Sparing of Methionine Requirements: Evaluation of Human Data Takes Sulfur Amino Acids Beyond Protein\textsuperscript{1,2}

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ABSTRACT The intimate relation between amino acids and protein and nitrogen requirements is well recognized. Nutrition research has focused on the capacity of food to meet the need for nitrogen and indispensable amino acids (IAA) and led to the conclusion that the quality, not just the quantity, of protein is critical. This is especially relevant in regard to the sulfur amino acids (SAA) methionine and cysteine because of the increased understanding of their role in relation to chronic diseases (e.g., cardiovascular disease, dementia, cirrhosis), immunomodulation, DNA transcription, and RNA translation. Considerable effort has been expended to determine whether and to what extent cysteine can spare the requirement for the IAA methionine. In vivo studies in humans generally concur that the dietary requirement of the SAA ranges between 13 and 16 mg\textper kilogram\textper day, but how much can be met by cysteine relative to methionine remains controversial. This review examines the current status of in vivo estimates of methionine and cysteine requirements in human adults and considers needs beyond what is necessary for protein synthesis. Factors influencing the utilization of methionine and cysteine, especially those conditions that lead to toxicity on the one hand or beneficial effects on the other, are discussed. Data on alternative dietary sources of methyl groups (e.g., betaine, choline, phosphatidylcholine, S-adenosylmethionine, S-methylmethionine) or sulfur (e.g., N-acetylcysteine or L-2-oxothiazolidine-4-carboxylic acid) support a role for the SAA "beyond protein." Other pathways may influence the specific requirement for methionine and/or cysteine, especially when the person is challenged by disease, inadequate availability of food, or environmental stress. J. Nutr. 136: 1676S–1681S, 2006.

KEY WORDS: • methionine • cysteine • methyl groups • sulfur amino acid requirement • amino acids

Methionine, an indispensable amino acid (IAA),\textsuperscript{4} is required for protein synthesis, as a precursor for cysteine and as a donor of methyl groups for numerous methylation reactions. The dietary requirement for methionine, originally based on early nitrogen balance studies (1–4), is usually reported as a component of the requirement for total sulfur amino acids (SAA) that includes cysteine/cystine. Estimates of adult SAA requirements range between 13 and 16 mg\textper kilogram\textper day (17 to 27 mg\textper gram\textper protein)\textsuperscript{(5)}. Controversy arose as investigators attempted to demonstrate in vivo in humans that a significant proportion of total SAA requirements could be met by dietary cyst(e)ine.\textsuperscript{5}

\textit{In vivo studies to determine human methionine requirements.} The 1985 FAO/WHO/UNU report on energy and protein requirements lists as the upper limit for total SAA [methionine plus cyst(e)ine] the value of 13 mg\textper kilogram\textper day. Using a variety of stable isotopically labeled amino acids and study designs, investigators at the Massachusetts Institute of Technology (MIT) led by the late Vernon R. Young supported the probable adequacy of this estimate for total SAA, with the qualification that this requirement could not be met by a substantially lower intake of methionine that was supplemented with a generous amount of cyst(e)ine (6,7). This led to the proposal that a prudent diet would supply methionine at an intake that approaches, if not equals, the FAO/WHO/UNU requirement for total SAA and a simultaneous supply of cyst(e)ine (CYS) because cysteine may be more effective at maintaining cysteine and glutathione homeostasis (8). Using the indicator amino acid oxidation (IAAO) approach to further examine this issue, Di Buono et al. (9–11) confirmed the mean requirement for methionine as 12.6 mg\textper kilogram\textper day in the absence of exogenous cysteine but noted that a safe level of intake of total SAA for the population was substantially higher at 21 mg\textper kilogram\textper day. Furthermore, when they held cysteine intake constant at 21

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\textsuperscript{4} Abbreviations used: CYS, cyst(e)ine; GSH-glutathione; HCY, homocysteine; IAA, indispensable amino acid; IAAO, indicator amino acid oxidation; MIT, Massachusetts Institute of Technology; MET, methionine; NAC, N-acetylcysteine; NAFLD, nonalcoholic fatty liver disease; OTZ, \textit{l}-2-oxothiazolidine-4-carboxylic acid; SAA, sulfur amino acids; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TM, transmethylation; TS, transsulfuration.

