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Development of methionine synthase, cystathionine-β-synthase and S-adenosyl-homocysteine hydrolase during gestation in rats

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The developmental onset of three homocysteine metabolizing enzymes in the rat conceptus was investigated. Cystathionine-β-synthase and methionine synthase were assayed from day 10 to day 20 of gestation in decidual and placental tissue, from day 10 to day 12 of gestation in embryonic tissue, from day 14 to day 20 of gestation in fetal liver and from day 14 to day 20 of gestation in fetal tissue without liver. On each day, material was obtained from at least four conceptuses from two dams. S-adenosylhomocysteine hydrolase was assayed in neurulating conceptuses in decidual tissue, parietal yolksac plus ectoplacental cone, visceral yolksac plus amnion and embryo proper. Conceptuses were pooled from seven (day 9.5 of gestation) or three (days 10.5 and 11.5 of gestation) dams. In embryonic and fetal tissue cystathionine-β-synthase first occurred in fetal liver. During the organogenic phase it was present only in decidual tissue. Methionine synthase was present in all tissues from the first gestational day investigated and S-adenosylhomocysteine hydrolase was present in all tissues throughout the neurulating period. Our results indicate that the homocysteine–methionine cycle, which is crucial to transmethylation reactions, is functional during the neurulating period in embryonic tissue. Owing to the absence of cystathionine-β-synthase at this stage of development in embryonic tissue, the homocysteinyl moiety is conserved in the homocysteine–methionine cycle.

Introduction

A disturbance of methionine–homocysteine metabolism may play a role in the aetiology of neural tube defects, and also in other obstetrical problems, such as recurrent spontaneous abortion and abruptio placentae (Steegers-Theunissen *et al.*, 1991, 1992; Schorah *et al.*, 1993; Steegers-Theunissen, 1993; Wouters *et al.*, 1993).

L-Homocysteine can be metabolized by several enzymes (Fig. 1). Methionine synthase (N^5-methyltetrahydrofolate-L-glutamate:L-homocysteine S-methyltransferase, EC 2.1.1.13) remethylates L-homocysteine to L-methionine. In this way the homocysteinyl moiety is conserved in methionine–homocysteine metabolism. At the same time methionine synthase is the only enzyme in mammals that can demethylate N^5-methyltetrahydrofolate. Since N^5-methyltetrahydrofolate is the major circulating form of folate, methionine synthase is the key enzyme to their active reduced folate supply, which is essential in one-carbon metabolism, and purine and pyrimidine synthesis. Therefore, methionine synthase is considered to be ubiquitous in mammals (Shane and Stokstad, 1983). During gestational development methionine synthase activity seems to progressively decline in several species (Gaull *et al.*, 1973; Sternowsky *et al.*, 1976). However, a developmental study, including the organogenic phase, has never been published.

Betaine:L-homocysteine S-methyltransferase (EC 2.1.2.5) can also remethylate L-homocysteine to L-methionine. Betaine:L-homocysteine S-methyltransferase activity is found in liver tissue of all mammals investigated and also occurs in kidney tissue (Ericson, 1960; Finkelstein *et al.*, 1971). In humans betaine:L-homocysteine S-methyltransferase activity is also present in both fetal and adult brain tissue, although activity is low (Gaull *et al.*, 1973). Since betaine:L-homocysteine S-methyltransferase is a typical liver enzyme, and the liver is not present at the time of neurulation, the period under investigation, no attempt was made in this study to measure its activity in rat embryonic and fetal tissue.

Cystathionine-β-synthase (EC 4.2.1.22) condenses L-serine with L-homocysteine to L-cystathionine. This is an irreversible step in the transsulfuration pathway, the main pathway for methionine and homocysteine catabolism. Generally cystathionine-β-synthase activity increases during fetal development; however, the developmental onset is unknown, and may differ between species (Volpe and Laster, 1970, 1972; Gaull *et al.*, 1972; Sturman *et al.*, 1976; Rassin *et al.*, 1981).

S-adenosylhomocysteine hydrolase (EC 3.3.1.1) is the only source of L-homocysteine in mammals as it hydrolyses S-adenosylhomocysteine to L-homocysteine and adenosine.

