The effect of feeding adequate or deficient vitamin B$_6$ or folic acid to breeders on methionine metabolism in 18-day-old chick embryos

J. Lu,[*] J. T. Weil,[†] P. Maharjan,[†] M. K. Manangi,[‡] S. Cerrate,[§] and C. N. Coon[†,1]

*Analytics & Data Science, Syngenta, Greensboro, NC 27409, USA; †Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA; ‡Research and Development, Novus International, Inc., St. Charles, MO, 63304, USA; and §Credinser LLC, Madison, AL 35756, USA

ABSTRACT Three isotopic tracers ([2,3,3-^{2}H$_3$]-L-serine, [^{2}H$_{11}$]-L-betaine, and [1-^{13}C]-L-methionine) were administered by amnion injection into 18-day-old chick embryos to investigate the kinetics of methionine metabolism. The embryos utilized were from eggs collected from 34-week-old Cobb 500 broiler breeders that were fed either a control diet containing folic acid (1.25 mg/kg diet) and pyridoxine HCl (5 mg/kg diet) or diets devoid of supplemental pyridoxine or folic acid. Intermediate metabolites of methionine metabolism and polyamines were analyzed in 18-day-old chick embryos. There were no differences in hepatic [^{2}H$_2$] methionine or [^{2}H$_3$] cysteine enrichments or in physiological concentrations of sulfur amino acids for chick embryos from breeders fed the control diet and embryos from breeders fed diets containing no pyridoxine or folic acid. Supplementation of B$_6$ or folic acid did not affect the production of methionine and cysteine in chick embryos. However, breeders fed the control diet with both folic acid and pyridoxine supplementation produced embryos with a two-fold reduction of hepatic homocysteine and increased spermine compared with embryos from breeders fed diets containing no supplemental pyridoxine or folic acid ($P < 0.05$). Hepatic S-adenosylmethionine for embryos from breeders fed no supplemental B$_6$ was half the concentration compared with embryos from breeders fed the control diet. Embryos from breeders fed the control diet were utilized to determine the proportion of homocysteine going through remethylation and transsulfuration and also to determine the pathway of remethylation. Sixty-five percent of the methyl groups used for homocysteine remethylation from control embryos was via the MFMT pathway. Alternatively, 61% of homocysteine from control embryos was remethylated via the MFMT and the BHMT reactions and 39% of homocysteine was catabolized to cysteine via the transsulfuration pathway. These data show that in embryos, intermediate metabolites of methionine and polyamines increase in concentration when pyridoxine levels are provided in deficient concentrations to the breeder hen. In addition, this research demonstrates that folic acid deficient embryos conserve methionine, rather than catabolize it to cysteine.

Key words: vitamin, broiler breeder, methionine metabolism, stable isotopes, chick embryos

INTRODUCTION

Previous studies have shown that methionine metabolism is critical for embryo and fetal growth and development as a result of involvement in protein synthesis, polyamine synthesis, formation of cysteine, regeneration of active folic acid, DNA synthesis, and DNA methylation (Sturman et al., 1970a,b; Gaull et al., 1972; Gaull et al., 1973; Rosenquist et al., 1996; Rosenquist and Finnell, 2001; Mudd et al., 2001; Stipanuk, 2004; Kalhan and Marczewski, 2012). Owing to its involvement in metabolic processes and oxidative stress, a nutritional imbalance of methionine can lead to a variety of metabolic problems. For instance, prior studies have suggested that altered methionine supplies impair normal methionine metabolism in laying hens and broilers and methionine metabolism plays an important role in chick embryo growth and development (Cao et al., 1995; Lu et al., 2020a, 2020b). In addition, Rosenquist and Finnell reported that increased homocysteine levels impaired chick embryonic development. In

separate studies with chick embryos and broiler chicks hatched from breeders fed control nutrient and vitamin formulations, Lu et al. (2020a, 2020b) indicated lower levels of cystathionine ß-synthase activity may be limiting cysteine formation during embryogenesis and during the early period of broiler grow-out. High levels of cystathionine found in the human fetus have been shown to be related to a limitation of the transsulfuration pathway because the cystathionase (EC 4.4.1.1, C-ase) activity does not appear until after birth (Gaull et al., 1972). Providing adequate methionine to broilers and breeders ensures a metabolic pathway of methionine metabolism as shown in Figure 1, unless nutritional diversities arise (Finkelstein, 1990).

Vitamin B_6 and folic acid are 2 key vitamins utilized as coenzymes in methionine metabolism. Pyridoxal phosphate, an active form of B_6, plays a key role as coenzyme for serine hydroxymethyltransferase (**SHMT**), cystathionine ß-synthase (EC 4.2.1.22, CBS), and C-ase (Finkelstein, 2000). Vitamin B_6 has been shown to alter homocysteine metabolism by decreasing SHMT activity and by suppressing homocysteine catabolism via the transsulfuration pathway (Selhub, 1999; Martinez et al., 2000). Folic acid is involved through the 5-methyltetrahydrofolate-homocysteine methyltransferase (EC.2.1.1.13, MFMT) reaction which is one of 2 remethylation pathways for converting homocysteine to methionine. Low levels of folate lead to a reduction in methylation, inhibiting the synthesis of methionine and S-adenosylmethionine (**SAM**) (Finkelstein, 1990; Stipanuk, 2004). Bottiglieri (1996) has reported that deficiencies of folate can decrease methylation of homocysteine, leading to elevated homocysteine levels in blood and urine.

