Homocysteine Remethylation in Young Broilers Fed Varying Levels of Methionine, Choline, and Betaine

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ABSTRACT Methionine is critical in amino acid nutrition for chickens, yet details of the flux of Met metabolites in the avian system are lacking. This study explored the interactions among dietary choline (CHO), betaine (BET), and sulfur amino acid levels on growth and hepatic homocysteine (HCY) remethylation. Graded levels (0, 0.07, 0.11, and 0.24%) of DL-Met were added to diets adequate in CHO and deficient in sulfur amino acids (0.26% digestible Met, 0.26% digestible Cys). Each Met level was tested alone or with the addition of CHO (0.25%) or BET (0.28%). Broilers were reared from 8 to 22 d in raised wire floor battery cages, and the 12 dietary treatments were fed to 3 replicate pens containing 5 birds per pen. Weight gain and feed efficiency were maximized ($P < 0.05$) with addition of 0.11% supplemental Met, whereas feed intake was maximized ($P < 0.05$) with addition of 0.07% supplemental Met. Overall, growth parameters were not affected ($P > 0.05$) by CHO or BET addition. Hepatic tissue primed by the different dietary treatments was subjected to a newly developed stable isotope methodology and HPLC-mass spectrometry to quantify the impact of diet on HCY remethylation. Dietary Met level did not ($P > 0.05$) affect HCY remethylation, but remethylation through the Met synthase pathway was increased ($P < 0.05$) by addition of CHO or BET to diets containing deficient or excess levels of Met. Minimal changes in hepatic HCY remethylation through the betaine-homocysteine methyltransferase pathway occurred in response to dietary changes; therefore, data failed to support previous suggestions that BHMT might have a regulatory role when diets containing deficient or excess Met levels are fed. In contrast to previous suppositions based on enzyme activity, under most dietary conditions, the quantity of HCY remethylated by Met synthase appeared to exceed that remethylated by the alternate betaine-homocysteine methyltransferase pathway.

Key words: broiler, methionine, homocysteine, remethylation

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INTRODUCTION

The liver is an important site for metabolism of Met, which is the first-limiting amino acid in commercial poultry diets. In its role as a methyl donor (transmethylation), Met is converted to homocysteine (**HCY**), which lies at the crossroads of sulfur amino acid (**SAA**) metabolism (Figure 1). Formation of Cys may occur if HCY proceeds through the irreversible transsulfuration pathway; alternatively, HCY may be converted back to Met after addition of a methyl group by folate-vitamin B_{12}-dependent Met synthase (**MS**) or betaine (**BET**)-HYC methyltransferase (**BHMT**). The methyl group provided by BHMT is derived from BET, a product of choline (**CHO**) oxidation. Enzymes involved in transsulfuration are found at significant activity levels in liver, pancreas, and kidney tissues in rats (Mudd et al., 1965); similarly, BHMT activity is found predominantly in the liver. Some activity is also present in kidney tissue (Finkelstein et al., 1971; Finkelstein and Martin, 1984; Cao et al., 1995). In contrast, MS is widely expressed in avian and mammalian tissues (Stipanuk, 2004).

Most commercial poultry diets are deficient in Met, so there is a tremendous need for supplemental Met in commercial poultry diets. Thus, continued evaluation of SAA metabolism is an important part of working toward optimum diet formulation. In particular, interrelationships of dietary CHO, BET, Met, and Cys need to be further explored in poultry at the metabolic level. Because of the liver's essential and largely unique role in SAA metabolism, it is appropriate to focus on the impact of diet on SAA metabolism in the liver. Finkelstein and Martin (1984) reported that, in mammals, approximately 54% of hepatic HCY is remethylated to Met, and Storch et al. (1988) estimated that in young adult men, approximately 38% of HCY was remethylated to Met. Information about HCY flux through the hepatic remethylation systems in poultry is lacking.