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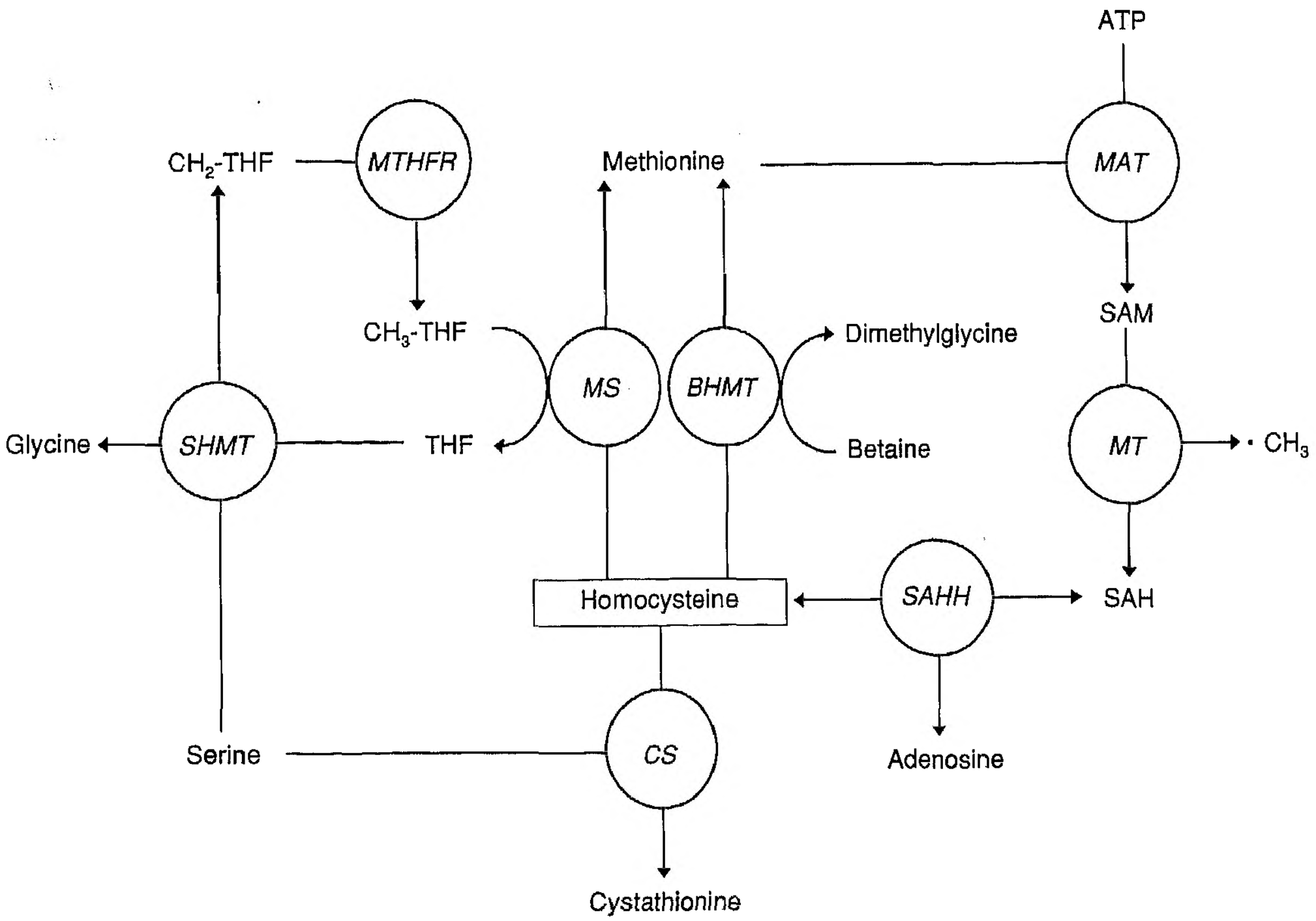

Fig. 1. Homocysteine and methionine metabolism and its relation to folate metabolism. BHMT: betaine-homocysteine methyltransferase; CS: cystathionine-β-synthase; MAT: methionine-adenosyl transferase; MS: methionine synthase; MT: various methyltransferases; MTHFR: methylenetetrahydrofolate reductase; SAHH: S-adenosyl-homocysteine hydrolase; SHMT: serinehydroxymethyltransferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine: THF: tetrahydrofolate; CH_2-THF: N^5,N^{10}-methylenetetrahydrofolate; CH_3-THF: N^5-methyltetrahydrofolate.

However, when L-homocysteine levels are high, S-adenosylhomocysteine hydrolase will synthesize S-adenosylhomocysteine, as the reaction is reversible and S-adenosylhomocysteine synthesis is energetically more favourable (Ueland, 1982). Since S-adenosylhomocysteine accumulation will inhibit crucial transmethylation reactions, in mammals, S-adenosylhomocysteine hydrolase is assumed to be ubiquitous as well. However, developmental studies have not been published.

The objective of this study was to investigate the gestational development of methionine synthase, cystathionine-β-synthase and S-adenosylhomocysteine hydrolase in the rat conceptus to provide a better understanding of the possible relationship between methionine–homocysteine metabolism and the aetiology of neural tube defects.

