sham-operated litter mates. The operated animals were kept with their mothers until assay.

Each adrenalectomized rat was given a daily intraperitoneal injection of 0.5mg. of triamcinolone diacetate (Lederco; Lederle Laboratories, Pearl River, N.Y., U.S.A.). Control animals received an equal volume of 0.9% NaCl. Other non-adrenalectomized newborn rats received 0.5mg. of triamcinolone/day intraperitoneally for 4 days; control animals received an equal volume of 0.9% NaCl. Enzyme assay of the liver was performed on the fourth day.

Experiments with puromycin. Puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was administered intraperitoneally to newborn rats at 3hr. intervals (0.25mg. of puromycin/dose) and the postnatal increase of arginine synthetase was studied at 12hr. Control animals received 0.9% NaCl.

Control experiments in which the effect of puromycin on the incorporation of [14 C]leucine (New England Nuclear Corp., Boston, Mass., U.S.A.) into liver protein was studied (Table 1) showed that the amount of puromycin used was only capable of totally inhibiting protein synthesis in the liver of the newborn rat for 1hr. after the injection.

Assay of urea-cycle enzymes. The enzymes involved in urea synthesis, carbamoyl phosphate synthetase, ornithine transcarbamoylase, argininosuccinase and arginase, were determined by the method of Brown & Cohen (1959) as modified by McLean & Gurney (1963). The activity of the arginine synthetase system (overall reaction described by Ratner, 1955) was also determined by a modification of the method described by Brown & Cohen (1959). The assay system included 1.25 μ moles of L-[ureido- 14 C]citrulline (New England Nuclear Corp.) (specific activity 1.8×10^4 counts/min./ μ mole), 1.25 μ moles of L-aspartate, 1.25 μ moles of ATP, 1.25 μ moles of MgSO_4 , 12.5 μ moles of potassium phosphate buffer, pH 7.8, and an excess of arginase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). The total volume was 300 μ l., and 50 μ l. of the tissue extract was used. The incubation was continued for 20 min. at 37° and stopped by boiling the tubes for 3 min. The reaction mixture was then transferred into sealed flasks, and urease was added to liberate CO_2 from the urea formed. The liberated CO_2 was trapped in Hyamine hydroxide and the radioactivity determined in a Packard Tri-Carb liquid-scintillation counter. Experiments with different concentrations of citrulline, ATP and L-aspartate with both foetal and adult liver preparations showed that the concentrations used in the assay gave optimum arginine

Table 1. *Effect of puromycin on incorporation of [14 C]leucine into liver protein in newborn rats*

Puromycin (0.25mg.) was administered intraperitoneally to newborn rats. Then 0.1 μ C of [14 C]leucine was given at the stated intervals after the puromycin injection and the incorporation of radioactivity into total liver protein was assayed after 5 min.

Time after puromycin injection (min.)	Incorporation (counts/min./mg. of liver protein)	Inhibition (%)
10	0	100
60	0	100
120	1.6×10^3	90
240	5.6×10^3	67
360	7.2×10^3	57
Control (no puromycin)	16.2×10^3	0

synthetase activity. The apparent K_m values for citrulline, as determined by the method of Lineweaver & Burk (1934), were 2.0×10^{-4} M for newborn rat liver and 2.8×10^{-4} M for adult rat liver.

The overall arginine synthetase system comprises a condensing enzyme, argininosuccinate synthetase, and a splitting enzyme, argininosuccinase. The argininosuccinase has a higher activity than the condensing enzyme in rat liver (Schimke, 1962; McLean & Gurney, 1963), and thus the assay system applied measures mainly the rate-limiting enzyme, argininosuccinate synthetase.

Enzyme units. The enzyme activities are all expressed in terms of units defined as the amount of enzyme catalysing the formation of 1 μ mole of product/hr. under the assay conditions used. The activities are given as units/g. wet wt. of liver. The protein content of the liver homogenates was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), but since the expression of enzyme activities/mg. of protein did not significantly alter the relative changes the activities are only presented per g. wet wt. of liver.

