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Effects of Methionine Supplement on Methionine Incorporation in Rat Embryos Cultured In Vitro

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ABSTRACT The effect of supplementary L-methionine (Met) on the incorporation of methionine was evaluated in 9.5-day rat conceptuses cultured in vitro. Parallel experiments with L-leucine (Leu) were performed for comparison. Conceptuses were cultured for 24 hr in the presence of ^{3}H-labeled Met or Leu, and the incorporation of radiolabel into the embryo and visceral yolk sac was measured. Supplementary Met proportionately increased the incorporation of Met, but supplementary Leu did not have as great an effect on the incorporation of Leu. A hypothesis is presented to explain these findings. It is proposed that Met, but not Leu, is a rate-limiting nutrient for organogenesis-stage rat embryos cultured in rat serum. The results are also discussed with reference to the established efficacy of supplementary folic acid in decreasing the incidence of neural tube defects in human populations and to claims that Met reverses certain teratogenic phenomena, both in vitro and in vivo. *Teratology 60:6–9, 1999.*

There is growing evidence that dietary supplementation with methionine may prevent the occurrence of some congenital malformations, including some neural tube defects. Axial defect (*Axd*) mice have a spontaneous 25–30% incidence of spina bifida; this is reduced to 10–15% by methionine supplementation (Essien and Wannberg, '93). Methionine supplementation has also been shown to ameliorate the pathologic effects of certain teratogens such as N_2O-induced neural tube defects (Fujinaga and Baden, '94), valproic acid-induced resorption (Nosel and Klein, '92), and spina bifida (Ehlers et al., '96), and retinoic acid- or corticosteroid-induced cleft palate in mice (Lau and Li, '95). In humans, spontaneous abortion and fetal wastage has been reported to be overcome by supplementation with amino acids, including methionine (Ferrari et al., '94). Most recently a correlation between dietary intake of methionine and the incidence of neural tube defects in a human population has been reported (Shaw et al., '97), although the data may equally support a correlation with several amino acids and perhaps other nutrients.

Organogenesis-stage rat embryos can be cultured in vitro in whole rat serum for at least 48 hr, with development and growth similar to that observed in vivo (Cockroft, '97). Clearly, rat serum provides an adequate supply of methionine and other nutrients. Methionine is present in rat serum as both the free amino acid and as protein, and it has recently been shown that more than 95% of the methionine incorporated into the conceptus in vitro, and probably in vivo, derives from protein (Lloyd et al., '96; Beckman et al., '96). As previously shown for leucine (Freeman et al., '81; Freeman and Lloyd, '83), the serum proteins are endocytosed by the epithelial cells of the visceral yolk sac (VYS), digested in the lysosomes and the resulting amino acids delivered to the embryo.

Because the reports of Lloyd et al. ('96) and Beckman et al. ('96) demonstrate that the free methionine in serum is of little quantitative importance in supplying methionine to the normally developing embryo, it is surprising to find reports that the development of embryos cultured in suboptimal conditions is improved by supplementing the culture medium with additional free methionine. Thus, 9.5-day rat conceptuses grown for 48 hr in dog or cow serum exhibited abnormal neural tube closure, which could be prevented by adding methionine (25 μg/ml; 168 μM) to the serum (Flynn et al., '87; Coelho et al., '89; Coelho and Klein, '90). In another study (VanAerts et al., '94) 9.5-day rat conceptuses were cultured for 48 hr in a medium comprising human serum (71%), rat serum (10%), and Tyrode's buffer (19%). The embryos did not develop normally and displayed a number of neural tube closure abnormalities. The addition of 1.0 mM methionine decreased the average number of dysmorphic features per conceptus from 4.4 to 0.3. Homocysteine, the demethylated derivative of methionine, was equally effective at the same concentration. Methionine at 100 μM was partially effective. Ferrari et al. ('94) also showed that methionine (25 μg/ml; 168 μM) could improve the development of rat embryos grown in serum drawn from a woman with a history of recurrent abortion.