Materials and Methods

Chemicals

Bovine serum albumin (BSA), N^5-methyltetrahydrofolate, complete *o*-phthaldialdehyde reagent solution, Coomassie Brilliant Blue G, dithiothreitol, L-homocysteinethiolactone, L-serine, L-cystathione, S-adenosylhomocysteine, S-adenosylmethionine, lubrol PX and pyridoxal phosphate were purchased from Sigma (St Louis, MO). Ascorbic acid and 2-mercaptoethanol were purchased from Merck (Darmstadt). T61 containing 20% (w/v) embutramide, 5% (w/v) mebezomiumiodide and 0.5% (w/v) tetracaine hydrochloride in water) was obtained from Hoechst Veterinär GmbH (Munich); hydroxycobalamin (vitamin B_{12b}) from Fluka Chemie (Buchs); [U-^{14}C]-L-serine (specific activity 166 mCi mmol^{-1}) from DuPont de Nemours (Dreiech); erythro-9[2-hydroxy-3-nonyl]adenine from Burroughs Wellcome (Research Triangle Park, NC); adenosine from USB (Cleveland, OH) and Triton X-100 from Serva Feinbiochemica (Heidelberg). All solutions were made in tap water, demineralized by a Milli-RO 10TS system from Millipore Corporation (Bedford, MA), except for the HPLC buffers, which were prepared with demineralized water additionally filtered by a Nanopure System from Sybron/Barnstaedt (De Bugue, IA). L-Homocysteine was prepared from L-homocysteinethiolactone by alkaline hydrolysis (5 mol NaOH l^{-1}, 5 min, 37°C). The solution was neutralized with HCl and diluted with the appropriate assay buffer.

Animals and dissection of tissues

Random bred Cpb:WU (Wistar) rats were housed in pairs or groups of three in cages and allowed to eat (MRH-TM pellets, Hope Farms B.V., Woerden) and drink (tap water) *ad libitum*. For animals from which material was obtained on days 10, 11, 12, 14, 16, 18 or 20 of gestation, lights were on from 12:00 h

until 00:00 h. On day 0, females (11–26 weeks old, 200–300 g) were brought together with males (1:1) from 09:00 h to 12:00 h. Animals from which material was obtained on days 9.5, 10.5 or 11.5 of gestation, were housed under the same conditions, except for the light regimen and the time of mating, which were shifted by 12 h.

When the conceptuses had reached the gestational age desired, the dams were killed by an intracardial injection of 0.4 ml T61. The uterus was removed and the conceptuses were explanted and placed in Hank's balanced salt solution. Conceptuses at day 10, 11 or 12 of gestation were dissected into (i) embryo proper and (ii) decidua. Extraembryonic membranes (amnion, visceral and parietal yolk sac and ectoplacental cone) were discarded. Conceptuses at day 14, 16, 18 and 20 of gestation were dissected into (i) fetal liver, (ii) rest of fetus and (iii) placenta plus extraembryonic membranes plus residual decidual tissue. Conceptuses at day 9.5 of gestation were dissected into (i) embryo proper plus amnion plus visceral yolksac, (ii) parietal yolksac plus ectoplacental cone and (iii) decidua. Conceptuses at day 10.5 or 11.5 of gestation were dissected into (i) embryo proper, (ii) amnion plus visceral yolksac, (iii) parietal yolksac plus ectoplacental cone and (iv) decidua. For reference, maternal liver (day 10 of gestation) was used. After dissection, tissues were immediately frozen in liquid nitrogen and stored at −80°C until further treatment.

Enzyme assays

Protein concentrations were assayed using Coomassie Brilliant Blue G according to Bradford (1965). BSA was used as a standard. All samples were analysed in triplicate.

The methionine synthase and the cystathionine-β-synthase assays were performed with all tissues obtained from dams on days 10, 11, 12, 14, 16, 18 or 20 of gestation. At each stage, material was obtained from two animals and at least two conceptuses from each animal were assayed in duplicate. The methionine synthase assay and measurement of the *o*-phthaldialdehyde derived methionine by HPLC and fluorometric detection were performed according to Garras *et al.* (1991).

The cystathionine-β-synthase assay was performed according to Fowler *et al.* (1978), except that tissues were homogenized (1:10, w:v) in ice-cold 50 mmol potassium phosphate buffer l^{-1} (pH 7.4) containing 0.1% (v/v) lubrol PX and the assay buffer (final volume 100 µl) contained 50 µl homogenate, 25 mmol potassium phosphate l^{-1}, 0.05% (v/v) lubrol PX, 108 mmol Tris–HCl l^{-1} (pH 8.6), 8 mmol l^{-1} L-serine (final specific activity 1563 µCi $mmol^{-1}$ [U-^{14}C] L-serine), 1 mol pyridoxal phosphate l^{-1}, 1.5 mmol dithiothreitol l^{-1} and 15 mmol L-homocysteine l^{-1}. L-Homocysteine stock solution (7.5 µl) was added to the incubation mixture after preincubation for 5 min at 37°C.

