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Studies on Cystathionase Activity in Rat Liver and Brain during Development

EFFECTS OF HORMONES AND AMINO ACIDS *IN VIVO*

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High activity of cystathionase was present in rat liver but only low amounts of activity in rat brain during development. Triamcinolone had no effect on liver cystathionase activity in foetuses but increased the enzyme activity significantly in postnatal rats. L-Thyroxine decreased liver cystathionase activity significantly in newborn rats; administration of pyridoxal 5′-phosphate did not prevent this effect. L-Methionine significantly increased liver cystathionase activity in newborn rats.

For the biosynthesis of cysteine the sulphur of methionine is transferred to the carbon skeleton of serine through the trans-sulphuration pathway. The last step in this pathway, cleavage of L-cystathionine into cysteine and homoserine, is catalysed by the vitamin B_6-dependent enzyme cystathionase [L-homoserine hydro-lyase (deaminating), EC 4.2.1.15].

Liver of adult rats, as well as of other mammals, has high activities of all trans-sulphuration enzymes, including cystathionase (Sturman *et al.*, 1970*a*). Injection of L-methionine into adult rats has been found to increase liver cystathionase activity (Chatagner & Trautmann, 1962). Addition of DL-methionine or casein to the diet increases liver cystathionase activity in adult rats (Chatagner & Trautmann, 1963; Finkelstein, 1967). Injection of cortisol into adult rats is without significant effect on liver cystathionase activity (Finkelstein, 1967). Thyroidectomy has been found to increase liver cystathionase activity in adult rats (Chatagner *et al.*, 1967), whereas injection of L-thyroxine into adult rats decreases liver cystathionase activity (Finkelstein, 1967; Chatagner *et al.*, 1962).

Studies on trans-sulphuration in developing mammalian brain have shown that the concentration of L-cystathionine increases markedly during the period of brain myelination (Volpe & Laster, 1970). This development, and the high concentration of free L-cystathionine in the white matter of brain, suggests that this amino acid has some relation to brain myelination. In rat brain the activity of cystathionine synthase increases together with the increasing concentration of L-cystathionine within the first 2 weeks of postnatal life (Volpe & Laster, 1972), but the concomitant development of cystathionase activity during this critical period of brain myelination has not been previously examined.

This communication reports the developmental study of cystathionase activity in rat liver and brain from foetal life to maturity. The response of cystathionase activity in perinatal rat liver to administration *in vivo* of one pharmacological dose of triamcinolone, glucagon, insulin, oestradiol or L-thyroxine was examined. The effect of one intraperitoneally injected dose of L-methionine and L-cystathionine on liver cystathionase activity in newborn rats was studied.

Materials and Methods

Materials

L-Cystathionine and dithiothreitol were obtained from Calbiochem (Los Angeles, Calif., U.S.A.); pyridoxal 5′-phosphate was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Hormones were also obtained commercially: cortisol sodium succinate (Solu-Cortef) from Upjohn Co. (Kalamazoo, Mich., U.S.A.); triamcinolone acetonide (Kenacort) from Squibb (Stockholm, Sweden); L-thyroxine from Sigma Chemical Co.; glucagon and short-acting insulin from Novo Industri (Copenhagen, Denmark); and oestradiol (Estrogen forte) from Orion (Helsinki, Finland).

Animals and experimental procedure

Rats of Wistar albino strain were used, the term 'adult' referring to 200–300g animals. The gestational age of foetuses was calculated from the mating date, known to have occurred within 16h.

In all experiments amino acids and hormones were injected intraperitoneally. For injections L-cystathionine and L-thyroxine were dissolved in 0.9% NaCl made alkaline with NaOH (final pH of the solution 8.5 and 8.2 respectively). The other agents were dissolved in 0.9% NaCl. Control animals received an equal volume of saline only.

In prenatal experiments injections of foetuses *in utero* were performed with the technique described by Yeung *et al.* (1967). Results of Yeung *et al.* (1967) and of Volpe & Laster (1972) indicate that the transfer of hormones from experimental to control animals via maternal–foetal circulation may occur, if a foetal experiment is performed on a single litter. Therefore, in this study foetal experiments were performed on pairs of litters: all foetuses of the experimental litter received injections of triamcinolone whereas foetuses of the control litter received injections of saline. Each postnatal experiment was performed on one litter with half of the littermates as an experimental group and the remaining animals as controls.