A stable isotope technique has been developed to allow the quantification of hepatic HCY remethylation via

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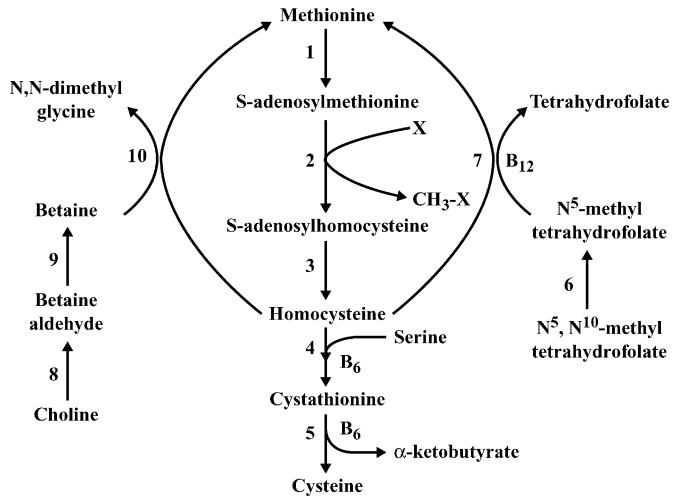

Figure 1. Metabolism of sulfur amino acids, choline, and betaine. Numerals indicate the following enzymes: 1) methionine adenosyltransferase, 2) various enzymes, 3) S-adenosylhomocysteine hydrolase, 4) cystathionine β-synthase, 5) cystathionine γ-lyase, 6) N^5,N^{10}-methylenetetrahydrofolate reductase, 7) methionine synthase, 8) choline dehydrogenase, 9) betaine aldehyde dehydrogenase, and 10) betaine-homocysteine methyltransferase.

Table 1. Composition of the sulfur amino acid deficient basal diet[1]

Ingredient	(%)
Corn	55.78
Peanut meal	32.10
Soybean oil	6.00
Dicalcium phosphate	2.00
Limestone	1.00
NaCl	0.40
Mineral mix[2]	0.15
Vitamin mix[3]	0.20
Choline Cl (60%)	0.12
L-Lys·HCl	0.75
L-Thr	0.31
L-Val	0.17
L-Trp	0.01
L-Ile	0.20
Corn starch	variable

[1]The diet was analyzed to contain 0.28% Met (0.26% digestible Met), 0.31% Cys (0.26% digestible Cys), and 19.0% CP; the diet was calculated to contain 3,200 kcal of ME/kg and was fed from 8 to 22 d posthatching.

[2]Provided (/kg of diet): 100 mg of Mn ($MnSO_4 \cdot H_2O$); 100 mg of Zn ($ZnSO_4 \cdot 7H_2O$); 50 mg of Fe ($FeSO_4 \cdot 7H_2O$); 10 mg of Cu ($CuSO_4 \cdot 5H_2O$); and 1 mg of I ($Ca(IO_3)_2 \cdot H_2O$).

[3]Provided (/kg of diet): 9,900 IU of vitamin A; 3,300 ICU of cholecalciferol; 13 IU of vitamin E; 0.013 mg of vitamin B_{12}; 6.6 mg of riboflavin; 66 mg of niacin; 1.1 mg of folacin; 1.1 mg of thiamin; 3.3 mg of pyridoxine; 0.11 mg of d-biotin; 0.20 mg of selenium; and 125 mg of ethoxyquin.

BHMT or MS in broilers. Previous research on enzyme activity indicated that dietary changes in SAA, CHO, or BET levels result in changes in the hepatic activity of BHMT in broilers and rats (Finkelstein et al., 1982; Emmert et al., 1996). Hence, we hypothesized that metabolite flux through remethylation pathways might, in some way be affected by nutritional status of the birds, as has been seen in previous studies in rats and humans (Storch et al., 1988). In this study an in vitro stable isotope method was employed to study HCY remethylation in avian hepatic tissue. The impact of SAA, CHO, and BET levels on growth parameters and HCY remethylation was explored.

MATERIALS AND METHODS

All procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee. Newly hatched commercial broiler chicks (Cobb 500, Cobb-Vantress, Inc., Siloam Springs, AR) were raised on floor pens and were fed a corn-soybean meal broiler starter diet that met or exceeded NRC (1994) recommendations. On d 8, chicks were allocated to battery cages (5 birds per pen) by weight to obtain similar ($P > 0.05$) mean initial BW. Each of the 12 dietary treatments was replicated with 3 pens; treatments were fed from 8 to 22 d. A corn-peanut meal basal diet (Table 1) was formulated to meet NRC (1994) recommendations for broilers with the exception of Met and Cys. Dietary SAA levels were designed to range from deficient (0.59% total SAA) to roughly adequate (0.83% total SAA), and dietary treatments consisted of graded levels of DL-Met (0, 0.07, 0.11, or 0.24%) added to the basal diet either alone or with excess isomethyl levels of CHO (0.25%) or BET (0.28%). Body weight, feed intake, and mortality were recorded, and BW gain, feed intake, and feed efficiency (adjusted for mortality) were calculated. At termination of the growth assay, hepatic tissue from the experimental birds were collected, pooled by pen, and stored at −80°C.