\textsuperscript{5} Cyst(e)ine denotes both cysteine and cystine.
mg·kg⁻¹·d⁻¹, they found a breakpoint in the rate of CO₂ release from L-[¹⁴C]phenylalanine, the indicator AA, at a methionine intake of 4.5 mg·kg⁻¹·d⁻¹ and concluded that dietary cysteine could “spare” the requirement for methionine. Differences in study design (e.g., length of dietary adaptation) and underlying assumptions of the model account for the varying opinions and are discussed in greater detail by Professor Ball in this issue (12). However, we are reminded by the commentary “The Feast of the Assumptions” by M. Wiseman that “there is no single right answer . . . and we should bear that in mind in applying our imperfect estimates, especially where the stakes are high.” He went on to quote Oliver Cromwell: “In the bowels of Christ, gentleman, I beseech you to remember you may be mistaken” (13). This humbling thought should be at the heart of the practice of nutrition scientists and health professionals, especially as new technology helps to better define “requirements.”

Cysteine sparing of SAA requirements: Is the drive the requirement for methyl groups? Animal studies have shown that ~50–60% of the SAA requirement could be met by dietary cysteine (1,8). Several studies in humans, summarized in Table 1, do not differ significantly in their outcomes but rather in the interpretation of the data. The consensus appears to be that the requirement for SAA ranges from 13 to 21 kg·d⁻¹, that the need for methionine alone is between 5 and 13 mg·kg⁻¹·d⁻¹, and that cyst(e)ine may spare the requirement for methionine. The magnitude of the effect, however, depends on the individual’s age, nutritional status, and health. The conclusions drawn are subject to the different experimental approaches and underlying assumptions, such as 1) length of dietary stabilization, 2) choice of isotope and modeling parameters, and 3) variation in the individual’s vitamin and methyl group status. However, one thing that is clear is that human SAA requirements must extend beyond the need of methionine and cysteine for protein synthesis. The determination of the actual requirement may be driven by the requirement for methyl groups for the synthesis of multiple other compounds.

The first step in methionine metabolism is the formation of S-adenosyl methionine (SAM) via activation of methionine by ATP. In addition to a recent review (8), Stipanuk et al. describe this in detail in this issue. SAM can subsequently transfer its methyl group via a variety of methyltransferases for the synthesis of cellular components such as creatine, epinephrine, and catecholamine, DNA, RNA, proteins, and choline. SAM availability can be modulated by the availability of other sources of methyl groups either endogenously or exogenously. As the methyl donor for almost all known biological reactions involving methylation, except for those related to the methylation of homocysteine (HCY), it is clear that methionine has a role beyond being a constituent amino acid in protein.

The other product of transmethylation is S-adenosylhomocysteine (SAH), which is hydrolyzed to adenosine and HCY. If HCY is not remethylated, it proceeds down the transsulfuration (TS) pathway to yield cysteine. The methionine-sparing effect of cysteine is believed to occur by reducing methionine breakdown through transsulfuration. The ability of cysteine to have this effect in vivo human studies depends on whether it replaces part of dietary methionine intake (i.e., keeping the molar amount of SAA constant) or whether the ratio of methionine to cysteine is increased (9,10). If one supposes that the regulatory drive is at the transmethylation locus (i.e., need for SAM) rather than at the TS locus, dietary manipulation of methyl group availability may alter the requirement for methionine.

Choline and betaine have been studied as alternate dietary sources of methyl groups. Choline is necessary for the structural integrity and signaling functions in cell membranes and directly affects cholinergic neurotransmission, transmembrane signaling, and lipid transport and metabolism (22). Betaine is a metabolite of choline and acts as the methyl donor in the remethylation of HCY to methionine. Betaine and/or choline contributes 2 methyl groups to the folate pool as well as providing a methyl group for the betaine-HCY-methyltransferase reaction. Together N-methyl tetrahydrofolate, betaine, and choline contribute to the regeneration of methionine from HCY and consequently to the formation of SAM for methylation reactions, with HCY as its product, thereby completing the cycle (23,24). Supplementation of low-dose betaine in the range of the normal dietary intake was reported to lead to immediate and long-term lowering of plasma HCY in healthy men and women (25). In a recent double-blind randomized trial of 6 incremental levels of folic acid daily or placebo carried out in 308 Dutch men and postmenopausal women (26), total HCY levels were found to be inversely related to betaine concentrations, and the relation was independent of age, sex, and concentrations of folate, creatine, and cobalamin. This led the authors to conclude that plasma betaine was an important determinant of fasting HCY in healthy older (50–75 y) adults. Unfortunately the impact of the intervention on methionine and cysteine levels and their kinetics was not available. In a related study, these investigators found that supplementation with phosphatidylcholine also decreased post-methionine-loading HCY concentrations in 26 healthy men (27). Taken together, these data emphasize the importance of methyl group availability in SAA homeostasis and raise the question of whether betaine and choline could contribute to methionine sparing. This would, parenthetically, imply that cysteine availability versus HCY catabolism might be affected by the dietary intake of alternate sources of methyl groups.