Stable isotope-labeled amino acids have been used to determine the kinetics of methionine, homocysteine, and cysteine in adult humans and rats (Storch et al., 1988; Young et al., 1997; Gregory et al., 2000; Martinez et al., 2000; Cuskelly et al., 2001; MacCoss et al., 2001). The stable isotope of an amino acid is a naturally occurring form of an amino acid (Sulzman, 2007). Several forms of isotopes exist, differing in the amount of neutrons (Ben-David and Flaherty, 2012). Positional variations in isotopes of atoms can exist and are known as isotopomers (Ben-David and Flaherty, 2012). The use of stable isotopes as metabolic tracers provides an opportunity for in vivo assessment of metabolic changes occurring in physiological systems. Vaccinations to chick embryos are often provided through in ovo injections in the amniotic fluid, considering its ability to deliver a substrate efficiently (Jochemsen and Jeurissen, 2002; Zhai et al., 2008; Keralapurath et al., 2010). This paper exploits the similar technique of vaccination to introduce isotopic molecules into the amniotic fluid to study methionine metabolism in 18-day-old embryos. To the authors' knowledge, the use of stable
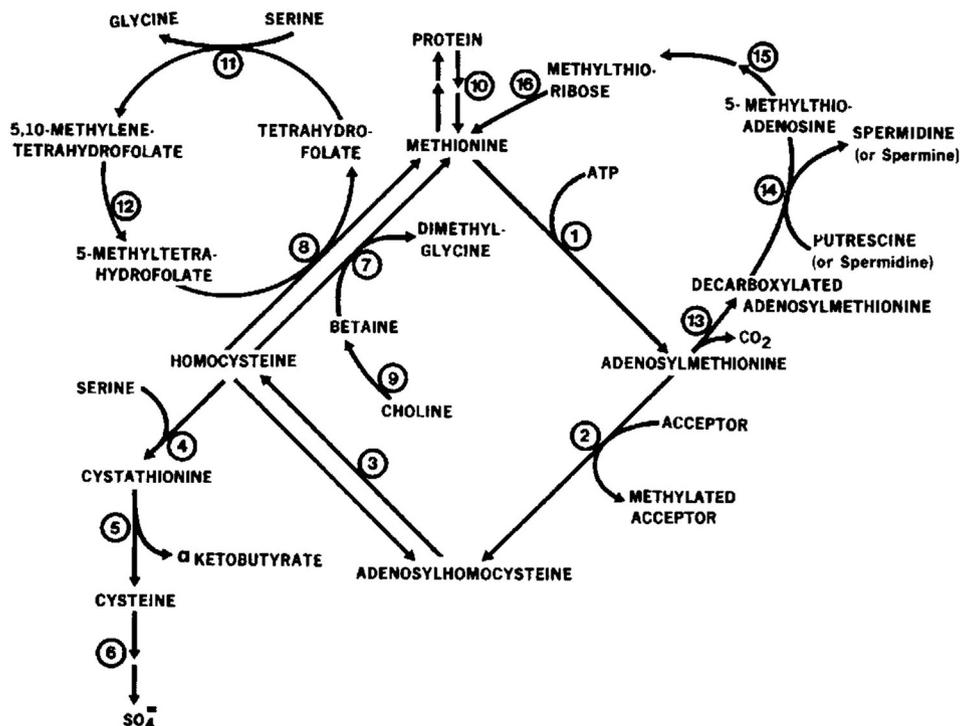
Figure 1. Methionine metabolism in mammals. The *numbers* represent the following enzymes or sequences: *1* methionine adenosyltransferase (EC 2.5.1.6), *2* AdoMet-dependent transmethylations, *3* adenosylhomocysteinase (EC 3.3.1.1), *4* cystathionine-b-synthase (EC 4.2.1.22), *5* g-cystathionase (EC 4.4.1.1), *6* further metabolism of cysteine, *7* betaine-homocysteine methyltransferase (EC 2.1.1.5), *8* methylfolate-homocysteine methyltransferase (EC 2.1.1.13), *9* choline + betaine aldehyde dehydrogenases (EC1.1.99.1 and EC1.2.1.8), *10* equilibrium between free and protein methionine, *11* serine hydroxymethylase (EC 2.1.2.1), *12* methylenetetrahydrofolate reductase (EC 1.7.99.5), *13* AdoMet decarboxylase (EC 4.1.1.50), *14* spermidine (spermine) synthase (EC 2.5.1.16 & EC 2.5.1.22), *15, 16* methylthioadenosine phosphorylase (EC 2.4.2.28) + methionine formation via methylthioribose-1-phosphate. Pathway drawing of methionine and cysteine metabolism is from the publication of Finkelstein, J. D., 1990. Permission to use figure granted by Dr. Finkelstein.

isotopes to study methionine kinetics for the purpose of evaluating the importance of folic acid and pyridoxine in chick embryos has not been reported at this time. Therefore, the objectives of the present study were 1) to investigate the effect of supplementing folic acid or pyridoxine in breeder diets on methionine metabolites and understand methionine kinetics evaluating remethylation and transsulfuration of homocysteine in liver tissue of 18-day-old chick embryos; 2) to determine the proportion of homocysteine remethylated to methionine by hepatic 5-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13, MFMT) compared with hepatic betaine-homocysteine methyltransferase (EC 2.1.1.5, BHMT) in 18-day-old chick embryos in folic acid and pyridoxine supplemented breeder diets.

MATERIALS AND METHODS

All procedures regarding the use of live animals in this study were conducted in accordance with the University of Arkansas Institutional Animal Care and Animal Use Protocol #03008.