Methods for assessing HCY remethylation were based on an in vitro stable isotope technique similar to that used by Storch et al. (1988, 1991) and van Guldener et al. (1999), with necessary modifications. Frozen liver tissue (2 g) from experimental birds was suspended in double the volume of a potassium buffer (4.7 g of KCl, 0.14 g of KH_2PO_4, 0.037 g of EDTA, 0.391 mL of L-2-mercaptoethanol, and water to total 500 mL), homogenized and centrifuged. The supernatant was then collected. The protein content of each of sample was estimated, and the protein contents in the different sample preparations (measured in mg/mL) were determined to be similar ($P > 0.05$; data not shown). Throughout the procedures, the temperature was maintained at 4°C to slow metabolism in the homogenate. Respective samples of homogenates (350 μL) were then incubated in a water bath at 37°C for 0 or 10 min after adding 50 μL of a 50 m*M* solution of HCY (Aldrich Chemical Company, Milwaukee, WI) and 20 m*M* solution of a stable isotope of betaine (Cambridge Isotope Laboratories, Inc., Andover, MA) (D^{11}-betaine) in K buffer. From preliminary studies (data not shown), the incubation time of 10 min was found to be within the linear response range for enzyme activity and substrate concentration. Adding 2 mL of 2N HCl to the tubes at the respective time periods terminated the reaction. The resulting mixture was centrifuged and then spiked with a stable isotope of phenylalanine (D^5-phenylalanine) by adding 100 μL of 25 μg solution of the D^5-phenylalanine in K buffer/mL to 400 μL of the supernatant of the incubate. The supernatant was then subjected to HPLC-mass spectrometry analysis for the quantification of Met isotopomers.

The Met isotopomers and the internal standard were separated and quantified using an HPLC-mass spectrometry system, employing reverse-phase chromatography and electro-spray ionization (Barnes et al., 1999). A Waters platform LCZ bench top mass spectrometer that was interfaced with a Waters Alliance 2690 HPLC was used for the quantification of Met isotopomers and the internal standard. The mass spectrometer was equipped with an electrospray ionization probe set to operate in a positive ion mode. The mass spectrometer operating parameters were optimized for detection of the isotopic enrichment. Separation of the underivitized amino acid isotopomers was accomplished using a Waters Symmetry C18 column (2.1 × 150 mm × 5 μm), employing a gradient elution profile of 0.1% trifluroacetic acid with water and 0.1% trifluroacetic acid with acetonitrile.

Once the peak area of the Met isotopomers and the phenylalanine internal standard were quantified, the Met isotopomer peak areas were standardized using the corresponding internal standard peak areas. The standardized peak area values from 0- and 10-min incubation periods were used to arrive at conclusions about remethylation of HCY. The Met formed by remethylation of HCY through the BHMT pathway had a molecular weight of 152 u (enriched MET) because it carried a methyl group received from the BET stable isotope, which is heavier by 3 a.m.u. than a regular methyl group; Met formed through the MS pathway (native Met) had a normal molecular weight (149 a.m.u.). Thus, in this reaction system, the remethylated Met that was formed through BHMT had a higher molecular weight than the Met that was formed through MS. During the 10-min incubation period, it is likely that a certain amount of Met turnover occurred in the reaction system; thus, the system does not measure all new Met per se; rather, the net Met change that occurred during the 10-min incubation was measured. The difference in the standardized peak areas of 0- and 10-min incubations gave a representation of the quantity of Met that was formed through remethylation, and these peak area values were used to arrive at conclusions about the behavior of the 2 remethylation pathways when the birds were fed different levels of SAA, CHO, and BET.

Data from the HPLC-mass spectrometry analysis was processed, and the peak areas from the 0- and 10-min incubation were used to calculate the following parameters.

- Net change in native Met. This is the difference between the native Met peak (molecular weight of 149) areas after 10- and 0-min incubations and represents the net change in Met formed through the MS pathway.
- Net change in enriched Met. This is the enriched Met peak area after a 10-min incubation period and represents the net change in Met formed through the BHMT pathway.
- Net change in total Met. This is the sum of the net change in native and enriched Met, representing total remethylation through the BHMT and MS pathways.
- Percentage of remethylation through the BHMT pathway. This represents the proportion of remethylation that occurred through the BHMT pathway, calculated by dividing enriched Met by total Met.

