Ontogeny of hepatic enzymes involved in serine- and folate-dependent one-carbon metabolism in rabbits

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Thompson, Henry R., Gayle M. Jones, and Michael R. Narkewicz. Ontogeny of hepatic enzymes involved in serine- and folate-dependent one-carbon metabolism in rabbits. Am J Physiol Gastrointest Liver Physiol 280: G873–G878, 2001.—Serine occupies a central position in folate-dependent, one-carbon metabolism through 5,10-methenyltetrahydrofolate (MTHF) and 5-formyltetrahydrofolate (FTHF). We characterized the ontogeny of the specific activity of key enzymes involved in serine, 5,10-MTHF, and 5-FTHF metabolism: methenyltetrahydrofolate synthetase (MTHFS), MTHF reductase (MTHFR), the glycine cleavage system (GCS), methionine synthase (MS), and serine hydroxymethyltransferase (SHMT) in rabbit liver, placenta, brain, and kidney. In liver, MTHFS activity is low in the fetus (0.36 ± 0.07 nmol-min−1·mg protein−1), peaks at 3 wk (1.48 ± 0.56 nmol-min−1·mg protein−1), and then decreases to adult levels (1.13 ± 0.32 nmol-min−1·mg protein−1). MTHFR activity is highest in early gestation (24.9 ± 2.4 nmol-h−1·mg protein−1) and declines rapidly by birth (4.7 ± 1.3 nmol-h−1·mg protein−1). MS is highest during fetal life and declines after weaning. Cytosolic SHMT activity does not vary during development, but mitochondrial SHMT peaks at 23 days. GCS activity is high in the fetus and the neonate, declining after weaning. In placenta and brain, all activities are low throughout gestation. Cytosolic and mitochondrial SHMT activities are low in kidney and rise after weaning, whereas MTHFS is low throughout development. These data suggest that the liver is the primary site of activity for these enzymes. Throughout development, there are multiple potential sources for production of 5,10-MTHF, but early in gestation high MTHFR activity and low MTHFS activity could reduce 5,10-MTHF availability.

Serine is a semiessential amino acid that, during fetal development, plays a central role in folate-dependent one-carbon metabolism in protein, phospholipid, cysteine, DNA, and RNA synthesis (5, 13, 14) (Fig. 1). Serine is unlike other amino acids in that it has a unique fetal metabolic pattern. In vivo studies in humans and sheep have shown that serine is poorly transported across the placenta and that, like glutamate, there is net production across the fetal liver (7, 8, 24, 28–30). However, unlike glutamate, the net hepatic synthesis of serine stops shortly after birth (20). Studies in primary cultures of fetal ovine hepatocytes have confirmed this net serine biosynthesis and demonstrated that the majority of serine produced by hepatocytes is derived from glycine via serine hydroxymethyltransferase (SHMT) and the glycine cleavage enzyme system (GCS) (36, 49). Although these in vivo and in vitro studies have shown that the majority of fetal hepatic serine synthesis occurs via SHMT (36, 49), the ontogeny of SHMT activity does not appear to regulate fetal serine synthesis (34).

Cetin et al. (6) have demonstrated that growth-retarded human fetuses have selectively lower plasma serine and glycine concentrations compared with normally grown fetuses. These data imply that serine may be a critical component in the support of normal fetal growth and development. Several studies have demonstrated a link between maternal folate deficiency (12, 47, 48) or fetal folate deficiency (2, 10, 39) and fetal growth retardation. Recent work in neuroblastoma cells showed that serine synthesis is controlled by the balance of 5,10-methenyltetrahydrofolate (5,10-MTHF) and 5-formyltetrahydrofolate (5-FTHF), two key cofactors in serine metabolism (16). Serine is linked directly to these coenzymes through the enzymes methenyltetrahydrofolate synthetase (MTHFS), MTHF reductase (MTHFR), GCS, and SHMT and to the methylation cycle by methionine synthase (MS) (Fig. 1).