RESULTS

The developmental changes in activity of the enzymes involved in urea synthesis in the rat liver are shown in Table 2. In 21-day-old foetal liver, only ornithine transcarbamoylase and arginase had activities exceeding 20% of adult activity. At birth, the arginine synthetase exhibits the lowest relative activity of all enzymes, being less than 10% of adult values. The postnatal increase is most rapid with arginine synthetase, the activity of which increases fivefold within 24hr. after birth. A second marked increase of the arginine synthetase activity was observed after the tenth day of age. Carbamoyl phosphate synthetase activity increases threefold during the first day, and the other three enzymes showed a twofold or less increase of activity.

The addition of foetal liver homogenates, foetal serum or placental homogenates to adult liver

Table 2. *Activities of urea-cycle enzymes in livers of rats during development*

All results are given as means $\pm$ s.d. with the numbers of animals studied in parentheses. In all studies on foetal animals, three livers were pooled for each determination. For further details see the text.

Age	Activity (units/g. wet wt. of liver)				
	Carbamoyl phosphate synthetase	Ornithine trans-carbamoylase	Arginine synthetase system	Argininosuccinase	Arginase
Foetal					
-3 days	30 (6)	2140 (6)	2.0 (6)	18 (6)	4940 $\pm$ 800 (18)
-2 days			5.0 $\pm$ 1.0 (9)		6000 (6)
-1 day	130 (6)	4750 (6)	6.9 $\pm$ 1.6 (12)	28 (6)	14700 (6)
Birth	131 $\pm$ 12 (15)	6800 $\pm$ 812 (15)	7.3 $\pm$ 1.4 (21)	64 $\pm$ 21 (15)	17000 $\pm$ 3400 (21)
6hr.			13.6 $\pm$ 5.1 (12)		
12hr.			19.3 $\pm$ 6.1		
1 day	300 $\pm$ 53 (15)	7340 $\pm$ 1720 (18)	35.2 $\pm$ 10.5 (18)	137 $\pm$ 22 (24)	26000 $\pm$ 5200 (21)
2 days	334 $\pm$ 100 (9)	9680 $\pm$ 960 (9)	31.6 $\pm$ 4.8 (15)	168 $\pm$ 24 (14)	37000 $\pm$ 11000 (21)
4 days	325 $\pm$ 52 (18)	9580 $\pm$ 2720 (9)	46.5 $\pm$ 10.2 (18)	291 $\pm$ 67 (12)	41500 $\pm$ 2580 (12)
10 days	582 $\pm$ 112 (13)	14700 $\pm$ 3720 (9)	47.1 $\pm$ 4.1 (15)	467 $\pm$ 45 (9)	65000 $\pm$ 11400 (12)
20 days	750 $\pm$ 100 (6)	16400 $\pm$ 3330 (5)	64.2 $\pm$ 22.9 (5)	330 $\pm$ 49 (7)	75200 $\pm$ 2680 (12)
Adult	782 $\pm$ 160 (11)	17400 $\pm$ 1560 (6)	102.0 $\pm$ 23.4 (18)	446 $\pm$ 37 (6)	81400 $\pm$ 3600 (9)

Table 3. *Effect of 24hr. starvation from birth on the postnatal increase of urea-synthesizing enzymes in the rat*

The starved newborn animals were kept at 30° for 24hr.; the control animals were kept with their mother. Seven animals were studied in each group. The results are given as means $\pm$ s.d.