The S-adenosylhomocysteine hydrolase assay was performed with all tissues obtained on day 9.5 (7 rats, 75 conceptuses), day 10.5 (3 rats, 32 conceptuses) or day 11.5 (3 rats, 40 conceptuses) of gestation. Material from each rat was pooled separately, except for day 9.5 of gestation when material from 2 or 3 rats was used for each pool. Thus, for each tissue and each gestational stage, tissues were assayed in triplicate. Tissues were sonicated at a protein concentration of approximately 10 mg ml^{-1} in ice-cold 22.5 mmol potassium phosphate buffer l^{-1} (pH 7.0) containing 1 mmol $Na_2EDTA{\cdot}2H_2O$ l^{-1}, 20 µmol erythro-9[2-hydroxy-3-nonyl]adenine l^{-1}, 1.64 ml Triton X-100 l^{-1} and 1.1 mmol dithiothreitol l^{-1}. The assay was performed at 37°C for 20 min in the same buffer (10 µl homogenate in a final volume of 200 µl) additionally containing 0.5 mmol L-homocysteine l^{-1} and 0.5 mmol adenosine l^{-1}. After the incubation, protein was precipitated with 12 µl 8 mol perchloric acid l^{-1}. After 10 min on ice, the mixture was centrifuged in an Eppendorf centrifuge for 5 min (13 000 *g*, 4°C) and 25 µl 4 mol dipotassium hydrogenphosphate l^{-1} was added to the supernatant. After another 10 min on ice, the precipitate was removed by centrifugation and the supernatant was frozen in liquid nitrogen. Samples were stored at −80°C until further analysis. S-adenosylhomocysteine was measured in duplicate by using HPLC and spectrophotometric detection according to DeAbreu *et al.* (1982). All enzyme activities were expressed in µU (pmol product formed min^{-1} mg^{-1} protein).

Statistical analyses

Linear regression analysis was used to test whether methionine synthase activity or cystathionine-β-synthase activity increased or decreased during gestation. A decrease or increase was regarded as significant if $P < 0.05$ for the null-hypothesis that the regression coefficient equalled zero. Differences in enzyme activity between gestational days and differences in S-adenosylhomocysteine hydrolase activity between tissues for each gestational day were tested using Student's *t* test with Bonferroni correction. With a nominal α of 0.05, differences were regarded as statistically significant at $P < 0.05\ k^{-1}$, where *k* is the number of comparisons made. All differences, increases and decreases, in enzyme activities mentioned in the results section met the degree of statistical significance as defined above.

Results

Methionine synthase was present in all tissues investigated. The highest specific activity was found in embryonic tissue on day 12 of gestation. On days 10 and 11 of gestation it was lower and from day 12 onward the activity decreased at an average rate of 33 µU day^{-1}. Methionine synthase specific activity of maternal liver was 180 µU, which was comparable to the average methionine synthase specific activity of fetal liver. Methionine synthase activity of the fetal liver decreased during gestational development at a rate of 26 µU day^{-1}. The lowest methionine synthase specific activity was found in decidual and placental tissue, and here it did not change significantly during gestation (Fig. 2).

During gestation, cystathionine-β-synthase activity was not present in embryonic tissue. In this organogenic phase of development, it was present only in the surrounding decidual or placental tissue. In the latter tissue, it decreased during gestation at a rate of 15 µU day^{-1} and was virtually absent at day 16 of gestation. In fetal tissue, activity first occurred in the liver, where it was present from the first day (day 14) that it