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Methionine supplements can also prevent the dysmorphic effects of certain teratogens in vitro. Thus, methionine (49 µg/ml; 328 µM) overcomes the embryopathic effects antibodies against laminin and certain laminin peptides (Chambers et al., '95a,b). Recent data from our own laboratory (Fawcett et al., '97, '98) demonstrate the efficacy of L-methionine in ameliorating the effects of teratogenic anti-VYS antiserum in 9.5-day rat embryos cultured in vitro.

In our experiments on the incorporation of [^{3}H]methionine into 9.5-day rat conceptuses in vitro it was found that when the radiolabel was presented in the form of serum protein, its incorporation into the tissues of the embryo was more efficient than the incorporation of [^{3}H]leucine in the same proteins (Lloyd et al., '96). We interpreted this as indicating that leucine, but perhaps not methionine, was available to the conceptus in amounts in excess of requirements; and that any insult that inhibits the uptake and degradation of protein by the VYS might decrease the methionine supply below the amount needed for normal development and growth. Under these circumstances, free methionine might assume an importance it does not have under normal conditions. The work we now report was undertaken to test this hypothesis: we have sought further evidence indicating whether the methionine supply to the rat embryo cultured in rat serum is barely sufficient. A preliminary report of this work has appeared (Pugarelli et al., '97).

MATERIALS AND METHODS

Materials

L-[4,5-^{3}H]Leucine (169 Ci/mmol) and L-[methyl-^{3}H]methionine (85 Ci/mmol) were from Amersham International. All chemicals and reagents were from Sigma or Aldrich.

Assays

The protein content of tissues pre-digested in 0.5 M NaOH was measured by the method of Lowry et al. ('51). The ^{3}H-radioactivity of culture medium or tissue samples was measured by adding 300 µl of glacial acetic acid and 10 ml scintillation fluid (Ecoscint, National Diagnostics) to a 50- to 250-µl sample. Vials were counted in Beckman LS 7500 liquid scintillation spectrometer with quench correction.

Normal rat serum

Male Wistar rats were anesthetized with halothane and exsanguinated via the abdominal aorta. The blood was immediately centrifuged at 2,000*g* for 10 min and heat-inactivated at 56°C for 30 min. Streptomycin (50 µg/ml) and penicillin (50 IU/ml) were added and the serum was stored at −70°C.

In vitro culture of 9.5-day rat conceptuses

Wistar rat conceptuses at 9.5 days postconception were cultured in vitro, as previously described (Lloyd et al., '96), with four conceptuses incubated in 3 ml heat-inactivated normal rat serum. In some experiments, supplementary methionine or leucine was added in a volume not greater than 50 µl. After 3 hr, the cultures were inspected morphologically and any unexpanded conceptuses noted. Cultures were then continued for a further 24 hr.

Uptake of radiolabeled amino acids by 9.5-day rat conceptuses cultured in vitro

At the 3-hr inspection time-point, a radiolabeled amino acid was added to the culture in a volume not greater than 5 µl, and the cultures continued for a further 24 hr. At the end of the culture period the conceptuses were removed from the vial, placed in phosphate-buffered saline (PBS) at 37°C and examined to confirm normal development, as described previously (Lloyd et al., '96). After draining off excess saline, all the acceptable embryos from a single vial were pooled, as well as all the VYSs.

The combined fetuses from one vial were incubated in 400 µl 0.5 M NaOH at 37°C for 1 hr to achieve complete dissolution. The VYSs were treated in an identical manner. Samples (50 µl) of tissue digest and of culture medium were assayed for ^{3}H-radioactivity, as described above.

To determine the acid-soluble and acid-insoluble radioactivities of embryo and VYS, 100 µl of tissue digest was added with 25 µl rat serum to 500 µl of 15% trichloroacetic acid. Incubation at 4°C for 1 hr and centrifugation for 10 min at 4,600*g* yielded a pellet and a supernate. The pellet was washed three times by resuspension in diethyl ether and centrifugation, dried under vacuum at room temperature, and dissolved in 250 µl of 1 M NaOH. The radioactivity of a 100-µl sample of the redissolved pellet, and of a 250-µl sample of the supernate, was measured as described above.