The dose of L-methionine injected corresponded on a weight basis to 3.3mmol/kg, used in adult rats by Lin & Knox (1958) and Chatagner & Trautmann (1962). Because of the poor solubility of L-cystathionine the dose used corresponded to only 2.2mmol/kg. Hormones were injected in the following doses per animal: cortisol 0.5–1.0mg; triamcinolone, 0.150–0.350mg; L-thyroxine, 2μg; glucagon, 0.1mg; insulin, 4 i.u.; oestradiol, 0.1mg. The time between the injection and the killing of animals was 4h in all experiments with amino acids (Lin & Knox, 1958) and 6–26h in experiments with hormones.

Bilateral adrenalectomies were performed immediately after birth as described by Räihä & Suihkonen (1968); control animals underwent a sham-operation.

All animals were killed by decapitation and liver and brain were excised without delay. If the fresh tissues were not analysed immediately for cystathionase activity, they were quick-frozen in liquid N_2 and stored at −70°C until examined (Gaull *et al.*, 1969).

Assay of cystathionase activity

Liver and brain tissue were homogenized in ice-cold 30mM-potassium phosphate buffer, pH6.9. Homogenates were centrifuged at 28000*g* for 30min and the supernatant fluid was assayed (Mudd *et al.*, 1965; Gaull *et al.*, 1969). The activity of cystathionase was assayed by the method of Gaull *et al.* (1969). The reaction mixture contained the following, in μmol, in a total volume of 0.5ml: Tris–HCl buffer, pH8.4, 50; pyridoxal 5′-phosphate, 0.125; L-cystathionine, 2; tissue extract in 0.1ml of 30mM-potassium phosphate buffer, pH6.9. The incubation time for rat liver enzyme was 30min and for rat brain enzyme 120min: under the conditions of standard assay the rate of formation of cysteine was linear with time. The reaction was stopped by cooling the tubes in an ice bath. Dithiothreitol (5μmol)/tube was added to bring all cysteine to the reduced form (Cleland, 1964), and the amount of cysteine was determined by the spectrophotometric method of Gaitonde (1967). The soluble protein concentration of tissue extracts was determined by the method of Lowry *et al.* (1951). For rat liver, the amount of cysteine formed under the conditions of the standard assay was found to be linear with concentration of soluble protein up to 0.6mg/tube; for rat brain linearity was found up to the soluble protein concentration of 2.0mg/tube.

Results

Liver and brain cystathionase activity during development of the rat is presented in Fig. 1. Already at the 16th day of gestation low cystathionase activity was found in foetal rat liver and during subsequent days of gestation a gradual increase in the enzyme activity was observed. During the last days before term a rapid 3-fold increase in liver cystathionase activity occurred. Birth was accompanied by a transient elevation in liver cystathionase activity; on the second postnatal day there was a diminution to values similar to those observed at term. During the first 6 postnatal weeks liver cystathionase activity remained at term values; until this age there was no significant difference in liver cystathionase activity among young female and male rats. The same values of liver cystathionase activity were observed in adult female rats, whereas in adult male rats significantly lower activities of this enzyme were found ($P<0.001$). Liver cystathionase activity in pregnant rats at the end of gestation (730 ± 80nmol of cysteine formed/60min per mg of soluble protein; mean ± S.D.) was lower than that in non-pregnant female rats of the same age (1200 ± 85nmol of cysteine formed/60min per mg of soluble protein); this difference was significant ($P<0.001$).

Because glucocorticoids are known to influence the development of other trans-sulphuration enzymes (Chase *et al.*, 1968) the effects of triamcinolone on cystathionase activity in perinatal rat liver was studied. At the 19th day of gestation, i.e. before the pre-term increase in foetal liver cystathionase activity occurred, four litters were injected with triamcinolone *in utero* whereas four control litters received injections of saline only. After exposure to this hormone for 6, 12, 18 and 24h there was no increase in enzyme activity apparent ($P>0.05$). Postnatally, one pharmacological dose of triamcinolone significantly increased liver cystathionase activity in newborn and 3-day-old animals (Table 1). Variation of the dose of triamcinolone from 0.150 to 0.350mg did not change the stimulatory effect of this hormone on liver cystathionase activity. The stimulatory effect was also independent of the time-interval between drug administration and liver excision within the range 18–26h. Cortisol administered in a single pharmacological dose of 0.5 or 1.0 mg/animal into postnatal rats increased liver