Statistical Analysis

Pen means were considered the experimental unit, and data were subjected to analysis of variance (SAS, 2004) as a completely randomized design; treatment means were separated using least significant difference multiple comparison procedures or Duncan's multiple range test. Single df contrasts were used to test overall effects of Met, CHO, and BET (when appropriate). The level at which differences were considered significant was $P < 0.05$.

RESULTS AND DISCUSSION

The overall impact of dietary treatment on growth performance is presented in Table 2. Weight gain and feed efficiency increased linearly ($P < 0.05$) up to 0.11% supplemental Met, whereas feed intake was maximized at 0.07% Met supplementation. Overall, excess CHO and BET did not ($P > 0.05$) impact growth performance. The response to supplemental Met was interesting because the experimental birds showed a maximum growth response at a level of Met supplementation (0.70% total SAA) lower than that recommended by NRC (1994). Previous observations in our laboratory (unpublished data) also indicate a potentially lower requirement for Met and Cys for broilers during the starter period; further research is needed to determine whether maximum growth performance can be obtained in currently available commercial broiler chicks with lower-than-recommended levels of Met and Cys.

Although the overall impact of excess CHO and BET on growth performance was not significant ($P > 0.05$; Table 2), small increases in BW gain did occur when CHO or BET were added to the diet devoid of supplemental Met. Several authors have reported that supplemental CHO or BET increased BW gain of birds fed diets marginally deficient in Met (Quillin et al., 1961; Pesti et al., 1980; Emmert et al., 1996). Thus, in marginally Met-deficient diets, it appears that CHO, BET, or Met can lead to an equivalent growth response in broilers and that CHO and BET can spare a small portion of the Met requirement, presumably by supplying the portion of the Met requirement that is needed for methyl groups.

As occurred with the current study, growth responses to supplemental CHO or BET have been previously noted in diets substantially deficient in MET or SAA (Emmert et al., 1996). However, with diets severely deficient in Met, growth responses to supplemental CHO or BET have been far below those observed with supplemental Met. The slight response to CHO or BET might have been due to increased HCY remethylation, and thus cellular Met levels, brought about by increased activity of remethylation enzymes. In the current study, HCY remethylation was indeed increased by supplemental CHO or BET in

Table 2. Impact of Met, choline (CHO), and betaine (BET) on growth[1]

DL-Met	CHO[2]	BET[2]	Gain	Feed intake	Gain:feed
	(%)		(g)		(g/kg)
0%	0	0	390[e]	715[d]	548[e]
	0.25	0	429[de]	723[d]	593[de]
	0	0.28	466[cd]	769[bcd]	607[cde]
0.07%	0	0	541[ab]	807[abc]	677[abcd]
	0.25	0	521[bc]	816[abc]	639[bcd]
	0	0.28	537[ab]	807[abc]	665[abcd]
0.11%	0	0	576[ab]	790[bc]	729[a]
	0.25	0	563[ab]	800[abc]	704[ab]
	0	0.28	580[ab]	815[abc]	712[ab]
0.24%	0	0	586[a]	853[a]	687[abc]
	0.25	0	518[bc]	759[cd]	683[abcd]
	0	0.28	578[ab]	819[ab]	707[ab]
Pooled SEM			21.5	20.4	30.9
			P		
Met effect			<0.0001	<0.0001	<0.0006
CHO effect			0.08	0.08	0.54
BET effect			0.07	0.13	0.43

[a–e]Means within the column that lack a common superscript differ ($P < 0.05$).

[1]Replicate pens contained 5 chicks per pen. The basal diet was analyzed to contain 0.28% Met (0.26% digestible Met) and 0.31% Cys (0.26% digestible Cys); experimental diets were fed from 8 to 22 d posthatching.

[2]Choline and BET were added on an isomethyl basis.

diets containing deficient levels of SAA (Table 3), but a growth response was only noted in the diet containing no supplemental Met, bringing into question the correlation between increased remethylation and growth. Further studies are needed to elucidate the mechanism underlying growth responses to CHO or BET in diets deficient in SAA.