	Weight (g.)	Activity (units/g. wet wt. of liver)				
		Carbamoyl phosphate synthetase	Ornithine trans-carbamoylase	Arginine synthetase system	Argininosuccinase	Arginase
At birth	4.0 $\pm$ 0.5	133 $\pm$ 6	4460 $\pm$ 1500	6.6 $\pm$ 0.5	49 $\pm$ 8	9600 $\pm$ 2110
Control at 24hr.	4.5 $\pm$ 1.1	302 $\pm$ 50	5690 $\pm$ 1140	32.4 $\pm$ 13.1	107 $\pm$ 19	23850 $\pm$ 2720
Starvation at 24hr.	3.8 $\pm$ 0.4	309 $\pm$ 54	6150 $\pm$ 1430	25.9 $\pm$ 3.3	120 $\pm$ 32	23400 $\pm$ 5280

homogenates had no effect on any of the enzyme activities of the latter. The addition of adult liver homogenates was also without effect on the foetal enzyme activities.

Starvation of newborn rats for 24hr. had very little effect on the postnatal increase of any of the enzymes studied, as shown in Table 3.

Administration of puromycin to newborn rats at birth had a marked inhibitory effect on the postnatal increase of the arginine synthetase activity. When newborn rats received 0.25mg. of puromycin intraperitoneally every 3hr. for 12hr. after birth, the arginine synthetase activity was 10.3 ± 3.0 units/g. of liver. Control animals had an enzyme activity of 20.2 ± 4.9 units/g. of liver. Ten animals were used in each group. The inhibition was only 77%, but this is consistent with the results given in Table 1, which show that puromycin inhibited protein synthesis totally in the liver when given

every hour, but when given every 3hr., as in this experiment, only a 70–80% inhibition could be expected.

The role of the adrenal cortex was studied by removal of the adrenals immediately after birth (Table 4). Adrenalectomy caused an almost complete inhibition of the postnatal increase of liver arginine synthetase at 24hr. after birth. At 48hr. after birth the inhibition was 72%. The administration of triamcinolone to adrenalectomized newborn animals was quite effective in preventing the decrease in enzyme activity caused by the removal of the adrenal glands (Table 4).

Treatment of unoperated newborn animals with 0.5mg. of triamcinolone/day resulted in a marked stimulation of the postnatal increase of arginine synthetase activity when assayed on the fourth postnatal day. The enzyme activity was 156.0 ± 24.9 units/g. of liver in the treated animals and

Table 4. Activity of the arginine synthetase system in liver of adrenalectomized newborn rats as compared with sham-operated animals and adrenalectomized animals who received daily injections of triamcinolone

The results are given as means $\pm$ S.D. with the numbers of animals studied in parentheses. For further details see the text.

	Arginine synthetase activity (units/g. wet wt. of liver)	
At birth	7.3 $\pm$ 1.4	(21)
1 day, sham operation	17.4 $\pm$ 4.0	(4)
1 day, adrenalectomy	7.9 $\pm$ 1.3	(5)
2 days, sham operation	31.6 $\pm$ 6.3	(5)
2 days, adrenalectomy	14.3 $\pm$ 4.3	(5)
2 days, adrenalectomy + triamcinolone	30.0	(2)

Fig. 1. Development of the activity of the arginine synthetase system in normal rats (●), in rats delivered prematurely (○) and in rats delivered 1 day postmaturely (Δ) and 2 days postmaturely (□). Vertical lines represent standard deviations. For further details see the text.

64.6 $\pm$ 16.1 units/g. of liver in the control animals. Six animals were used in each experimental group.

The effect of premature and delayed delivery on the arginine synthetase activity and on the postparturition change of activity is shown in Fig. 1. Animals delivered 24 hr. before normal term did not exhibit the usual postnatal increase of enzyme activity, and activity 24 hr. after birth was equal to that of control animals at the moment of normal birth. When pregnancy was prolonged for 24 hr. and 48 hr. the enzyme activity at the moment of birth was similar to that of control animals exhibiting the postnatal activity increase (Fig. 1).

Injections of progesterone to pregnant rats

between the fifteenth and the twentyfirst day of gestation did not affect the arginine synthetase activity found at birth.