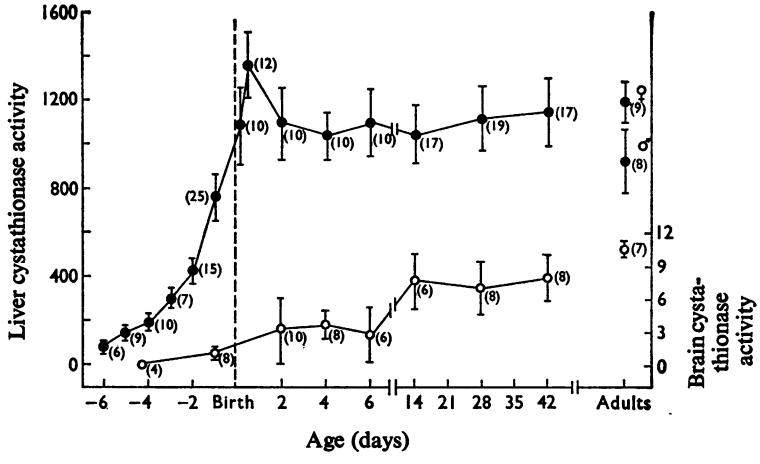

Fig. 1. *Cystathionase activity in rat liver and brain during development*
Each point represents the mean value for the number of animals indicated in parentheses. Vertical lines indicate S.D. Cystathionase activity is expressed as nmol of cysteine formed/60min per mg of soluble protein. ●, Liver cystathionase activity; ○, brain cystathionase activity.

Table 1. *Liver cystathionase activity in newborn rats after intraperitoneal injection of one dose of* L-*methionine,* L-*cystathione,* L-*thyroxine,* L-*thyroxine plus pyridoxal 5′-phosphate and triamcinolone*

Each experiment was performed on a single litter with half of the animals as an experimental group and the remaining litter-mates as controls. The following doses per animal were used: L-methionine, 17μmol and L-cystathionine, 11μmol (corresponding on a weight basis to 3.3mmol/kg and 2.2mmol/kg respectively); L-thyroxine, 2μg; pyridoxal 5′-phosphate, 2mg (divided into four doses and injected within a time-period of 24h); triamcinolone, 0.150–0.350mg. Results are expressed as means ± S.D. with numbers of animals examined in parentheses. Each determination represents one animal. *P* values were calculated by Student's *t* test; n.s., not significant ($P>0.05$).

Age (days)	Agent	Time after injection (h)	No. of litters examined	Activity of liver cystathionase (nmol of cysteine/60min per mg of soluble protein)		*P*
				Control	Experimental	
+1	L-Methionine	4	3	1050 ± 90 (15)	1530 ± 100 (18)	<0.001
+1	L-Cystathionine	4	2	1040+70 (14)	1160 ± 200 (14)	n.s.
+1	L-Thyroxine	24	3	1100 ± 80 (15)	790 ± 120 (15)	<0.001
+1	L-Thyroxine + pyridoxal 5′-phosphate / L-Thyroxine (controls)	24	3	700 ± 110 (14)	815 ± 105 (10)	n.s.
+1	Triamcinolone					
	0.150mg	24	1	1060 ± 70 (4)	1580 ± 73 (5)	<0.001
	0.250mg	24	1	1090 ± 50 (4)	1520 ± 200 (4)	<0.01
	0.350mg	24	3	1020 ± 73 (4)	1480 ± 140 (4)	<0.01
+3	Triamcinolone					
	0.350mg	24	1	1060 ± 55 (5)	1500 ± 170 (4)	<0.01

cystathionase activity, but this effect was not significant ($P>0.05$). The effects of bilateral adrenalectomy on liver cystathionase activity of newborn rats was studied to elucidate whether adrenal steroid secretion was necessary for the maintenance of normal postnatal concentrations of cystathionase. Half of the newborn rats of four litters were adrenalectomized at birth and the remaining litter-mates were

sham-operated. No difference in liver cystathionase activity could be observed between adrenalectomized and sham-operated animals at the ages of 2 and 4 days.

The effect of one pharmacological dose of L-thyroxine on liver cystathionase activity in newborn rats was examined. At 24h after exposure to this hormone a significant diminution in liver cystathionase activity was observed (Table 1). The effect of pyridoxal 5'-phosphate administration on liver cystathionase activity of thyroxine-treated newborn rats was examined. Two litters received injections of L-thyroxine at birth: half of the animals of each litter received massive doses of pyridoxal 5'-phosphate (2mg/animal divided into four doses and injected intraperitoneally within the time-interval of 24h). As indicated in Table 1, administration of pyridoxal 5'-phosphate did not prevent the decrease in liver cystathionase activity which followed the exposure to L-thyroxine. A single pharmacological dose of insulin, glucagon or oestradiol had no effect on liver cystathionase activity *in utero* or in postnatal animals.

Effects of a single intraperitoneal injection of L-cystathionine or L-methionine on liver cystathionase activity in newborn rats was studied. An injection of L-cystathionine had no effect on liver cystathionase activity whereas an injection of L-methionine significantly increased the activity of this enzyme (Table 1).