Flock Management and Experimental Diets

Fifty-four Cobb 500 females (34-week-old) with similar body weights were randomly selected from a flock of Cobb broiler breeder hens (first egg, 180 d; peak egg production, 83%). The females were randomly assigned to 54 individual breeder cages and received one of the following dietary treatments for a 12-week period before egg collection: control, control-deficient pyridoxine, or control-deficient folic acid (Table 1). The breeder control diet (Table 1) was formulated to meet Cobb 500 female nutrient requirements with supplemental folic acid (1.25 mg/kg diet) and pyridoxine HCl (5 mg/kg diet) (Cobb-Vantress, 2016). Requirements of folic acid and pyridoxine for broiler-breeders in production, as published by Cobb-Vantress, are 3 and 6 mg/kg, respectively (Cobb-Vantress, 2018). Supplemental pyridoxine was omitted in the pyridoxine-deficient treatment. For the folic acid–deficient treatment, supplemental folic acid was removed from the diet. Amino acid analyses of the feed were conducted at the University of Missouri Agricultural Experiment Station Chemical Laboratories following AOAC Official Method 982.30. To determine folic acid concentration in the diets, AOAC Official Methods 960.46 and 992.05 (2005) were followed. In addition, pyridoxine HCl was measured by a commercial laboratory using a modification of AOAC Official Method 961.15. Feed allocation was 144 g per bird during the first week of the experiment. Feed was reduced 1 g per week until the hens were receiving 133 g during the last week of the experiment. The 144 g feed amount provided 420 kcal of ME/hen/day, which is lower than the energy recommendations by Cobb-Vantress (Cobb-Vantress, 2016). The amount of feed supplied to the hens was reduced because of the lower energy expenditure for hens housed individual cages, than for those in floor pens. This knowledge is based on our previous

findings (Reyes and Coon, 2003). Twenty-nine Cobb males that were the same age as the hens were used to artificially inseminate each breeder, once each week, with approximately 50 million sperm by using the procedure fully described by de Beer and Coon (2007). In short, this procedure involved massaging the saddle region of the male, collecting the semen, pooling the semen, and measuring the sperm cell concentration using an IMV MicroReader I (IMV Technologies, Minneapolis, MN). During the last week of the experiment, hatching eggs were collected from the inseminated hens and set in incubators. Eggs were incubated 18 d in Jamesway incubators (Jamesway Incubator Company, Cambridge, Ontario, Canada) at the University of Arkansas Poultry Research Farm before sampling. Incubation settings followed standard industry conditions in which the optimum temperature of the incubator was 100.5 degrees Fahrenheit. Relative humidity inside the incubator ranged from 50 to 55%.

Isotopic Infusion Procedures

Stable isotopes of L-serine (2,3,3-$^2H_3$, 98%; 15N 98%), L-betaine ($^2H_{11}$, 98%), and L-methionine (1-^{13}C, 99%) (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) were administered as a single bolus dose by amnion injection into the 18-day-old embryos from each treatment (control, basal-B6, and basal-folic acid). To administer the isotope, the egg was first removed from the incubator, candled for fertility, and weighed to determine body weight. Doses were 380, 380, and 95 μmol/kg of egg weight for [2H3] serine, [2H11] betaine, and [1-13C] methionine, respectively. The eggs were injected in ovo with the appropriate treatment using a 2.54-cm 21-gauge needle. To ensure that isotopic solution entered the amnion, the length of the needle was inserted entirely into egg. Six embryos per treatment were sampled at 0, 30, 60, and 120 min after injection. Liver tissues were then separated from sampled embryos. The 3 isotopic tracers described above were also separately administered by amnion injection into 18-day-old chick embryos from broiler breeders fed control diets. Doses are 3,420, 3,420, and 2,280 μmol/kg egg weight for L-serine (2, 3, 3-2H3, 98%; 15N 98%), L-betaine (2H11, 98%), and L-methionine (1-13C, 99%), respectively (Cambridge Isotope Laboratories, Inc., Tewksbury, MA). Four embryos were sampled at 0, 30, 60, 120, 240, and 480 min after injection to understand methionine formation via remethylation and transsulfuration and relative contributions of BHMT and MFMT pathways. Liver tissues from the 18-day-old embryos were collected. Immediately after removal, the liver samples were frozen and stored at −79°C until analysis.

Amino Acid Assay

Hepatic free methionine, cysteine, cystathionine, serine, and glycine analysis was completed using high-pressure liquid chromatography (HPLC) with AccQ-Tag derivatization and fluorescence detection (Cohen and Micheaud,

1993). Hepatic free homocysteine was determined following the procedure described by Gilfix et al. (1997) with tissue sample preparation modified by Uthus et al. (2002). Hepatic SAM and S-adenosylhomocysteine levels were obtained by using the procedure described by Wang et al. (2001). Hepatic concentrations of putrescine, spermidine, and spermine were determined with the procedure described by Merali and Clarkson (1996).

Determination of Isotopic Enrichments

The isotopic enrichment of methionine and cysteine were determined by HPLC-MS using reverse-phase chromatography and electro-spray ionization. The benchtop mass spectrometer (MS) was a Waters Quadrapole LCZ equipped with an electrospray ionization probe and interfaced with a Waters Alliance 2690 HPLC. The MS was operated in positive ion mode using the scan mode collecting mass data. Separation of the methionine isotopomers was accomplished following the procedure described by Pillai et al. (2006). Separation of the cysteine isotopomers was accomplished based on procedures reported by Lu et al. (2006) with modifications using a Synergi Fusion-RP 80 column (250 × 2 mm × 4 μm) (Phenomenex Co., Torrance, CA). The mobile phase followed a gradient elution profile of 0.1% trifluoroacetic acid with water (solvent A) and 0.1% trifluoroacetic acid with methanol (solvent B). The column equilibration time was 8 min between injections and the solvent flow rate was maintained at 0.1 ml per minute. The elution gradient profile followed was 0 min – 3% B; 8 min – 3% B, 18 min – 95% B; 25 min – 95% B; 27 min – 3% B; 35 min – 3% B.