SAA requirements in health maintenance. As discussed earlier, the drive for methionine requirements may rest in the need for methionine beyond its use for protein synthesis, especially when considering the evolution of disease. The need for methyl groups has been viewed as a separate dietary need, although for humans, the major source of methyl group in foods comes from methionine (~10 mmol methyl/d), from N-methyltetrahydrofolate (~5 to 10 mmol methyl/d), and from choline (~30 mmol methyl/d). The interrelationship of these 3 components of the diet makes it imperative that all 3 be assessed in an attempt to determine the requirement of each. Because ~15 to 30% of the population may have increased dietary methyl needs because of genetic polymorphisms in enzymes related to methyl group metabolism (28,29), it is logical that the requirement for methionine as well as cysteine may be affected by the need for methyl groups.

In an earlier review (23), we discussed the role of DNA methylation in the maintenance of health and the evolution of disease and raised the question whether dietary manipulation of methyl group availability could alter methylation patterns that promote health or disease. Interest in the optimal “methylation diet” stems from the role of DNA-methylation changes in overall adult-onset diseases and during development (29,30). Some of these changes are key to a better understanding of certain birth defects (e.g., neural tube defects) and the long-term consequences of early environmental influence on gene expression (i.e., metabolic programming). Van den Veyer (29) suggested that even small decreases in dietary factors influencing methylation could result in significant long-term health effects that accumulate over the years, leading to disorders such as cardiovascular disease and cancer. Recently, Geisel et al. (31) reported that a vegetarian lifestyle characterized by lower vitamin B-12 intake, relatively low methionine content, and slightly higher homocysteine concentrations did not appear to influence whole-genome methylation. It was SAH that appeared to have an inhibitory effect on whole-genome methylation.
### TABLE 1