RESULTS

Normal rat serum contains 70 µM methionine and 250 µM leucine (Milakofski et al., '84). Adding L-methionine to increase the concentration 11-fold, to 770 mM, had so significant effect on the in vitro development of 9.5-day rat conceptuses; likewise, the addition of L-leucine, to achieve a final concentration of 2,750 µM, was without effect on any of the developmental parameters. The protein content of embryos grown in the leucine-supplemented serum was 30.8 ± 7.1 (s.d.) µg (n = 11 vials); in methionine-supplemented serum the value was 33.6 ± 5.8 (s.d.) µg (n = 12 vials).

Table 1 shows the results of experiments in which 9.5-day rat conceptuses were cultured for 24 hr in vitro in rat serum containing [^{3}H]leucine or [^{3}H]methionine. The uptake of radiolabel is expressed as a clearance (µl/mg protein) and also as nmol of leucine (or methionine) per mg protein. The uptake of leucine by the embryo and by the VYS increased 4-fold when the leucine concentration increased 11-fold but was unaffected by an 11-fold increase in the methionine concen-

TABLE 1. Incorporation of amino acids into 9.5-day rat conceptuses cultured in vitro for 27 hr*

Radiolabeled moiety added to culture	Amino acid added	No. of vials	Embryo[c] Clearance (µl/mg tissue protein)	Embryo[c] Uptake (nmol/mg tissue protein)[d]	Visceral yolk sac[c] Clearance (µl/mg tissue protein)	Visceral yolk sac[c] Uptake (nmol/mg tissue protein)[d]
[^{3}H]Leucine	None[a]	4	262 ± 78	66 ± 20	269 ± 126	67 ± 31
	Leu and Met (×2)[b]	6	246 ± 64	123 ± 32	308 ± 42	154 ± 21
	Leu (×11)	6	95 ± 8	261 ± 22	91 ± 16	250 ± 44
	Met (×11)	6	344 ± 25	86 ± 6	294 ± 28	74 ± 7
[^{3}H]Methionine	None[a]	4	166 ± 25	12 ± 2	160 ± 52	11 ± 4
	Leu and Met (×2)[b]	5	193 ± 39	27 ± 5	190 ± 27	27 ± 4
	Leu (×11)	5	207 ± 43	14 ± 3	226 ± 33	16 ± 2
	Met (×11)	6	128 ± 17	99 ± 13	180 ± 27	139 ± 21

*The radiolabeled and supplementary amino acids were present for the final 24 hr of culture.
[a]Data from Lloyd et al. ('96) Table 1.
[b]Data from Lloyd et al. ('96) Table 2.
[c]Values are mean ± s.d.
[d]Calculated by multiplying the clearance by the concentration of leucine or methionine in the culture medium. For leucine, this was 250 pmol/µl (no added leucine), 500 pmol/µl (×2), 2,750 pmol/µl (×11); for methionine, 70 pmol/µl (no added methionine), 140 pmol/µl (×2), 770 pmol/µl (×11).

TABLE 2. Acid-insoluble radioactivity in 9.5-day rat embryos after culture in vitro for 27 hr*

Radiolabeled moiety added to culture	Amino acid added[b]	No. of vials	Acid-insoluble radioactivity[a] Embryo	Acid-insoluble radioactivity[a] Visceral yolk sac
[^{3}H]Leucine	Leucine	5	0.87 ± 0.03	0.78 ± 0.06[c]
	Methionine	6	0.95 ± 0.01	0.84 ± 0.02
[^{3}H]Methionine	Leucine	5	0.60 ± 0.06	0.44 ± 0.04
	Methionine	7	0.54 ± 0.05	0.38 ± 0.04

*The radiolabeled and supplementary amino acids were present for the final 24 hr of culture.
[a]Values are the acid-insoluble radioactivity divided by the total (insoluble plus soluble) radioactivity; mean ± s.d.
[b]Leucine was added to give a concentration of 2.7 mM; methionine to give a concentration of 0.77 mM.
[c]Data are for six vials.

tration. The accumulation of methionine increased 8-fold (embryo) and 12-fold (VYS) when the concentration of methionine increased 11-fold but was not significantly affected by an 11-fold increase in the leucine concentration.