Determination of Metabolic Pathways

Isotopic enrichment measurements for methionine and cysteine were performed to understand the remethylation and transsulfuration pathways (Lu et al., 2006; Pillai et al., 2006). Briefly, the relative contribution to methionine formation via homocysteine remethylation by MFMT pathway or BHMT pathway was calculated for control group based on the value of Met $(M + 2/M + 0)$/Met $(M + 3/M + 0)$. This expression was based on the assumption that [2H3] methionine was formed from betaine via BHMT and $[^2H_2]$ methionine was formed from 5-methyltetrahydrofolate, via MFMT, originally from serine. Both the contributions from BHMT pathway or MFMT pathway to methionine formation was expressed in percentage. Furthermore, the derivations on total remethylation and transsulfuration

pathways for homocysteine were made. The relative distribution of labeled serine between remethylation via MFMT and transsulfuration was calculated based on the value of $[^2H_2]$ methionine $(M + 2/M + 0)$/$[^2H_3]$ cysteine $(M + 3/M + 0)$. The relative distribution of homocysteine undergoing the remethylation pathway (via both MFMT and BHMT) compared with the transsulfuration pathway was measured by separately determining the expressions for remethylation or transsulfuration:

$$\text{Remethylation (via MFMT and BHMT)}, \% = A+B/(1 + A + B),$$

where

$$A = [^2H_2] \text{ Met } (M + 2 /[^2H_3]\text{cysteine}(M + 3 /M + 0);$$

$$B = A * 1 / \{[^2H_2]\text{Met } (M + 2 / M + 0) / [^2H_3]\text{Met } (M + 3 / M + 0)\};$$

$$\text{Transsulfuration}(\%) = 100$$
$$- \text{ Remethylation via MFMT and BHMT}(\%).$$

Measurement of Time-Course Labeling of Metabolites

The time course labeling of hepatic free methionine (^{13}C-Met) pool was measured over the 480 min after exposure of $[^{13}C]$ methionine for control group. Similarly, hepatic free cysteine enrichment ($^2H_{3\_}$ Cysteine) and hepatic free methionine ($^2H_2$- Met) were also measure at different time points over 120 min after exposure of $[^{13}C]$ methionine, [2, 3, 3-$^2H_3$]-serine, and $[^2H_{11}]$ betaine for all 3 treatment groups.

Statistical Analysis

All the data were subjected to one-way analysis of variance using SAS procedures (SAS Institute, Cary, NC). Differences between means for amino acids analyzed were compared using the Duncan multiple range test where control-deficient pyridoxine, or control-deficient folic acid was compared with the control group. Differences in mean enrichment values between treatments for time course of labeling for hepatic free methionine $(M + 1)$ pool, hepatic free cysteine $(M + 3)$ pool, and hepatic free methionine $(M + 2)$ pool were compared using all pairs, Tukey HSD test. Differences with an α level of $P < 0.05$ were considered to be statistically significant.
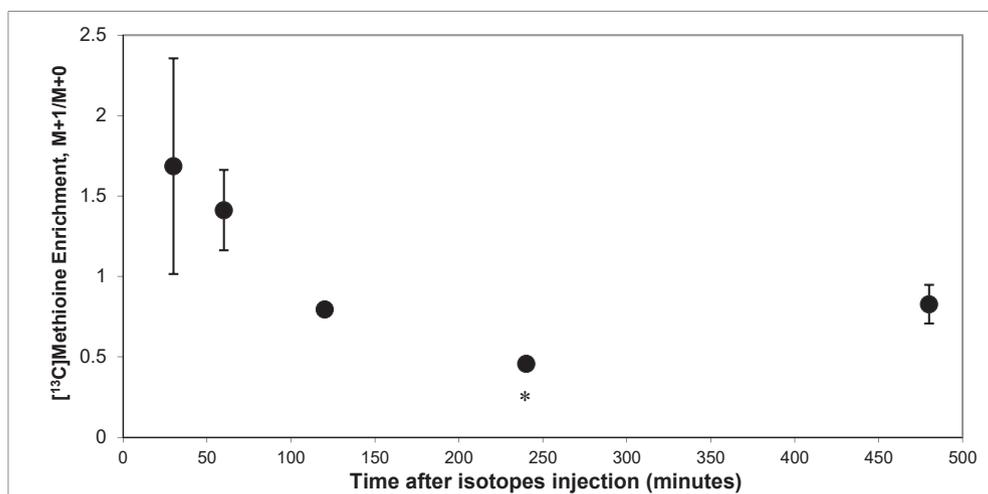
Figure 2. Time course of labeling hepatic free methionine (M + 1) pool from [^{13}C] methionine administered by amnion injection into 18-day-old chick embryos in control diet. *Note:* Data points are means ± SEM (n = 4). Asterisk (*) denotes significantly different mean value compared to other mean values at different time points ($P < 0.05$).

RESULTS

Hepatic Amino Acids, Cosubstrates, and Polyamines

The results showed there was no significant effect on embryonic hepatic levels of methionine, cysteine, serine, or glycine from the breeders fed the diet devoid of synthetic vitamin pyridoxine or folic acid (Table 2; $P > 0.05$). Although no differences in the analyzed amino acids were observed, significantly lower hepatic SAM levels from vitamin B$_6$ treatment and significantly higher hepatic homocysteine and lower spermine levels from both B$_6$ and folic acid treatment were observed (Table 2; $P < 0.05$).