*In vivo human studies examining cysteine-sparing of methionine requirements*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study subjects</th>
<th>Diets</th>
<th>Design</th>
<th>Findings and conclusion</th>
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<tr>
<td>Storch et al., 1990 (14)</td>
<td>Healthy young men (4 per diet group)</td>
<td>Semi-synthetic formulation with nitrogen requirement provided as an L-amino acid mixture: 1. Adequate MET (2.4% by weight, no CYS) 2. SAA devoid 3. MET-free, 2% CYS 5 days dietary adaptation</td>
<td>L-[1-13C]; methyl-2H5]MET tracer infusion after 5 days of diet with hourly intake of 1/12 total daily dietary intake</td>
<td>SAA-devoid diet reduced flow of MET through TM and TS pathways; addition of CYS to SAA-devoid diet reduced MET oxidation by ~50%. Conclusion: Dietary CYS can lower MET requirements.</td>
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<td>Young et al., 1991 (15)</td>
<td>Healthy young men (n = 5)</td>
<td>Purified diet as above with MET content 1.3% by weight, no CYS 5–7 days of diet</td>
<td>At end of dietary period, L-[1-13C]; methyl-2H5]MET tracer infusion with hourly intake of 1/12 total daily dietary intake on day 5 and postabsorptive infusion on day 7</td>
<td>Based on estimates of MET oxidation and MET balance, concluded that upper range of requirement for MET plus CYS probably exceeds 13 mg.kg⁻¹.d⁻¹</td>
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<td>Hiramatsu et al., 1994 (16)</td>
<td>Healthy young men (n = 8)</td>
<td>Purified diet as above with varying MET/CYS content, respectively (mg.kg⁻¹.d⁻¹): Diet 1: 13/0 Diet 2: 6.5/0 Diet 3: 6.5/5.2 Diet 4: 6.5/10.5 Diet 5: 6.5/20.9</td>
<td>At end of dietary period, isotope study with a L-[1-13C]; methyl-2H5]MET and L-[3,3-2H2]cysteine given IV</td>
<td>In absence of CYS, MET requirement is ~13 mg.kg⁻¹.d⁻¹, and possibly between 6.6–13 mg.kg⁻¹.d⁻¹. CYS does not appear to significantly affect MET requirement. Conclusion: Route of tracer administration may influence outcome because IV tracer may not detect changes in splanchnic SAA metabolism.</td>
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<td>Fukagawa et al., 1998 (17)</td>
<td>Healthy older men (n = 5) and women (n = 7)</td>
<td>Purified diet as above with varying amounts of MET/CYS, respectively, (mg.kg⁻¹.d⁻¹): Diet 1: 13/0 Diet 2: 6.5/5.2 Diet 3: 6.5/21</td>
<td>At end of dietary period, isotope study with a L-[1-13C]; methyl-2H5]MET and L-[3,3-2H2]cysteine given IV</td>
<td>Conclusion: Total SAA requirement in older individuals may be not met if CYS content accounts for as much as half the total SAA content. However, under these conditions, there is a modest sparing effect of CYS on MET oxidation.</td>
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<tr>
<td>Raguso et al., 1997 (6)</td>
<td>Healthy young men (n = 6) and women (n = 2)</td>
<td>Purified diet as above with varying amounts of MET/CYS, respectively, (mg.kg⁻¹.d⁻¹): Diet A: 13 / 0 Diet B: 5 / 0 Diet C: 6 / 6.5 6 days of dietary adaptation</td>
<td>At end of dietary period, isotope study with a L-[1-13C]; methyl-2H5]MET and L-[3,3-2H2]cysteine given orally at 30-min intervals in fasted and fed states</td>
<td>No major effect of CYS on irreversible loss of MET when MET intake was ~40% of recommended amount (determined in the absence of dietary cysteine). Conclusion: clear and significant sparing effect of dietary CYS on MET metabolism was NOT detected under these conditions.</td>
</tr>
<tr>
<td>Raguso et al., 2000 (7)</td>
<td>Healthy men (n = 10) and women (n = 2)</td>
<td>Experiment 1: protein-free diet for 6 days Experiment 2: 3 separate 7-d diet periods 1. High MET, 13 mg.kg⁻¹.d⁻¹; 2. Low MET, 6.5 mg.kg⁻¹.d⁻¹; 3. MET, 5 mg.kg⁻¹.d⁻¹ with CYS 6.5 mg.kg⁻¹.d⁻¹</td>
<td>Expt 1: 8-h IV isotope tracer infusion at end of dietary period with L-[1-13C]; methyl-2H5]MET and L-[3,3-2H2]cysteine; Expt 2: IV isotope tracer with L-[1-13C]cysteine. 3-hr fasted and 5 hr fed state studies</td>
<td>Exp 1: MET and cysteine oxidation rates similar to losses predicted from obligatory nitrogen losses. Exp 2: SAA balance less negative on high MET diet than on low MET or combination of MET and CYS. Conclusion: Maintenance of SAA balance best achieved by supplying MET at ~13 mg.kg⁻¹.d⁻¹.</td>
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<td>Raguso et al., 1999 (18)</td>
<td>Healthy young adults (n = 11)</td>
<td>5-d dietary adaptation of protein-free, SAA-free or leucine-free diets</td>
<td>24-h constant IV infusion of tracers</td>
<td>Obligatory oxidative losses support previous findings that there is limited MET sparing by dietary CYS</td>
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<td>Kurpad et al., 2003 (19)</td>
<td>Twenty-one healthy or undernourished Indian men</td>
<td>7-d dietary adaptation; 7 test MET intakes (3, 6, 9, 13, 18, 21, 24 mg.kg⁻¹.d⁻¹) without CYS</td>
<td>24-hr indicator amino acid oxidation and balance approach with L-[1-13C]leucine tracer</td>
<td>Conclusion: MET requirement in absence of cysteine is 15 mg.kg⁻¹.d⁻¹; similar in well-nourished and chronically undernourished Indian men</td>
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<tr>
<td>Kurpad et al., 2004 (20)</td>
<td>Twenty-one healthy Indian men</td>
<td>7-d dietary adaptation with 2 test diets (5 or 12 mg.kg⁻¹.d⁻¹ CYS) with varying MET (3, 6, 9, 13, 18, 21, 24 mg.kg⁻¹.d⁻¹)</td>
<td>24-hr indicator amino acid oxidation and balance approach with L-[1-13C]leucine tracer</td>
<td>Conclusion: CYS may spare MET requirement in healthy men but the amount is difficult to quantify.</td>
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The relation between SAA and multiple diseases in humans has been recognized, especially with respect to inborn errors of metabolism and to the potentially detrimental effects of high levels of HCY.