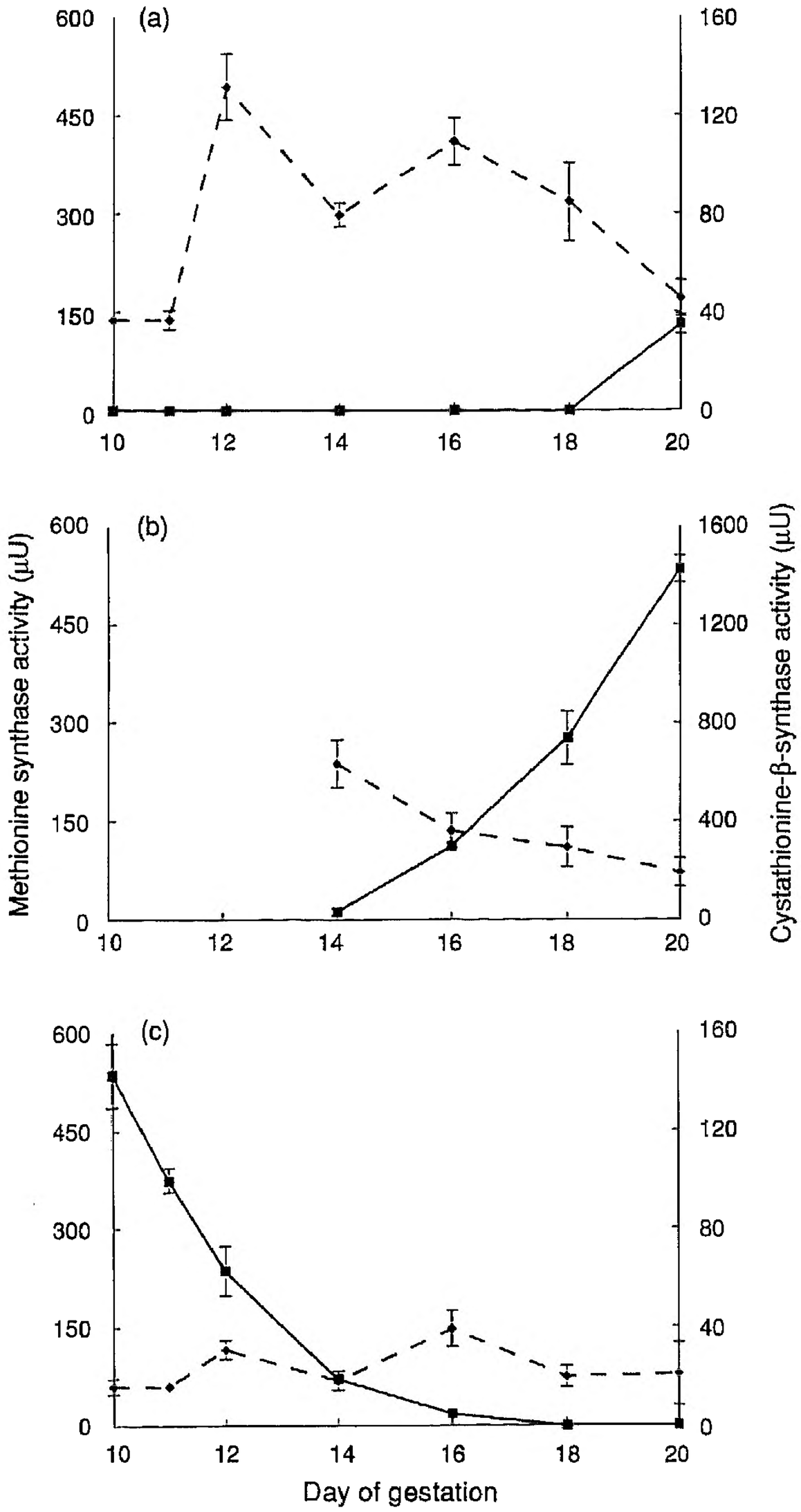

Fig. 2. Methionine synthase activity (– – –) and cystathionine-β-synthase activity (——) in (a) embryonic and fetal tissue without liver, (b) fetal liver and (c) decidual and placental tissue during the second half of gestation in rats. Each point represents the mean of four conceptuses taken from two dams. Error bars represent SEM.