Metabolic Pathways

The relative contribution to methionine formation by homocysteine remethylation via betaine-homocysteine methyltransferase (EC 2.1.1.5, BHMT) was 25 to 49% and via 5-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13, MFMT) was 51 to 75% 30 to 120 min after labeled betaine injection (Table 3). In addition, the relative distribution of labeled serine between remethylation pathway via 5-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13, MFMT) and transsulfuration pathway was obtained with the ratios of methionine (M + 2/M + 0) to cysteine (M + 3/M + 0). No significant change in the relative distribution of labeled serine between the remethylation pathway (via MFMT) and transsulfuration pathway was observed, although there was a decreased trend for the MFMT pathway and an increased trend for transsulfuration pathway with time (Table 4). A final calculation was conducted to determine the relative distribution of homocysteine between the remethylation pathway via MFMT and BHMT and the transsulfuration pathway (Tables 3

and 4). Sixty-one percent of homocysteine was remethylated via both the MFMT and BHMT reactions back to methionine in 18-day-old chick embryos (Table 5).

Stable Isotope and Isotopomer Enrichment

In the present study, a decrease in [^{13}C] methionine enrichment was found over time (Figure 1). In addition, [^{13}C] methionine enrichment at 240 min (0.45) after injection was significantly lower than at 30 min (1.8) (Figure 2; $P < 0.05$). Along with the isotopic enrichment, isotopomers were analyzed. Results showed that the M + 3 isotopomer of free cysteine did not appear within 30 min after injection; however, the enrichment was observed at 60 and 120 min after injection (Figure 3). No significant difference in cysteine enrichment (M + 3/M + 0) among dietary treatments was observed (Figure 3; $P > 0.05$).

Examination of the [^{2}H$_2$] methionine enrichment revealed significantly greater enrichment in chick embryos from breeders fed without pyridoxine supplementation 30 min after injection, as compared with enrichment from breeders fed control diets (Figure 4; $P < 0.05$). Alternatively, no significant changes after 1 or 2 h in hepatic [^{2}H$_2$] methionine in embryos from breeders fed the diets devoid of folic acid or pyridoxine supplementation was observed (Figure 4; $P > 0.05$) although both treatment groups were numerically higher than the control group.

DISCUSSION

The increased levels of hepatic homocysteine from the pyridoxine-deficient treatment compared with control diet indicated impairment in methionine and homocysteine metabolism (Table 2). The increase in hepatic homocysteine in chick embryos, as observed in the present study, agreed with previous reports of the elevations of
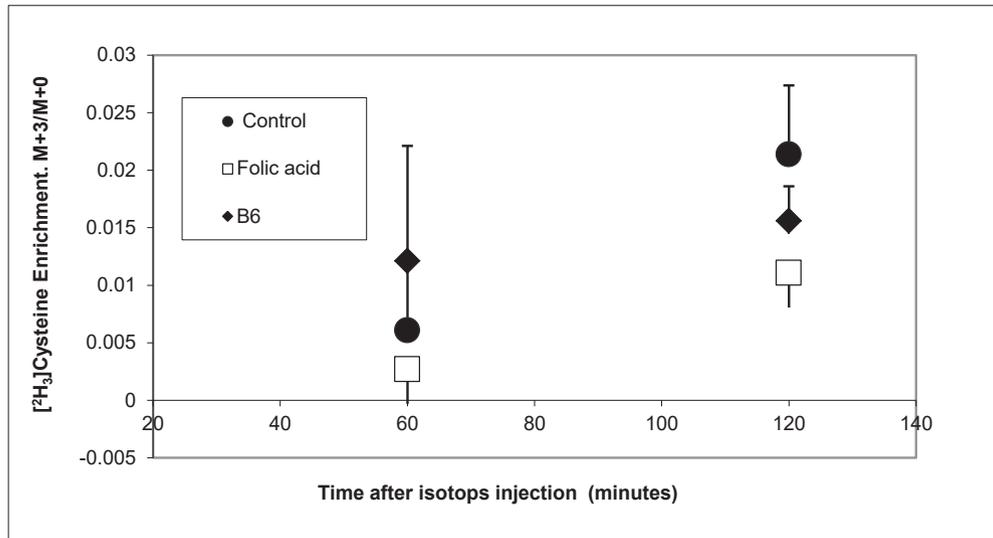
Figure 3. Time course of labeling of hepatic free cysteine pool from $[^{13}C]$ methionine, $[2,3,3-^2H_3]$-serine and $[^2H_{11}]$ betaine administered by amnion injection into 18-day-old chick embryos from breeders fed test diets devoid of folic acid or pyridoxine supplementation. *Note:* Data points are means $\pm$ SEM (n = 6).

plasma homocysteine in pigs, rats, and elderly humans that were deficient in vitamin B_6 (Smolin et al., 1983; Selhub, 1999; Martinez et al., 2000). Two-fold elevations in hepatic homocysteine of chick embryos from breeders fed deficient folic acid diets also indicated an impaired methionine/homocysteine metabolism, agreeing with previous reports in humans and in rats (Table 2) (Miller et al., 1994; Selhub, 1999; Cuskelly et al., 2001). Embryonic hepatic SAM levels in embryos decreased when breeders were fed a pyridoxine-deficient diet (Table 2). This was most likely due to an increase in phosphatidylethanolamine N-methyltransferase (**PEMT**)