Table 2 shows that almost all of the [^{3}H]leucine incorporated into the embryo is acid-insoluble, although the fraction is slightly lower when culture was in the presence of additional leucine. By contrast, only around one-half of the [^{3}H]methionine is acid-insoluble. The fraction of acid-insoluble radioactivity is consistently lower in the VYS than in the embryo.

DISCUSSION

We showed earlier (Lloyd et al., '96) that, when 9.5-day rat conceptuses are cultured for 24 hr in rat serum, only 3% of the leucine and methionine incorporated derives from free amino acid, the remainder arising from the endocytosis of serum proteins. By increasing the concentration of free leucine from its normal level (250 µM) to 11 times this value (2,750 µM), the incorporation increased only fourfold. Our earlier experiment (Lloyd et al., '96) suggested that the degradation of endocytosed protein in the VYS generates amounts of leucine in excess of requirements. Because it is unlikely that the endocytic uptake and degradation of protein by the VYS are affected by the higher concentration of leucine in the culture medium, we conclude that the additional leucine adds further to the overabundance of leucine available to the embryo.

An 11-fold increase in the free methionine concentration, from 70 µM to 770 µM increased incorporation of free methionine by 9- to 12-fold. Taken together with our earlier finding (Lloyd et al., '96) that protein-derived methionine was incorporated into the tissues of the conceptus more efficiently than the leucine arising from the same proteins, these data suggest that the proteins and free amino acid of normal rat serum supplies barely enough methionine for the embryo's needs.

Our data, taken together with other data from our own and other laboratories, are compatible with the following hypothesis, which we present for critical evaluation and testing through further experimental work. Because of its continuous net protein synthesis, the early organogenesis rat embryo requires a steady supply of all 20 amino acids. These arise chiefly by degradation of maternal protein in the VYS and, to a minor extent, from maternal free amino acids. In addition to its role in protein synthesis, methionine is required by the embryo for some methionine-specific metabolic pathways. We propose that methionine, but not leucine, is a rate-limiting nutrient when organogenesis-stage rat embryos are cultured in rat serum. Thus, the embryopathic effects of decreasing amino acid supply from protein degradation, for example by a relatively mild inhibition of VYS endocytosis, can be reversed by supplementary free methionine, but not supplementary free leucine.

One explanation that has been advanced for the efficacy of dietary folic acid supplements in reducing the incidence of neural tube defects in human populations is that a folate deficiency decreases the embryo's supply of methionine. This was proposed because the regeneration of methionine from homocysteine, following methyl group donation by S-adenosyl methionine, is a folate-dependent reaction. Decreased methionine regeneration would increase the demand for exogenous methionine and, if methionine is rate-limiting in human embryonic development, this demand might go unmet. Conversely, a primary methionine deficit would lead to reduced utilization of S-adenosyl methionine, and therefore to a decrease in the amount of homocysteine available for remethylation. This latter reaction is important not only for the regeneration of methionine but also for the conversion of methyltetrahydrofolate into tetrahydrofolate, the form of folate needed for most of the single-carbon donation reactions in which folate participates. Thus, the metabolic pathways of folate and methionine are intimately interconnected, and the separation of cause and effect is not a simple matter.

LITERATURE CITED

Beckman DA, Brent RL, Lloyd JB. 1996. Sources of amino acids for protein synthesis during early organogenesis in the rat. 4. Mechanisms before envelopment of the embryo by the yolk sac. Placenta 17:635–641.

Chambers BJ, Klein NW, Nosel PG, Khairallah LH, Romanow JS. 1995a. Methionine overcomes neural tube defects in rat embryos cultured on sera from laminin-immunized monkeys. J Nutr 125: 1587–1599.