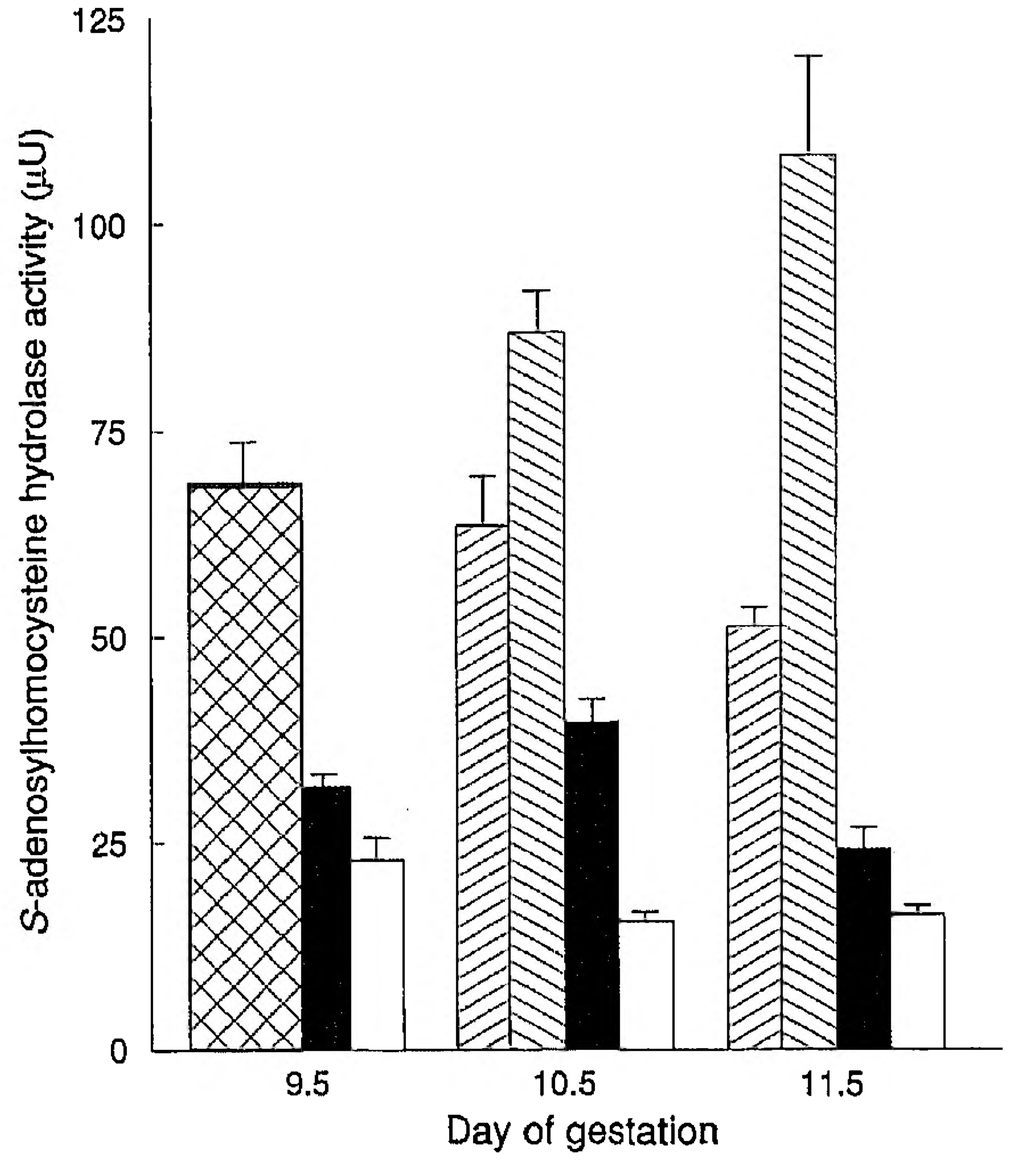

Fig. 3. *S*-adenosylhomocysteine hydrolase activity in neurulating rat conceptuses. Enzyme activity was measured in the direction of synthesis. Conceptuses were separated into embryo proper (▨), visceral yolksac (+ amnion) (▧), parietal yolksac (+ ectoplacental cone) (■) and decidua (□), except for embryos from day 9.5 of gestation, in which embryos and visceral yolksac were pooled together (⊠). Bars and error bars represent the mean and SEM of three pools of conceptuses.

was measured, although initially activity was low. Cystathionine-β-synthase specific activity of fetal liver increased during the last trimester of gestation, at a rate of 235 μU day^{-1} and reached, on day 20 of gestation, 68% of cystathionine-β-synthase specific activity of maternal liver, which was 2100 μU. In fetal tissue without liver, cystathionine-β-synthase, activity occurred only at days 18 and 20 of gestation (Fig. 2).

S-adenosylhomocysteine hydrolase was present in neurulating rat conceptuses on all days investigated (days 9.5 to 11.5 of gestation), both in the embryos proper and in the surrounding tissues. However, specific activities differed markedly between tissues (Fig. 3). On day 9.5 of gestation S-adenosylhomocysteine hydrolase specific activity was greater in embryonic plus visceral yolksac tissue than it was in decidual or parietal yolksac tissue. On day 10.5 of gestation, the investigated tissues could be ordered in sequence of increasing S-adenosylhomocysteine hydrolase activity as follows: decidua < parietal yolksac < embryo < visceral yolksac. On day 11.5 of gestation, S-adenosylhomocysteine hydrolase activity was greater in visceral yolksac tissue than it was in any other tissue. In addition, embryonic S-adenosylhomocysteine hydrolase activity was greater than decidual S-adenosylhomocysteine hydrolase activity. Interday differences in S-adenosylhomocysteine hydrolase activity were limited. Only S-adenosylhomocysteine hydrolase activity in parietal yolksac tissue on day 11.5 of gestation was lower as compared with that on day 10.5 of gestation. Maternal liver S-adenosylhomocysteine hydrolase specific activity (on day 10 of gestation) was 262 ± 48 (mean ± SEM) μU.