activity, allowing the conversion of phosphatidylcholine at the expense of SAM. Shields et al. (2005) has reported that there is a high correlation between PEMT activity and plasma homocysteine. Thus, in the present study, the elevated homocysteine concentration from pyridoxine-deficient breeder diets could increase the activity of PEMT. In addition, it has been found that SAM levels were lower in rat livers that were deficient in B_6, further supporting the results observed in the present study (Martinez et al., 2000). The significantly lower levels of spermine from both pyridoxine and folic acid treatments may indicate a state of diapause, considering
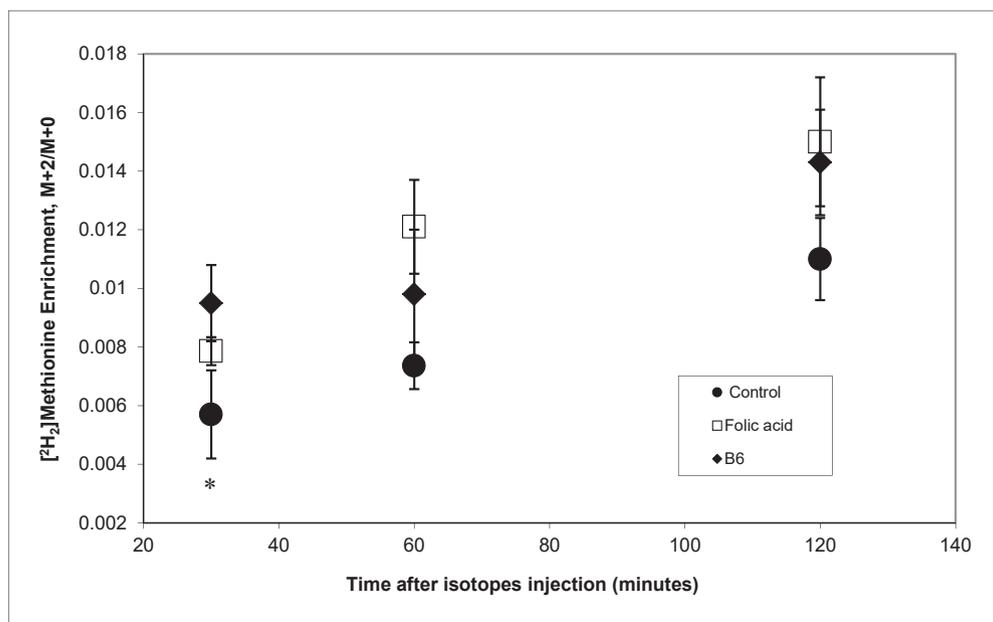
Figure 4. Time course of labeling of hepatic free methionine (M + 2) pool from $[^{13}C]$ methionine, $[2,3,3-^2H_3]$-serine and $[^2H_{11}]$ betaine administered via amnion injection into 18-day-old chick embryos from breeders fed test diets devoid of folic acid or pyridoxine supplementation. *Note:* Data points are means $\pm$ SEM (n = 6). Asterisk (*) denotes significantly different mean value between treatments within the time point ($P < 0.05$).

Table 1. Composition of broiler breeder diets[5] with supplemental folic acid and pyridoxine.

Ingredients	Unit	Control	No pyridoxine	No folic acid
Corn, ground	%	72.21	72.21	72.21
Soybean meal, 49% CP	%	17.20	17.20	17.20
Dicalcium phosphate, 18.5%	%	1.59	1.59	1.59
Calcium carbonate	%	7.12	7.12	7.12
Poultry fat	%	0.73	0.73	0.73
Selenium premix 0.06%	%	0.02	0.02	0.02
Ethoxyquin 66%	%	0.02	0.02	0.02
Kemin Myco Curb® 50% propionic acid	%	0.05	0.05	0.05
Sodium chloride	%	0.38	0.38	0.38
DL-Methionine	%	0.16	0.16	0.16
L-Lysine HCl	%	0.07	0.07	0.07
L-Threonine	%	0.04	0.04	0.04
Vitamin premix[1]	%	0.01	0.01	0.01
Copper sulfate	%	0.05	0.05	0.05
Trace mineral premix[2]	%	0.06	0.06	0.06
Choline chloride, 60%	%	0.20	0.20	0.20
Folic acid, 98%	mg/kg	1.25	1.25	-
Pyridoxine HCl	mg/kg	5.0	-	5.0
Calculated nutrient composition				
ME	kcal/kg	2,915		
CP	%	16.0		
Calcium	%	3.1		
Available P	%	0.41		
Methionine	%	0.45		
Methionine + cystine	%	0.74		
Nutrient analysis				
Folic acid[3]	mg/kg	0.97		
Pyridoxine HCl[4]	mg/kg	6.57		

[1]The vitamin premix provided the following per kilogram of diet: vitamin A, 11,000 IU; vitamin D_3, 1,750 IU; vitamin E, 40 IU; vitamin K, 5 mg; thiamin, 2.5 mg; niacin, 45 mg; biotin, 0.2 mg; pantothenic acid, 20 mg; vitamin B_{12}, 20 μg; riboflavin, 10 mg.

[2]The mineral premix provided the following per milligram per kilogram of diet: Mn, 74.5; Fe, 242.3; Zn, 86.8; Cu, 14.3.

[3]Folic acid was measured by commercial laboratories using Official Methods of Analysis of AOAC International 960.46 and 992.05, 2005.

[4]Pyridoxine HCl was measured by commercial laboratories using Official Methods of Analysis of AOAC International 961.15 (Modified), 2000.

[5]Diets are presented on "as is" basis in the diet table.

polyamines are necessary for DNA synthesis and cell proliferation. However, this mechanism of impaired spermine synthesis needs further study before conclusions are drawn.