Chambers BJ, Klein NW, Conrad SH, Ruppenthal GC, Sackett GP, Weeks BS, Kleinman HK. 1995b. Reproduction and sera embryotoxicity after immunization of monkeys with the laminin peptides YIGSR, RGD, and IKVAV. Proc Natl Acad Sci USA 92:6818–6822.

Cockroft DL. 1997. A comparative and historical review of culture methods for vertebrates. Int J Dev Biol 41:127–137.

Coelho CND, 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, Hoagland TA. 1989. Whole rat embryos require methionine for neural tube closure when cultured in cow serum. J Nutr 119:1716–1725.

Ehlers K, Elmazar MMA, Nau H. 1996. Methionine reduces the valproic acid-induced spina bifida rate in mice without altering valproic acid kinetics. J Nutr 126:67–75.

Essien FB, Wannberg SL. 1993. Methionine but not folinic acid or vitamin B-12 alters the frequency of neural tube defects in *Axd* mutant mice. J Nutr 123:27–34.

Fawcett LB, Pugarelli JE, Brent RL. 1997. Methionine supplementation reduces teratogenic antiserum-induced embryopathy in whole rat embryo culture. Teratology 55:35.

Fawcett LB, Pugarelli JE, Lloyd JB, Brent RL. 1998. Supplemental methionine prevents neural tube defects in rat embryos treated with teratogenic anti-visceral yolk sac serum. Teratology 57:203.

Ferrari DA, Gilles PA, Klein NW, Nadler D, Weeks BS, Lammi-Keefe CJ, Hillman RE, Carey, SW, Ying Y-K, Maier D, Olsen P, Wemple DW, Greenstein R, Muechler EK, Miller RK, Mariona FG. 1994. Rat embryo development on human sera is related to numbers of previous spontaneous abortions and nutritional factors. Am J Obstet Gynecol 170:228–236.

Flynn TJ, Friedman L, Black TN, Klein NW. 1987. Methionine and iron as growth factors for rat embryos cultured in canine serum. J Exp Zool 244:319–324.

Freeman SJ, Lloyd JB. 1983. Evidence that protein ingested by the rat visceral yolk sac yields amino acids for synthesis of embryonic protein. J Embryol Exp Morphol 73:307–315.

Freeman SJ, Beck F, Lloyd JB. 1981. The role of the visceral yolk sac in mediating protein nutrition by rat embryos cultured in vitro. J Embryol Exp Morphol 66:223–234.

Fujinaga M, Baden JM. 1994. Methionine prevents nitrous oxide-induced teratogenicity in rat embryos grown in culture. Anesthesiology 81:184–189.

Lau EV, Li Z-Q. 1995. Protection of mice from teratogen-induced cleft palate by exogenous methionine. Proc Soc Exp Biol Med 209:141–145.

Lloyd JB, Brent RL, Lloyd JB. 1996. Sources of amino acids for protein synthesis during early organogenesis in the rat. 3. Methionine incorporation. Placenta 17:629–634.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurements with the Folin phenol reagent. J Biol Chem 193:265–275.

Milakofsky L, Hare TA, Millerand JM, Vogel WH. 1984. Comparison of amino acid levels in rat blood obtained by catheterization and decapitation. Life Sci 34:1333–1340.

Nosel PG, Klein NW. 1992. Methionine decreases the embryotoxicity of sodium valproate in the rat: in vivo and in vitro observations. Teratology 46:499–507.

Pugarelli JE, Brent RL, Lloyd JB. 1997. Effects of methionine and leucine supplements on the incorporation of these amino acids into early organogenesis rat embryos cultured in vitro. Teratology 55:35–36.

Shaw GM, Velie EM, Schaffer DM. 1997. Is dietary intake of methionine associated with a reduction in risk for neural tube defect-affected pregnancies? Teratology 56:295–299.

VanAerts LAGJM, Blom HJ, DeAbreu RA, Trijbels FJM, Eskes TKAB, Copius Peereboom-Stegeman JHJ, Noordhoek J. 1994. Prevention of neural tube defects by and toxicity of L-homocysteine in cultured postimplantation rat embryos. Teratology 50:348–360.