With the report of the epidemic of obesity and diabetes mellitus worldwide (38), investigators have begun to explore many facets of associations and possible causalities. In humans, nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of abnormal liver function tests and is associated with obesity, type II diabetes mellitus, and hypertriglyceridemia, components of the “metabolic syndrome” (38,39). In mouse models of NAFLD, diets deficient in methionine and choline lead to fatty livers, the progenitor to steatohepatitis and cirrhosis (40). Other investigators have shown that mice lacking phosphatidylethanolamine N-methyltransferase (PEMT) also develop steatohepatitis (41) and that humans with a polymorphism in the PEMT gene have increased susceptibility to NAFLD (42). These data highlight a very close relation between risk factors for the metabolic syndrome NAFLD and methyl group metabolism.

Because supplemental methionine has been shown to reduce acetaldehyde levels after ethanol ingestion (43), investigators have explored the potential beneficial effects of supplemental methionine and its transmethylation (TM) product SAM, (44). However, concern about high methionine intake exists because methionine is the precursor for HCY, and HCY has been shown to be significantly associated with cardiovascular, hepatic, and cognitive diseases. In early studies (45,46), Anderson et al. concluded that variation in daily methionine intake did not influence responses to the methionine loading test used to determine the rise in HCY concentrations. Ward et al. (47) administered weekly graded doses of methionine in several groups of men and found that high HCY levels were induced only if the intake was > 5 times the usual intake. Because it is unlikely that one could consume this level of methionine, the authors concluded that HCY concentrations should not be affected by long-term changes in methionine intake. In another series of studies, Verhoef et al. examined the effects of a high-protein diet (48) and of free and dietary methionine on total homocysteine (tHCY) concentrations (49). The high-protein diet influenced tHCY concentration but not fasting levels. Furthermore, free methionine increased tHCY more than dietary methionine, and this effect could be attenuated by

### TABLE 1 (continued)

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<tr>
<td>Di Buono et al., 2001 (10)</td>
<td>Six healthy men</td>
<td>2-d dietary adaptation; liquid formula diet with crystalline amino acid mixture with graded (0, 6.5, 13.0, 19.5, 26.0, 32.0 mg.kg⁻¹.d⁻¹) MET; uncertain CYS intake</td>
<td>6-hr indicator amino acid oxidation approach with L-[1-¹³C]phenylalanine tracer administered orally</td>
<td>Based on ¹³CO₂ release in breath, mean MET requirement and population-safe intake of total SAA reported as 12/6 and 21 24 mg.kg⁻¹.d⁻¹, respectively.</td>
</tr>
<tr>
<td>Di Buono et al., 2001 (9)</td>
<td>Six healthy men</td>
<td>Graded (0, 2.5, 5.0, 7.5, 10.0, and 13.0 mg.kg⁻¹.d⁻¹) MET intake with no cysteine or an excess of cysteine (21 mg.kg⁻¹.d⁻¹)</td>
<td>6-hr indicator amino acid oxidation approach with L-[1-¹³C]phenylalanine tracer administered orally</td>
<td>Dietary cysteine can spare 52–64% of the MET requirement. Conclusion: Dietary requirement of total SAA could be met with both dietary MET and cysteine.</td>
</tr>
<tr>
<td>Di Buono et al., 2003 (11)</td>
<td>Five healthy men</td>
<td>Oral L-[1-¹³C; methyl-²H₂]MET tracer study at end of dietary period</td>
<td>Conclusion: Ratio of dietary cysteine to MET regulates SAA metabolism and when constant at 24 mg.kg⁻¹.d⁻¹, a sparing effect of cysteine is found at a MET intake of 5 mg.kg⁻¹.d⁻¹.</td>
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cysteine and serine. Hence, it appears that higher levels of methionine intake, especially if in the form of dietary protein, are not as detrimental as previously believed. In studies using a mouse model of atherosclerosis (apolipoprotein E−/−), Troen et al. (50) demonstrated that excess methionine increased atherogenesis but not directly through an elevation in HCY. This is consistent with the view that high HCY levels may not be directly causal but rather a marker of disease. Drs. Refsum and Selhub discuss this in greater detail in this issue (51,52). In summary, the nature of AA delivery (i.e., free vs. in protein) will influence the biological endpoints of interest. The risk of deleterious effects of high methionine intake in the free form relates to alterations in the thiol redox balance and could be reduced by supplementary cyst(e)ine. In fact, supplementation with the cysteine precursor N-acetylcysteine (NAC) has been shown to partially attenuate the excessive increase in HCY in plasma and oxidized HCY in whole blood (53). Although dietary cyst(e)ine may have only a small effect on total SAA requirements, it plays an important role in balancing the potentially toxic effects of free methionine.