The reduction of [13C] methionine enrichment with time in liver potentially indicates an increase in net protein synthesis or protein deposition of whole-body embryo with an assumption that chick embryos utilize amnion fluid at a constant rate (Figure 2). This observation is consistent with the previous report by Muramatsu et al. (1987) that suggests the chick embryo has a quick increase in protein content from the time of incubation. Considering the [13C] methionine enrichment decreased over time, an analysis on the specific pathways was conducted for clarity of the nutritional and metabolic end points of this particular amino acid. Examination of samples revealed that the amount of remethylation from BHMT pathway (25–49%) was similar to the 16 to 48% remethylation determined in broiler chick livers with in vitro isotope betaine administration (Pillai et al., 2006). N5-methyltetrahydrofolatehomocysteine accounted for about 65% of methionine formation via remethylation pathway in the chick embryo, which was in agreement with human research showing the MFMT pathway is the primary pathway for

remethylation (Table 3) (Selhub, 1999). In the embryo study presented herein, [2, 3, 3-$^2H_3$]-serine was selected as a tracer to provide indications of remethylation flux through MFMT by [$^2H_2$] methionine and transsulfuration flux by [$^2H_3$] cysteine. The relative shift from remethylation to transsulfuration over time after [2, 3, 3-$^2H_3$]-serine

Table 2. The effect of a breeder diet devoid of folic acid or pyridoxine supplementation on hepatic amino acid concentrations in 18-day-old chick embryos (nmole/g liver).

Compound	Control	No pyridoxine	No folic acid
Methionine	342.2 ± 32.1	377.1 ± 27.7	430.2 ± 39.5
Cysteine	128.7 ± 20.0	125.5 ± 14.3	153.8 ± 24.3
Serine	1,721.8 ± 127.4	1,792.3 ± 109.8	2,004.7 ± 171.6
Glycine	1,749.3 ± 126.9	1,776.9 ± 98.5	1,935.2 ± 166.2
SAM	120.6 ± 3.3	70.4 ± 2.1*	111.7 ± 6.1
SAH	37.0 ± 1.2	37.9 ± 0.5	40.5 ± 1.3
SAM/SAH	3.28 ± 0.2	1.86 ± 0.1	2.79 ± 0.2
Homocysteine	1.63 ± 0.2	4.10 ± 0.8*	3.54 ± 0.6*
Cystathionine	98.6 ± 10.4	65.0 ± 3.6	90.9 ± 11.6
Putrescine	90.0 ± 6.3	100.3 ± 2.5	96.5 ± 5.2
Spermidine	259.2 ± 21.4	257.6 ± 7.7	213.6 ± 9.6
Spermine	315.3 ± 27.4	241.0 ± 17.2*	157.2 ± 10.0*

Note: Each value is means ± SEM (n = 6). *Means significantly differ with that of the control in each row ($P < 0.05$).

Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Table 3. The relative contributions to methionine formation via remethylation between BHMT pathway and MFMT pathway.

Time (Min)	$[^2H_2]$Met (M+2/M+0)/$[^2H_3]$ met (M+3/M+0)[1]	MFMT[2] (%)	BHMT[2] (%)
30	3.04	75.25	24.75
60	1.02	50.57	49.42
120	2.76	73.41	26.59
Average	2.27	65	35

[1]Calculated from means of Met $(M + 2/M + 0)$ $(n = 4)$/means of Met $(M + 3/M + 0)$ $(n = 4)$. At each time point, 4 embryos received $[2, 3, 3-^2H_3]$-L-serine and another 4 embryos received $[^2H_{11}]$-L-betaine.

[2]$[^2H_3]$ methionine was formed from betaine via BHMT and $[^2H_2]$ methionine was formed from 5-methyltetrahydrofolate, via MFMT, originally from serine. The relative contribution to methionine formation via homocysteine remethylation by MFMT pathway or BHMT pathway was calculated based on the ratio of Met $(M + 2/M + 0)$/Met $(M + 3/M + 0)$.

amnion administration may indicate great demand of serine for synthesis of cysteine during chick embryogenesis because of the limitation in transsulfuration (Table 4).

Sixty-one percent of homocysteine was remethylated via both MFMT and BHMT reactions back to methionine in 18-day-old chick embryos (Table 5), which was higher than the values reported by Finkelstein and Martin (1984) and Storch et al. (1988) who conducted research on rats and humans, respectively. Storch et al. (1988) reported about 40% of homocysteine molecules formed were remethylated to methionine by using a constant intravenous infusion protocol, occurring over a 5-hour period, with [methyl-$^2H_3$]- and [1-^{13}C]-methionine in the postabsorptive state in human subjects. The 50% elevation in remethylation in chick embryos compared with adult humans may suggest there is a greater demand for methionine during embryogenesis due to higher protein synthesis rate, polyamines synthesis, and active methyl donor requirements. Storch et al. (1988) suggested that the conservation of methionine during the post-absorptive state is associated with protein synthesis, which is consistent with the suggestion by Lu et al. (2020a) that methionine conservation is primary in chick embryos and young hatched chicks for organ growth and protein deposition. Finkelstein and Martin (1984) demonstrated that remethylation accounts for 54% of homocysteine consumed in an in vitro system which approximated the in vivo condition in rat liver.

The M + 3 isotopomer of free cysteine did not occur until 60 or 120 min after injection for all test embryos (Figure 3). The slow formation of cysteine either with or without adequate vitamins may indicate slower cysteine synthesis if the cysteine requirement has not been adequately met for chick embryos at the late embryonic stage. The transsulfuration pathway may be limiting in 18-day-old chick embryos. The synthesis of cysteine from serine and homocysteine via transsulfuration pathway is catalyzed by cystathionine ß-synthase (EC 4.2.1.22) and cystathionase, and both of these enzymes require vitamin B_6. Lu et al. (2020b) recently reported lower levels of cystathionine ß-synthase in the E10-E21 embryo liver and from day 1 to day 7 in the liver of hatched chicks compared with cystathionine ß-synthase in 35 to 49 d broilers. No significant difference in cysteine enrichment $(M + 3/M + 0)$ from the pyridoxine-deficient treatment (Figure 3) was observed, which is consistent with the findings of Davis et al. (2005) that vitamin B_6 restriction in young men and women did not change cysteine flux from transsulfuration pathway. This was determined by using a protocol of primed, constant infusion of $[^{13}C_5]$ methionine, $[3-^{13}C]$ serine, and $[^2H_2]$ cysteine (Davis et al., 2005). Martinez et al. (2000) reported enrichment data of cysteine $(M + 3/M + 0)$ with a dose-flooding protocol with $[^2H_3]$ leucine, $[1-^{13}C]$ methionine, and $[^2H_3]$ serine. A significant increase in cysteine $(M + 3/M + 0)$ enrichment was observed in a rat liver with a vitamin B_6 deficient diet. However, a significant reduction in cysteine $(M + 3/M + 0)$ enrichment was

Table 4. The relative distribution of labeled serine undergoing the MFMT remethylation pathway compared with metabolism through the transsulfuration pathway.