Results from the stable isotope study are presented in Table 3. Dietary Met level did not significantly ($P > 0.05$) affect BHMT- or MS-dependent HCY remethylation or the relative proportion of HCY that was remethylated by BHMT. Addition of CHO to the basal diet (0% supplemental Met) significantly ($P < 0.05$) increased hepatic capacity for total HCY remethylation (as indicated by peak total MET levels), but in broilers fed supplemental Met, only small numerical increases in total HCY remethylation were observed. In contrast, addition of BET to diets containing 0, 0.07, or 0.24% supplemental Met increased ($P < 0.05$) total HCY remethylation. Remethylation through the MS pathway appeared to account for most changes

Table 3. Effect of Met, choline (CHO), and betaine (BET) on hepatic homocysteine (HYC) remethylation[1]

Dietary treatment			Estimate of HYC remethylation			
Met	CHO[2]	BET[2]	Native Met[3]	Enriched Met[4]	Total Met[5]	BHMT contribution[6]
	(%)		Peak area in millions			(%)
0%	—	—	1.9[d]	1.0[bc]	2.9[c]	35.8[abc]
	0.25	—	5.6[b]	1.4[abc]	7.0[b]	20.8[cd]
	—	0.28	6.9[ab]	1.4[abc]	8.3[ab]	16.3[d]
0.07%	—	—	1.8[d]	0.8[c]	2.6[c]	34.1[abc]
	0.25	—	2.7[cd]	0.7[c]	3.5[c]	20.2[cd]
	—	0.28	7.9[a]	1.6[ab]	9.5[a]	16.4[d]
0.11%	—	—	1.6[d]	1.2[abc]	2.7[c]	48.3[a]
	0.25	—	2.7[cd]	0.7[c]	3.5[c]	22.4[bcd]
	—	0.28	2.9[cd]	0.8[c]	3.7[c]	23.7[bcd]
0.24%	—	—	1.4[d]	0.9[c]	2.3[c]	38.0[ab]
	0.25	—	3.0[cd]	1.2[abc]	4.2[c]	29.3[bcd]
	—	0.28	5.0[bc]	1.8[a]	6.8[b]	28.1[bcd]
Pooled SD			1.24	0.36	1.40	8.45

[a–d]Means within a column lacking a common superscript differ ($P < 0.05$).

[1]Replicate pens contained 5 chicks per pen. The basal diet was analyzed to contain 0.28% Met (0.26% digestible Met) and 0.31% Cys (0.26% digestible Cys); experimental diets were fed from 8 to 22 d posthatching.

[2]Choline and BET were added on an isomethyl basis.

[3]Represents HCY remethylated through the Met synthase pathway.

[4]Represents HCY remethylated through the BHMT (BET-HYC methyltransferase) pathway.

[5]Represents total HCY remethylation through both remethylation pathways (sum of native and enriched Met).

[6]Remethylation through BHMT pathway expressed as a percentage of total remethylation.

in total remethylation. Remethylation of HCY by MS was significantly increased ($P < 0.05$) by CHO addition to the basal diet (0% supplemental Met) and numerically increased by CHO addition to diets containing supplemental Met. Remethylation of HCY by MS was significantly ($P < 0.05$) increased by BET addition to diets containing 0, 0.07, and 0.24% supplemental Met and was numerically increased by BET addition to the diet containing 0.11% supplemental Met. Diet appeared to have little impact on BHMT-dependent remethylation; the only significant changes were slightly increased ($P < 0.05$) HCY remethylation in response to addition of BET to diets containing 0.07 and 0.24% supplemental Met.

In comparing peak values for Met formation (Table 3), it appears that remethylation of HCY by BHMT was lower in magnitude under all dietary conditions, which suggests that MS is the predominant route of remethylation in the avian hepatic system regardless of the dietary content of Met or the presence of excess CHO or BET. The relative importance of MS becomes more evident when looking at remethylation through the BHMT pathway as a percentage of total remethylation (Table 3). Increasing dietary Met did not significantly ($P > 0.05$) impact the proportion of HCY that was remethylated through the BHMT pathway, even though there was a numerical increase at the 0.11% addition of Met. Addition of excess CHO or BET tended to reduce ($P < 0.05$) the proportion of remethylation through the BHMT pathway, in some cases significantly; and the impact of BET was more pronounced than CHO when added to diets containing 0 or 0.07% supplemental Met.