MTHFS (EC 6.3.3.2) is the only enzyme known to catalyze the conversion of 5-FTFH to 5,10-methenyltetrahydrofolate (5,10-methenyl-THF) (3). In human neuroblastoma cells, 5-FTFH has been shown to inhibit SHMT (serine biosynthesis) and other folate-dependent enzymes (16). Polyglutamated 5-FTFH is a slow, tight binder of SHMT, acting as an inhibitor (46). As serine synthesis and homocysteine remethylation compete for one-carbon units in the cytoplasm, inhibition of SHMT by 5-FTFH could reduce homocysteine concentrations. Bertrand et al. (3) have shown that inhibition of MTHFS results in an increase in 5-FTFH, which inhibits de novo purine synthesis and cell
growth. This implies that MTHFS may play a central role in the regulation of folate-dependent, one-carbon metabolism and that alterations in enzyme activity may be responsible for the link between folate deficiency, neural tube defects, and growth retardation in human fetuses. The ontogeny and regulation of this important enzyme in the liver has not been studied.

MTHFR (EC 1.1.1.68) catalyzes the reduction of 5,10-MTHF to 5-methyltetrahydrofolate (5-methyl-THF), a coenzyme for the conversion of homocysteine to methionine by MS. This reduces the amount of 5,10-MTHF available for DNA and RNA synthesis by committing one-carbon units to methionine production. Low levels of MTHFR result in hyperhomocysteinemia, which has been associated with cardiovascular disease, neural tube defects, and fetal growth retardation (31, 44, 47). Maternal hyperhomocysteinemia, associated with defects in MTHFR, has been proposed as one of the mechanisms linking maternal folate deficiency and fetal neural tube defects (44).

SHMT (EC 2.1.2.1) exists as both a cytosolic and a mitochondrial isoenzyme, each catalyzing the reversible interconversion of serine and tetrahydrofolate to glycine and 5,10-MTHF (Fig. 1). The use of serine for biosynthesis of nucleotides is initiated by SHMT. Recently, a second catalytic activity of SHMT, the irreversible hydrolysis of 5,10-methenyl-THF to 5-FTHF, has been demonstrated (45). Mitochondrial SHMT (mSHMT) is required for glycine synthesis, whereas cytosolic SHMT (cSHMT) is adequate for serine synthesis (1, 35, 37). We have recently demonstrated in sheep that hepatic mSHMT does not vary with gestation and that the cSHMT increases during the third trimester (34). However, this pattern of hepatic SHMT development did not appear to explain the changes in fetal and neonatal hepatic serine metabolism (34). Although Snell (43) has studied total SHMT liver activity in the rat, there is no study of postnatal development of SHMT isoenzymes.

GCS (EC 2.1.1.10) is a mitochondrial enzyme system that catalyzes the oxidation of glycine with the release of 5,10-MTHF, CO₂, ammonia, and NADH (18, 19, 23). GCS is the major pathway of glycine oxidation. SHMT and GCS act together in serine catabolism or synthesis (49).

MS (5-methyltetrahydrofolate-homocysteine S-methyltransferase; EC 2.1.1.13) catalyzes the conversion of homocysteine to methionine, committing 5-methyl-THF to the methylation cycle. This enzyme has been suggested to play a role in neural tube defects and fetal growth restriction (26, 27).

We hypothesized that provision of 5,10-MTHF may be a regulatory mechanism for the control of fetal hepatic serine synthesis. As a first step in the examination of this hypothesis, we determined the ontogeny of the specific activities of MTHFS, MTHFR, GCS, MS, and SHMT in rabbit tissues to assess their possible contribution to the regulation of fetal and perinatal serine and folate metabolism.

**MATERIALS AND METHODS**

All aspects of the animal care and use in these studies were reviewed and approved by the institutional animal care and use committee. Timed-pregnant New Zealand White rabbits were obtained from Myrtle’s Rabbitry (Thompson Station, TN). Animals were housed in individual nesting cages with unlimited access to food and water. Animals were studied at fetal ages 223, 215, 29, and 22 days, postnatal days 11, 13, 17, and 115, 123, and 128 (123 and 128 are after weaning), and as adults. For 23, 15, and 9 days, tissues from single pregnancies were pooled and used as a single mean data point.