Time (min)	$[^2H_2]$ met (M+2/M+0)/$[^2H_3]$cysteine (M+3/M+0)[1]	Remethylation via MFMT (%)[2]	Transsulfuration (%)[2]
30	1.23 ± 0.67	55.16	44.84
60	0.81 ± 0.11	44.75	55.25
120	1.07 ± 0.59	51.69	48.31
240	0.26 ± 0.07	20.63	79.37
480	0.14 ± 0.02	12.28	87.72
Average	0.70	36.90	63.10

[1]Each value is means ± SEM $(n = 4)$. At each time point, 4 embryos received $[2, 3, 3-^2H_3]$-L-serine.

[2]The relative distribution of labeled serine between remethylation via MFMT and transsulfuration was calculated based on the value of $[^2H_2]$ methionine $(M + 2/M + 0)$/$[^2H_3]$ cysteine $(M + 3/M + 0)$.

Table 5. The relative distribution of homocysteine undergoing the remethylation pathway (via both MFMT and BHMT) compared with the transsulfuration pathway.

Time (min)	Remethylation via MFMT and BHMT[1] (%)	Transsulfuration[1] (%)
30	62.04	37.96
60	61.6	38.4
120	59.31	40.69
Average	61	39

[1]Calculated based on the data in Tables 3 and 4 in 18-day-old chick embryos. Remethylation via MFMT and BHMT (%) = A + B/(1+A +B), where A = [$^2H_2$] Met (M + 2/M + 0)/[$^2H_3$]cysteine (M + 3/M + 0), B = A*1/{[$^2H_2$]Met (M + 2/M + 0)/[$^2H_3$]Met (M + 3/M + 0)}; Transsulfuration (%) = 100 - Remethylation via MFMT and BHMT (%).

shown in rat plasma (Martinez et al., 2000). Martinez et al. (2000) suggested that increasing hepatic cysteine production may be the result of the system compensating for impaired whole-body transsulfuration. Ubbink et al. (1996) reported that a vitamin B_6 deficiency may contribute to impaired transsulfuration in humans, in accordance with the response of plasma concentration of homocysteine, cystathionine, and cysteine after a methionine load. The finding of unchanged cysteine (M + 3/M + 0) in 18-day-old chick embryos from breeders fed without pyridoxine supplementation may be attributed to a limited transsulfuration ability in chick embryos suggested by Lu et al. (2020a, 2020b). Moreover, in accordance with the dissociation constants of CBS (0.7 umol/L) and SHMT (27.5 umol/L) (Jones and Priest, 1978; Kery et al., 1999), it seems that during vitamin B_6 deficiencies, the SHMT can be affected, but little or no effect on CBS is observed.

The observation of significantly greater hepatic [$^2H_2$] methionine enrichment in embryos from hens fed pyridoxine-deficient diets, than in those from hens fed control diets, 30 min after injection (Figure 4), was consistent with the previous findings in rats that were subject to a dose-flooding procedure at 30 min (Martinez et al., 2000). However, at time points 60 or 120 min after injection, the [$^2H_2$] methionine enrichment was similar between those fed the control and deficient B6 diets. The results observed in this study were different than those reported by Martinez et al. (2000) in which rats, 60 or 120 min after injection, had a significantly reduced hepatic [$^2H_2$] methionine for those fed the B_6 deficient diet compared with those fed adequate B_6. Studies have shown that [$^2H_2$] methionine enrichment in the plasma was similar between adequate and deficient B_6 in humans (Cuskelly et al., 2001) and rats (Martinez et al., 2000). This behavior was similar in the present study where the deficiency of pyridoxine did not affect the homocysteine remethylation in livers of chick embryos.

The observation that there were no significant changes in hepatic [$^2H_2$] methionine enrichment from folic acid treatment (Figure 4) was consistent with the enrichment data of Cuskelly et al., in which humans were supplied with a constant infusion. However, Cuskelly et al. (2001) believed that folic acid deficiencies depressed the relative synthesis of methionine by homocysteine remethylation when $R_{appearance}$ Met/$R_{appearance}$ Leu was considered as the relative synthesis rate for methionine, based on the assumption that proteolysis of body protein would release methionine in direct proportion to the release of leucine.

CONCLUSIONS

Although the findings in the present study indicate that folic acid and pyridoxine are important for methionine metabolism and play a critical role in breeder performance, and thereafter, chick embryonic development, further research should be conducted to create a more representative database regarding methionine and cysteine kinetics in chick embryos. Studies with stable isotope amino acids are required to determine whether (or how) pyridoxine nutritional status affects the synthesis of cysteine in chick embryos. Research from the present study indicates the chick embryo prefers methionine conservation, rather than catabolism of methionine to cysteine. Therefore, supplying adequate folic acid and pyridoxine in broiler breeder diets is necessary for chick embryonic methionine metabolism.

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DISCLOSURES

The authors have declared that no competing interest exists.

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