DISCUSSION

The changes in the activity of arginine synthetase during development are closely parallel to the capacity of liver slices to form urea on incubation with optimum substrate concentrations (Räihä & Kretchmer, 1965). Thus it seems evident that the rapid increase in activity of arginine synthetase initiates the ability to produce urea in the rat after birth. This finding is consistent with the arginino-succinate synthetase being rate-limiting in the formation of urea and shows how the function of a multi-enzyme pathway can be controlled by the activity of the rate-limiting enzyme.

According to Illnerová (1967) the excretion of urea starts to increase in the rat after the fourteenth day. This time corresponds to the second increase of arginine synthetase activity. A similar developmental curve to that of arginine synthetase with two distinct periods of activity increase is seen for the enzyme ornithine-keto acid aminotransferase (EC 2.6.1.13) in the rat (N. C. R. Räihä & M. Kekomäki, unpublished work). It can be postulated that the immediate postnatal increase of these enzymes concerned with nitrogen catabolism represents a 'primary' increase brought about by some as yet unknown factor and the later increase an adaptive change in enzyme activity due to a shift in the balance between the intake of nitrogenous compounds and their utilization for growth. Such a shift could occur at about 14 days after birth, when the nutrition of the rat changes from the species-specific maternal milk protein to the laboratory diet.

The postnatal increase of arginine synthetase could be blocked by puromycin, suggesting that the rise in enzyme activity after birth is due to the formation of new enzyme protein. The dose used in our experiments blocked the increase of enzyme activity by 77%. From the results of the experiments on the incorporation of [14 C]leucine into liver protein in the newborn rat it is, however, obvious that the dose of puromycin used should be given every hour in order to exert a total block on protein synthesis. However, when puromycin was injected every hour the newborn animals did not survive for more than a few hours.

Nemeth (1959) showed that the postnatal increase of tryptophan pyrrolase activity in the rabbit is initiated by the birth process, and he suggested that some factor in the uterine environment represses enzyme formation. In the rat, however, tryptophan pyrrolase activity did not increase until about 15 days after birth (Nemeth, 1959). Arginine

synthetase activity was not initiated in our experiments with the rat by premature delivery on the twentyfirst day of gestation, and control experiments showed that starvation of newborn rats for 24 hr. after term birth did not prevent the postnatal rise of enzyme activity. On the other hand, when pregnancy was prolonged beyond normal term by progesterone, the arginine synthetase activity increased in the postmature foetal liver almost as rapidly as after normal delivery. Progesterone failed to increase enzyme activity in foetal animals when given before term. It is difficult to speculate on the meaning of these results, but they suggest that the postnatal increase of enzyme activities in rat liver in some cases is not necessarily regulated by the process of birth, but rather by some time-dependent phenomena.

In adult animals adrenal steroid hormones can increase significantly the activity of many enzymes such as tryptophan pyrrolase, tyrosine transaminase and enzymes involved in gluconeogenesis (Kenney, Wicks & Greenman, 1966; Weber, Srivastava & Singhal, 1965). McLean & Gurney (1963) reported an increase in activity of some urea-cycle enzymes in adult rats produced by cortisone acetate and a marked decrease in activity of the arginine synthetase system caused by adrenal-ectomy. Increased synthesis of hepatic enzymes due to steroids is preceded by an increase in the rate of synthesis of nuclear RNA (Kenney *et al.* 1966). It is interesting to note the difference in adrenal steroid effect on some of these enzymes during development as compared with that found in adult animals. Steroid hormone administration does not produce any elevation in activity of tryptophan pyrrolase (Nemeth, 1959) or tyrosine trans-

aminase (Sereni, Kenney & Kretchmer, 1959) before the natural post-parturition increase has occurred. We have found similar results with ornithine-keto acid aminotransferase.

The results in the present study, however, indicate that intact adrenal glands are necessary for a normal postnatal increase of arginine synthetase activity.

This study was supported by the Association for the Aid of Crippled Children, New York, and Signe och Ane Gyllenbergs Stiftelse, Helsingfors. The very capable assistance of Miss Edith Sommer is gratefully acknowledged.

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