As a contrast with the high cystathionase activity observed in rat liver from late foetal life to maturity, rat brain cystathionase activity remained low throughout all stages of development (Fig. 1). At the end of the gestation brains of foetal rats had no detectable cystathionase activity. During the first weeks of postnatal life a slow increase in the activity of brain cystathionase occurred, and after the age of 6 weeks the low adult values were reached. No difference could be observed in cystathionase activity of cerebrum and cerebellum in adult rats.

Discussion

Present observations of rat liver cystathionase activity indicate that high concentrations of this enzyme are present in foetal liver at the end of gestation. At the same developmental stage foetal rat liver also has considerable activities of other trans-sulphuration enzymes (Sturman *et al.*, 1970*a*). Thus the biosynthesis of cysteine through the trans-sulphuration is possible in foetal rat liver, in contrast with the human foetus, which lacks liver cystathionase activity (Sturman *et al.*, 1970*b*).

The developmental pattern of rat liver cystathionase activity is characterized by a sharp pre-term elevation to approximately adult values. A similar pre-term increase was also observed in rat liver cystathionine synthase activity by Volpe & Laster (1972) whereas another trans-sulphuration enzyme in rat liver, methionine-activating enzyme, was found to increase to adult activities within the first 2 days after birth (Volpe & Laster, 1969). As an explanation of the pre-term increase in rat liver cystathionase activity effects of intrauterine inhibitors were considered. Foetal rat liver is exposed to high amounts of conjugated oestrogen (Levitz *et al.*, 1961); just before term a diminution in their concentration occurs. Oestrogens administered *in utero* are known to repress the activity of other trans-sulphuration enzyme, methionine-activating enzyme (Chase *et al.*, 1968). Conjugated oestrogens *in vitro* inhibit activity of pyridoxal 5'-phosphate-dependent enzyme kynurenine transaminase (Mason & Gullekson, 1960). Thus the fall in the amounts of conjugated oestrogens before term could explain the pre-term increase in foetal liver cystathionase activity. However, a single dose of oestradiol had no effect on rat liver cystathionase activity *in utero* or after birth.

L-Thyroxine has been found to increase the activity of rat liver NADPH dehydrogenase, which has a similar pattern of development to rat liver cystathionase (Greengard & Dewey, 1968). Present results show that administration of L-thyroxine to newborn rats was accompanied by a marked diminution in liver cystathionase activity. Decreased amounts of pyridoxal 5'-phosphate in tissues of thyroxine-treated rats have been observed by Mascitelli-Coriandoli & Boldrini (1959). However, massive doses of pyridoxal 5'-phosphate were incapable of reversing the repressing effects of L-thyroxine on cystathionase activity in perinatal rat liver. Thus simple depletion of cofactor is not sufficient to explain the observed decrease in liver cystathionase activity after L-thyroxine administration.

Glucocorticoids administered *in utero* before the pre-term elevation of liver cystathionase activity had no effect on the development of this enzyme. Postnatally a single injection of glucocorticoid (triamcinolone) increased liver cystathionase activity significantly in newborn and 3-day-old animals. The lack of response of the enzyme in foetal animals suggests that the developmental increase before term is not initiated by glucocorticoids and that these hormones are capable of influencing the regulatory mechanisms only when the competence to synthesize the enzyme has matured. Maintenance of normal postnatal amounts of liver cystathionase after bilateral adrenalectomy performed at birth further suggests that adrenal steroids had no major effect on the development of liver cystathionase activity. As reported by Chase *et al.* (1968) and Volpe & Laster (1972), glucocorticoids administered *in utero* were also incapable of inducing prematurely the normal developmental increase in the activity of other trans-sulphuration enzymes, methionine-

activating enzyme and cystathionine synthase, in rat liver.

A single injection of L-methionine into newborn rats resulted in a significant increase in liver cystathionase activity. This adaptive response of liver cystathionase to increasing substrate concentrations is in agreement with the observations of Chatagner & Trautmann (1962, 1963). Administration of L-cystathionine into newborn rats could be expected to increase cystathionase activity, but no effect was found. Similarly results of Frimpter *et al.* (1969) show that intraperitoneal administration of L-cystathionine into adult rats (in doses which on a weight basis equalled those used in this study) had no effect on liver cystathionase activity. It is possible that the high renal excretion rate of L-cystathione (Frimpter & Greenberg, 1967) militates against reaching an effective intracellular substrate concentration necessary for enzyme adaptation.

Volpe & Laster (1972) observed an increase in cystathionine synthase activity and L-cystathionine concentration concomitantly with the progression of myelination in developing rat brain. The present results show that only minimal activity of cystathionase was present in rat brain during this critical period of development. This finding further explains the rapid accumulation of free L-cystathionine in developing rat brain and supports the concept that this amino acid has an important but still undefined role in brain myelination.

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