Isotopes used were 5-[14C]methyl-THF and N-5,10-[14C]MTHF (Amersham Pharmacia Biotech, Piscataway, NJ) and 1-[14C]glycine (ICN Pharmaceuticals, Costa Mesa, CA). Xylazine was from Ben Venue Labs (Bedford, OH), ketamine was from Fort Dodge Labs (Fort Dodge, IA), and pentobarbital was from Abbott Labs (Chicago, IL). Folate coenzymes were from Schircks Laboratories (Jona, Switzerland). All other chemicals and supplies were of the highest grade available and were obtained from Sigma (St. Louis, MO).

**Tissue preparation.** Adult rabbits were anesthetized with intramuscular xylazine and ketamine before receiving intravenous pentobarbital. Rabbit pups were delivered via cesar-
ontogeny of serine and folate enzymes

Fig. 2. Developmental pattern of hepatic MTHFR (●) and MTHFS (○) activities. *P < 0.01 vs. all other ages; **P < 0.01 vs. adult values; ***P < 0.05 vs. −23, −15, −9 and −2 days.

Fig. 3. Developmental pattern of hepatic cytosolic (●) and mitochondrial (■) SHMT activities. *P < 0.05 vs. −2, +3, and +7 days.

RESULTS

Hepatic activity. The developmental patterns of the hepatic activities of MTHFS and MTHFR are shown in Fig. 2. MTHFS activity is low at the earliest measurable time point (−23 days, 0.25 ± 0.13 nmol·min⁻¹·mg protein⁻¹) and increases thereafter to sustained levels from 3 days to adulthood, with a peak at 23 days. In contrast, MTHFR activity is highest early in gestation (−23 days, 0.42 ± 0.04 nmol·min⁻¹·mg protein⁻¹; P < 0.01 vs. all other time points) and decreases rapidly to achieve steady, low levels by 2 days (0.07 ± 0.02 nmol·min⁻¹·mg protein⁻¹) that continue into adulthood (0.03 ± 0.02 nmol·min⁻¹·mg protein⁻¹).

The developmental pattern of SHMT isoenzyme activity is presented in Fig. 3. Liver mSHMT activity is concentrations in the assay mix were 125 mM KCl, 5 mM NaCl, 2.5 mM MgCl₂, 2.5 mM K₂HPO₄, 10 mM MOPS, 7 mM Tris-HCl, 1 mM EGTA, 1 mM ADP, 0.2 mM NAD, 0.2 mM DTT, 0.8 mM CaCl₂, 0.02 mM pyridoxal 5-phosphate, and 0.833 μCi of [14C]-glycine. The 14CO₂ was released with 0.3 ml of 30% perchloric acid, followed by incubation for one additional hour with a 10% KOH trap. Activity is expressed as nanomoles of glycine oxidized per minute per milligram protein.

MS was assayed by the nonradioactive assay of Drummond et al. (9), and activity is expressed as nanomoles per minute per milligram protein. SHMT was assayed by the binding assay of Geller and Kotb (15), which measures the binding of N-5,10-[14C]MTHF to DEAE-cellulose paper. Activity is expressed as nanomoles per minute per milligram protein.

Citrate synthase was assayed by the method of Shepherd and Garland (42). Protein was determined by the method of Lowry as modified by Hartree (17).

Data analysis. Values are means ± SD. Comparisons between activities at different gestational ages were by ANOVA, with significance at P < 0.05.

shMT was assayed by the method of Jois et al. (21), with the addition of calcium for maximal activity. Final

Fig. 2. Developmental pattern of hepatic MTHFR (●) and MTHFS (○) activities. *P < 0.01 vs. all other ages; **P < 0.01 vs. adult values; ***P < 0.05 vs. −23, −15, −9 and −2 days.

GCS activity was assayed by the method of Jois et al. (21), with the addition of calcium for maximal activity. Final

Fig. 3. Developmental pattern of hepatic cytosolic (●) and mitochondrial (■) SHMT activities. *P < 0.05 vs. −2, +3, and +7 days.