Discussion

The data on the developmental onset of cystathionine-β-synthase in rat fetuses extend those of Volpe and Laster (1972) and show that this enzyme first appears in the fetal liver and that, before this organ is formed, cystathionine-β-synthase activity is present only in the surrounding decidual and

placental tissue. The presence of methionine synthase activity in rat embryos and fetal liver (Baden *et al.*, 1984, 1987; Hansen and Billings, 1986) is confirmed; however, owing to the decrease of methionine synthase activity of fetal liver towards the end of gestation, it appears that the observation from Baden *et al.* (1984, 1987) that fetal liver activity is 50–65% of maternal liver activity is valid only for fetal liver at the end of gestation. The activity of methionine synthase in embryonic and fetal tissues from day 12 to day 18 of gestation was higher relative to maternal liver activity, which is in contrast to the results of Hansen and Billings (1985) that, at day 14 of gestation, activity in embryonic and fetal tissues was the same as the activity of maternal liver. The observations presented here extend those of Hansen and Billings (1985) and, for the first time, demonstrate that methionine synthase activity is present in neurulating rat embryos. In studies on the teratogenicity of nitrous oxide, a methionine synthase inhibitor, the presence of methionine synthase in neurulating embryos was only presumed (Baden *et al.*, 1983; Baden and Fujinaga, 1991; Fujinaga and Baden, 1994).

This is also the first report to demonstrate S-adenosylhomocysteine hydrolase activity in neurulating rat embryos. The higher activity of this enzyme in embryonic and visceral yolksac tissue relative to the activity in the surrounding parietal yolksac and decidua indicates that S-adenosylhomocysteine metabolism occurs mainly in the embryonic and visceral yolksac compartments. The presence of S-adenosylhomocysteine hydrolase and methionine synthase, and the absence of cystathionine-β-synthase, in the embryo proper indicates that the homocysteine–methionine cycle is functional at this stage of development, but that there is no diversion into the transsulfuration pathway in the embryo. It is calculated that in adult rat liver tissue about half of the homocysteine is transsulfurated, whereas the other half is remethylated to methionine (Finkelstein and Martin, 1986; Finkelstein, 1990). The proportion of homocysteine that will be remethylated can be increased or decreased depending on the need for S-adenosylmethionine. The conservation of the homocysteinyl moiety in the homocysteine–methionine cycle as a result of the absence of cystathionine-β-synthase in the neurulating embryo emphasizes the importance of this cycle in embryonic development at this stage. The relevance of this notion is also supported by the work of Klein and coworkers (Coelho *et al.*, 1989; Coelho and Klein, 1990; Ferrari *et al.*, 1993), who show that methionine deficiency leads to neural tube defects in cultured rat embryos and that the teratogenicity of human sera in the whole embryo culture could be reversed by addition of methionine. Homocysteine–methionine metabolism has a unique function in metabolism of one carbon compounds, namely by its metabolite S-adenosylmethionine, which is a universal methyl group donor in transmethylation reactions. It has been suggested that decreased DNA methylation (Li *et al.*, 1992; Matsuda and Yasutomi, 1992) and reduced protein methylation (Coelho and Klein, 1990) are teratogenic mechanisms. The constant withdrawal of homocysteinyl moieties by incorporation of methionine into proteins and the use of S-adenosylmethionine in polyamine synthesis dictate a need for constant replenishment of homocysteinyl moieties, preferably in the form of methionine. The embryo seems to economize in this respect by not expressing cystathionine-β-synthase during this phase of gestation.

References

Baden JM and Fujinaga M (1991) Effects of nitrous oxide on day 9 rat embryos grown in culture *British Journal of Anaesthetics* **66** 500–503

Baden JM, Rice SA, Serra M, Kelley M and Mazze R (1983) Thymidine and methionine syntheses in pregnant rats exposed to nitrous oxide *Anesthesia and Analgesia* **62** 738–741

Baden JM, Serra M and Mazze RI (1984) Inhibition of fetal methionine synthase by nitrous oxide *British Journal of Anaesthetics* **56** 523–526

Baden JM, Serra M and Mazze RI (1987) Inhibition of rat fetal methionine synthase by nitrous oxide. An *in vitro* study *British Journal of Anaesthetics* **59** 1040–1043

Bradford MM (1965) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding *Analytical Biochemistry* **72** 248–254

Coelho CND and Klein NW (1990) Methionine and neural tube closure in cultured rat embryos: morphological and biochemical analyses *Teratology* **42** 437–451

Coelho CND, Weber JA, Klein NW, Daniels WG and Hoagland TA (1989) Whole rat embryos require methionine for neural tube closure when cultured on cow serum *Journal of Nutrition* **119** 1716–1725

DeAbreu RA, van Baal JM, DeBruyn CHMM, Bakkeren JAJM and Schretlen EDAM (1982) High-performance liquid chromatographic determination of purine and pyrimidine bases, ribonucleosides, deoxyribonucleosides and cyclic ribonucleotides in biological fluids *Journal of Chromatography* **227** 45–52

Ericson LE (1960) Betaine-homocysteine methyltransferase I. Distribution in nature *Acta Chemica Scandinavica* **14** 2102–2112

Ferrari DA, Gilles PA, Klein NW, Nadler D, Weeks BS, Lamikeefe CJ, Hillman RE, Carey SW, Ying YK, Maier D, Olsen P, Wemple DW, Greenstein G, Muechler EK, Miller RK and Mariona FG (1993) Rat embryo development on human sera is related to numbers of previous spontaneous abortions and nutritional factors *American Journal of Obstetrics and Gynecology* **170** 228–236