Because findings suggest that certain protein-rich foods may have a lower HCY response on the basis of their ratio of methionine:serine:cysteine, the potential public health implications of attempts to alter specific amino acid content of proteins (54) are large. Earlier reports that a high-protein, high-methionine diet does not raise HCY compared to low-protein, low-methionine diets (55) will lead to consideration of the healthy benefits of specific proteins and possible alternate sources of cysteine. Vegetarian diets have been reported to have protective effects against chronic degenerative diseases related to a lower content of methionine, lysine, and tryptophan. However, the possibility that proteins containing high methionine balanced with appropriate cyst(e)ine content may be engineered is an intriguing thought. Finally, a brief word about 2 alternate sources of cysteine that have been used clinically. The first, N-acetylcysteine, increases cysteine availability through its deacetylation (56) and is the primary antidote for acetaminophen poisoning, NAC was recently used in vivo to alter the thiol redox balance after methionine loading (53). However, in a recent randomized controlled trial, NAC did not prevent postoperative renal dysfunction, additional interventions, complications, or mortality in high-risk patients undergoing coronary artery bypass surgery (57). Furthermore, it was recently reported that NAC failed to boost hepatic glutathione levels in diabetic animals, as hypothesized (58), but appeared to restrain GSH oxidation and heme oxygenase 1 expression, both markers of cellular oxidative stress. The bioavailability of NAC is uncertain, especially when administered orally (59). However, in vitro studies do show an effect of NAC (60), but these effects are not easily extrapolated to the in vivo situation. It is apparent that the underlying disorder plays an important role in determining the response to exogenous NAC, and it is conceivable that other forms of NAC that enter cells more readily would be more effective.

The other cysteine precursor, L-2-oxothiazolidine-4-carboxylic acid (OTZ), has received less attention in human studies. Early work focused on OTZ’s ability to increase GSH concentrations (35,61,62) and the utility of labeled OTZ as a probe to assess precursor mobilization for GSH synthesis (63). In Wistar rats, Limuro et al. (64) found that those receiving OTZ supplementation were protected against the deleterious effects of ethanol and related this to elevation in circulating GSH levels. In vitro studies reported that OTZ can attenuate the cytotoxicity of high glucose concentrations on mesothelial cells and suggested that OTZ may improve the biocompatibility of peritoneal dialysis fluid (65,66). Furthermore, Han et al. described the antidiabetic effect of OTZ through CD38 dimerization and internalization and suggested that OTZ might be a novel antidiabetic drug (67). More recently, Lee et al. found that OTZ reduced inflammatory responses (68) and modulated vascular permeability in murine models of asthma (69). The future for alternative approaches to modulate SAA utilization and requirements beyond its role as an IAA for protein synthesis is exciting and bright. Defining the upper limits of safe levels of SAA intake and interrelationships between SAA and alternate sources of methyl group and cysteine precursors will take the focus of SAA requirements beyond protein.

LITERATURE CITED
HUMAN METHIONINE REQUIREMENT SPARED BY CYSTEINE?  