Cytosolic and mitochondrial fractions were prepared as previously described (32). Samples were snap frozen on dry ice and stored at −70°C until the time of assay. Mitochondrial samples were stored in an equal volume of mitochondrial storage buffer (0.25 M sucrose, 0.01 M HEPES, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.001 M dithiothreitol (DTT), pH 7.4). Tissue was homogenized in 4 parts vol/wt (kidney, brain, and placenta) or 6 parts vol/wt (liver) of isolation buffer using a Teflon/glass (Potter) motor-driven homogenizer. Cytosolic and mitochondrial fractions were prepared as previously described (32). Samples were snap frozen on dry ice and stored at −70°C until the time of assay. Mitochondrial samples were stored in an equal volume of mitochondrial storage buffer (0.25 M sucrose, 0.01 M HEPES, 0.1 mM EGTA, 0.02 g% BSA, 4.9 mM MgCl₂, and 0.015 M DMSO) (38).

Assays. MTHFS activity was determined in cytosolic extracts by the assay of Girgis et al. (16) and Bertrand et al. (4) following the formation of 5,10-methenyl-THF at 30°C. The final concentration of the reaction mix was 50 mM MES, 10 mM magnesium acetate, 0.01 mM dithiothreitol (DTT), pH 7.4. Tissue was homogenized in 4 parts vol/wt (kidney, brain, and placenta) or 6 parts vol/wt (liver) of isolation buffer using a Teflon/glass (Potter) motor-driven homogenizer. Cytosolic and mitochondrial fractions were prepared as previously described (32). Samples were snap frozen on dry ice and stored at −70°C until the time of assay. Mitochondrial samples were stored in an equal volume of mitochondrial storage buffer (0.25 M sucrose, 0.01 M HEPES, 0.1 mM EGTA, 0.02 g% BSA, 4.9 mM MgCl₂, and 0.015 M DMSO) (38).

Data analysis. Values are means ± SD. Comparisons between activities at different gestational ages were by ANOVA, with significance at P < 0.05.

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The developmental pattern of SHMT isoenzyme activity is presented in Fig. 3. Liver mSHMT activity is
low throughout fetal and neonatal development, with a peak activity, like MTHFS, at 23 days. In contrast, there are no significant changes in cSHMT activity throughout development.

The ontogeny of hepatic MS activity is presented in Fig. 4. MS activity is elevated during the fetal period, declining after birth to reach adult levels by 15 days.

Hepatic GCS activity is highest during the fetal (14.3 ± 4.9 nmol·min⁻¹·mg protein⁻¹) and neonatal (28.8 ± 6.7 nmol·min⁻¹·mg protein⁻¹) periods before declining to adult levels (9.4 ± 1.1 nmol·min⁻¹·mg protein⁻¹; P < 0.01 vs. fetal and neonatal periods) after weaning.

Placental activity. The placenta has low activities of all enzymes except GCS throughout gestation. The mean activities are (in nmol·min⁻¹·mg protein⁻¹): MTHFS, 0.06; MTHFR, 0.07 ± 0.02; MS, 0.97; cSHMT, 0.25 ± 0.32; mSHMT, 0.07 ± 0.3; and GCS, 15. For all but GCS, the levels of activity are 4- to 50-fold lower than the corresponding hepatic values.

For activity in the brain and kidney, there were consistent values during the fetal (−23, −15, −9, and −2 days), neonatal (1, 3, 7, and 15 days), and post-weaning (23 days, 28 days, and adult) periods. Thus the values are grouped together and presented for these three developmental time periods.

Kidney. In the kidney, there were no changes in MTHFS or MTHFR activity throughout development (Table 1). Fetal kidney mSHMT activity was low compared with either neonatal or postweaning kidney. MS and cSHMT were higher in postweaning kidney than in fetal or neonatal kidney.

Brain. There was an increase in MTHFS and a decrease in MTHFR after weaning (Table 2). There was a peak in activity of MS in the neonatal time period. There were no significant changes in the activities of cSHMT, mSHMT, or GCS during development.