Finkelstein JD (1990) Methionine metabolism in mammals *Journal of Nutrition and Biochemistry* **1** 228–237

Finkelstein JD and Martin JJ (1986) Methionine metabolism in mammals. Adaptation to methionine excess *Journal of Biological Chemistry* **261** 1582–1587

Finkelstein JD, Kyle WE and Harris BJ (1971) Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue *Archives of Biochemistry and Biophysics* **146** 84–92

Fowler B, Kraus J, Packman S and Rosenberg LE (1978) Evidence for three distinct classes of cystathionine-β-synthase in cultured fibroblasts *Journal of Clinical Investigation* **61** 645–653

Fujinaga M and Baden JM (1994) Methionine prevents nitrous oxide-induced teratogenicity in rat embryos grown in culture *Anesthesiology* **81** 184–189

Garras A, Djurhuus R, Christensen B, Lillehaug JR and Ueland PM (1991) A nonradioactive assay for N^5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) based on *o*-phthaldialdehyde derivatization of methionine and fluorescence detection *Analytical Biochemistry* **199** 112–118

Gaull G, Sturman JA and Räihä NCR (1972) Development of mammalian sulfur metabolism: absence of cystathionase in human fetal tissues *Pediatric Research* **6** 538–547

Gaull GE, von Berg W, Räihä NCR and Sturman JA (1973) Development of methyltransferase activities of human fetal tissues *Pediatric Research* **7** 527–533

Hansen DK and Billings RE (1985) Effects of nitrous oxide on maternal and embryonic folate metabolism in rats *Developmental Pharmacology and Therapeutics* **8** 43–54

Hansen DK and Billings RE (1986) Effects of nitrous oxide on macromolecular content and DNA synthesis in rat embryos *Journal of Pharmacology and Experimental Therapeutics* **238** 985–989

Li E, Bestor TH and Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality *Cell* **69** 915–926

Matsuda M and Yasutomi M (1992) Inhibition of cephalic neural tube closure by 5-azacytidine in neurulating rat embryos *in vitro Anatomy and Embryology* **185** 217–223

Rassin DK, Sturman JA and Gaull GE (1981) Sulfur amino acid metabolism in the developing rhesus monkey brain: subcellular studies of the methylation cycle and cystathionine-β-synthase *Journal of Neurochemistry* **36** 1263–1271

Schorah CJ, Habibzadeh N, Wild J and Smithells RW (1993) Possible abnormalities of folate and vitamin B_{12} metabolism associated with neural tube defects. In *Maternal Nutrition and Pregnancy Outcome: Annals of the New York Academy of Sciences* **678** 81–91

Shane B and Stokstad ELR (1983) The interrelationships among folate, vitamin B_{12} and methionine metabolism *Advances in Nutrition Research* **5** 133–170

Steegers-Theunissen RPM (1993) *Homocysteine, Vitamins and Neural-tube Defects* PhD Thesis, Catholic University of Nijmegen

Steegers-Theunissen RPM, Boers GHJ, Trijbels FJM and Eskes TKAB (1991) Neural-tube defects and derangement of homocysteine metabolism *New England Journal of Medicine* **324** 199–200

Steegers-Theunissen RPM, Boers GHJ, Blom HJ, Trijbels FJM and Eskes TKAB (1992) Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae *Lancet* **339** 1122–1123

Sternowsky HJ, Räihä NCR and Gaull G (1976) Methionine adenosyltransferase and transmethylation in fetal and neonatal lung of the human, monkey, and rabbit *Pediatric Research* **10** 545–550

Sturman JA, Gaull GE and Niemann WH (1976) Cystathionine synthesis and degradation in brain, liver and kidney of the developing monkey *Journal of Neurochemistry* **26** 457–463

Ueland PM (1982) Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase *Pharmacological Reviews* **34** 223–253

Volpe JJ and Laster L (1970) Trans-sulfuration in primate brain: regional distribution of cystathionine-β-synthase, cystathionine and taurine in the brain of the rhesus monkey at various stages of development *Journal of Neurochemistry* **17** 425–437

Volpe JJ and Laster L (1972) Transsulfuration in fetal and postnatal mammalian liver and brain. Cystathionine-β-synthase, its relation to hormonal influences, and cystathionine *Biology of the Neonate* **20** 385–403

Wouters MGAJ, Boers GHJ, Blom HJ, Trijbels FJM, Thomas CGM, Borm GF, Steegers-Theunissen RPM and Eskes TKAB (1993) Hyperhomocysteinaemia: a risk factor in women with unexplained recurrent early pregnancy loss *Fertility and Sterility* **60** 820–825