Reports from research with rats and human beings (Mudd et al., 1970; Finkelstein and Martin, 1984) indicted that remethylation of HCY may be equally partitioned through the MS and BHMT pathways. In their study of the response of dietary changes in Met, CHO, and BET on hepatic remethylation enzyme activities in chicks, Saunderson and Mackinlay (1990) observed that the activity of BHMT was higher than that of MS in avian liver. Those researchers concluded that, in chickens, the transfer of methyl groups from BET principally accomplishes the remethylation of HCY. Our results differ from these findings under most of the dietary conditions used in this experiment. The proportion of HCY remethylated by BHMT in most cases was <40% and in some cases was <20% (Table 3). It was interesting, however, that for broilers fed 0.11% supplemental Met (the level at which growth performance was maximized) without excess CHO or BET, the proportion of HCY remethylated by MS or BHMT was roughly equivalent. Thus, for broilers fed diets containing apparently adequate SAA levels without excess methyl donors (CHO or BET), HCY appears to be partitioned equally between the remethylation pathways.

Most previous studies have measured enzyme activity as a means of assessing the impact of diet on hepatic SAA metabolism. In chicks and rats, hepatic BHMT activity increased when Met-deficient diets containing adequate or excess CHO and BET were fed (Emmert et al., 1996; Park et al., 1997; Park and Garrow, 1999). Finkelstein et al. (1982) also observed increased BHMT activity when a Met-free diet or a diet containing excess Met was fed to rats. They reported that activity of BHMT increases with a higher intake of protein and Met, whereas the activity of MS increases when the body is in need of Met. They speculated that BHMT activity functions to conserve Met under conditions of dietary deficiency. This result suggested a role for BHMT that agrees with the observations of Frontiera et al. (1994), who studied regulation of Met metabolism in human subjects and hypothesized that metabolic changes occur at extreme excess or extreme deficiency of dietary Met in an attempt to maintain or stabilize cellular Met levels.

In the current study in which actual HCY flux was assessed, only minimal changes in BHMT-dependent HCY remethylation were noted in response to dietary changes in Met, CHO, or BET levels that, based on previous research, would be expected to increase BHMT activity. Therefore, it does not appear that BHMT was acting to conserve cellular MET levels when dietary Met was deficient or to dispose of excess HCY when dietary Met was in excess, regardless of dietary CHO and BET levels. Rather, MS appeared to be more responsive to dietary changes, but only when excess CHO or BET was fed. It is important to note that our basal diet was deficient in both Met and Cys, whereas the diets of Emmert et al. (1996) and Finkelstein et al. (1982) contained adequate or excess Cys. Dietary Cys levels have been shown to impact transsulfuration (Yamamoto et al., 1995); thus, Cys level could potentially impact hepatic HCY remethylation.

The mechanism by which excess dietary CHO or BET could impact hepatic HCY remethylation via MS is not immediately clear, but several observations may be made. Activity of BHMT is inhibited by N,N-dimethylglycine, one of the by-products of the BHMT reaction (Stipanuk, 2004). Perhaps in diets containing deficient Met and excess CHO or BET, conditions previously shown to increase BHMT activity in broilers (Emmert et al., 1996), the increased flux that would be expected from higher activity is countered by increased production of N,N-dimethylglycine, resulting in increased inhibition of BHMT. There is also a link between N,N-dimethylglycine and the MS pathway. Oxidative demethylation of N,N-dimethylglycine results in the formation of sarcosine, which can infuse methyl groups into the folate pool.

Hepatic serine levels may also be relevant. In a study on hepatic HCY remethylation, Cuskelly et al. (2001) noted that methyl groups from 5,10-methylenetetrahydrofolate may be used either for the synthesis of Ser (from Gly) or for the remethylation of HCY. When the availability of labile methyl groups is minimal, methyl groups from 5,10-methylenetetrahydrofolate may be directed toward Ser synthesis. In contrast, when adequate methyl groups are present, HCY remethylation may be favored. Gregory et al. (2000) also studied the role of Ser on HCY remethylation and observed that 2 pools of Ser exist in one-carbon metabolism. In the presence of excess methyl groups in the system, Ser metabolism may provide methyl groups for the production of more 5-methylenetetrahy-

drofolate, thereby resulting in a greater amount of remethylation through MS. In our study, dietary treatments that increased HCY remethylation by MS contained excess methyl groups from excess dietary CHO or BET, thereby perhaps exhibiting an indirect influence on MS-dependent remethylation through the combined effects of N,N-dimethylglycine and Ser.

In conclusion, the in vitro stable isotope technique using hepatic tissue primed with dietary treatments appeared to be useful for studying HCY remethylation. A significant correlation of HCY remethylation and growth in broiler starter birds that were fed diets varying in SAA with or without excess CHO or BET was not observed. Further, contrary to indications from previous research based on enzyme activity, BHMT did not exhibit a regulatory role, and a greater proportion of HCY appeared to be remethylated by MS under most dietary conditions.

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