DISCUSSION

Serine and folic acid are important for fetal growth and development. Despite this knowledge, to date, there are no data on the coordinated ontogeny of the multiple enzymes involved in the metabolism of serine and its related folate coenzymes. In this study, we have presented data on the ontogeny of these enzymes.

The first observation from our study is that there is a coordinated developmental pattern of MTHFR, MTHFS, and MS activity during the prenatal and postnatal periods. In the placenta and brain, these enzymes do not appear to play an important role. In the kidney, there is an increase in MS and both cSHMT and mSHMT after weaning. However, the specific activity of the renal enzymes is not as high as the hepatic activities.

It has already been shown that the liver is the site of the majority of serine metabolism in the fetus (7, 8). As demonstrated by the ontogeny of the activity of the key enzymes of folate and serine metabolism in this study, the liver is also an important site for the interaction between serine and folate metabolism. In neuroblastoma cells and fetal hepatocytes, the balance between 5,10-MTHF and 5-FTHF controls serine biosynthesis (16, 33). SHMT, MTHFS, and GCS are key enzymes in the availability of 5,10-MTHF. The bioavailability of 5,10-MTHF is protected by duplicity of key enzyme pathways (high GCS, MTHFR, and SHMT) at all of the developmental stages after midgestation. However, at midgestation, only elevated GCS activity would provide adequate levels of 5,10-MTHF. Following midgestation, there is a decrease in MTHFR activity with an increase in MTHFS activity that could provide adequate levels of 5,10-MTHF.
creased availability of 5,10-MTHF during the rapid growth of late term and the postnatal period. This apparently developmentally integrated and coordinated enzyme system would ensure an adequate supply of 5,10-MTHF to meet the rapidly growing fetus’s metabolic demands throughout gestation and after birth.

MTHFR, MS, and MTHFS have been determined in human liver tissue from preterm, term, and 1-year-old infants. In this study, MS and MTHFR specific activity was highest in the preterm infant livers and declined with time (22). MTHF dehydrogenase (measured by an assay that would include MTHFS) increased with increasing age (22). Thus our data in rabbit liver are similar to the limited data available from humans.

The ontogeny of total hepatic SHMT has been determined in the rat, in which there was a slight increase in specific activity in adults (43). In contrast, in sheep liver, we have demonstrated a significant increase in cSHMT in late gestation (34). However, postnatal liver was not studied. There is precedent for species differences in SHMT, even between the rat and the rabbit (43), that would suggest that the differences between our sheep data and rabbit and rat data may be related to species. However, no previous study has compared the ontogeny of these closely related enzymes of serine and folate metabolism.

The placental activities are low for all except GCS. Thus the placenta does not appear to have a major role in the provision of folate coenzymes. Similarly, the brain has low levels of specific activity throughout development. This would suggest that the brain is dependent to a large extent on the exogenous supply of folate coenzymes. During the fetal and postnatal time periods, the kidney has low specific activity for all of the enzymes measured. After weaning, there is an increase in SHMT isoenzymes and MS specific activities. This is consistent with a role of the kidney in glycine metabolism in the adult animal (25, 41).

These observations are important because of their potential to provide a cellular explanation for intrauterine growth retardation and neural tube defects. The association of neural tube defects and inadequate folate intake is well established (11, 27, 50–52). It is unclear what the underlying defect or defects are that cause this association. To date, there have been no data on the ontogeny of all of these enzyme systems in one animal model. We feel that the increase in 5,10-MTHF availability through a variety of enzymatic activity changes is critical for rapid fetal and postnatal growth and development. It is of note that in midgestation, there is a lack of the duplicity of enzyme activities for 5,10-MTHF metabolism that are present at other times in development. Alterations in the relationship between MTHFS, MTHFR, GCS, and SHMT activity could explain some cases of neural tube defects and intrauterine growth retardation in humans. Further studies of the mechanism of the regulation of the ontogeny of these enzymes involved in the interaction of serine and folate coenzymes should focus on the trio of enzymes MS, MTHFR, and MTHFS.

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